HASAC RESEARCH UPDATE
DISEASE TRANSMISSION BY BIOTECHNOLOGY OF REPRODUCTION

****DISEASE TRANSMISSION BY IN VIVO DERIVED EMBRYOS****

Cattle

Bovine immunodeficiency virus

Three experiments were conducted to determine whether the lentivirus, bovine immunodeficiency virus (BIV) is likely to be transmitted via embryo transfer. In the first experiment, embryos collected from BIV-negative heifers were exposed in vitro to BIV for 24 h, washed and then tested for the presence of the provirus. In the second experiment, embryos obtained from BIV-negative heifers were transferred to the uterine horns of BIV-infected heifers; 24 h later these embryos were recovered and tested for the presence of BIV. In the third experiment, embryos were collected from heifers experimentally infected with BIV and then transferred to BIV-negative recipients. In all three experiments, (BIV) proviral DNA was not detected by PCR in association with any oocytes, embryos, follicular fluid, oviductal or uterine washes. Twelve single embryos collected from BIV experimentally infected donors were transferred to BIV-negative recipients resulting in the birth of 7 calves all of which were also negative for BIV; the recipients remained BIV-negative throughout the experiment. In conclusion, this study demonstrates that it is possible to produce transferrable stage embryos from donors infected with BIV and that such embryos are unlikely to transmit this agent either to the recipients or the resulting offspring. (**Bielanski A, et al. Theriogenology 2001;55:641-648.)**

Bovine leukemia virus (BLV)

A summarization of embryo transfer results (Di Giacomo et al, 1986, 1990; Eaglesome et al, 1982; Hare et al, 1985; Kaja et al, 1984; Krolinski et al, 1992; Olson et al, 1982; Parodi et al 1983; Steffani et al, 1987; Coulthard, unpublished data) shows that over 1300 zona pellucida-intact (ZP-I) embryos have been collected from BLV-seropositive dams bred by, or inseminated with semen from, either BLV-seropositive or BLV-seronegative sires, washed and then transferred to BLV-seronegative recipients. The transfers have produced over 618 pregnancies with 599 live calves. None of the recipients seroconverted and all of the calves remained seronegative. BLV was not identified from 60 ZP-I embryos and 26 ZP-I unfertilized eggs, collected from BLV-seropositive donors, washed and then co-cultivated with fetal lamb spleen cells, based on syncytium induction and immunofluorescence. **(Bouillant et al, Ann Rech Vet 1981;12:385-395.)**

BLV was identified using the above procedures in 4/25 (16%) flush fluids from BLV-seropositive donors, probably as a result of blood cell contamination. **(Bouillant et al, Ann Rech Vet 1981;12:385-395.)**

Twenty-four, day 6, untreated ZP-I embryos; 10, day 6, pronase treated, ZP-I (but weakened) embryos, and 14, day 12, hatched embryos, collected from BLV-seronegative donors that were inseminated with semen from BLV-seronegative bulls, were washed and then layered

onto cultures of BLV-infected fetal lamb kidney cells for 24 hrs at 37°C. They were washed again and then transferred in the above groups to three recipients. No pregnancies resulted and the recipients did not seroconvert, nor could BLV-antigen be detected up to 120 days post transfer. **(Hare et al, Can Vet J 1985;26:231-234.)**

Day 7 embryos were collected non-surgically and washed 3X prior to being bisected and transferred to recipient animals (not stated if bisected embryos were transferred naked or within a ZP). Sixty split embryo calves resulted. Of these, 19 originated from BLV^{+ve} dams and BLV^{-ve} sires, 4 from BLV^{+ve} sires and BLV $^{-ve}$ dams, 9 from BLV^{+ve} dams and sires, and 28 from BLV^{-ve} dairy herd. All calves were repeatedly tested serologically negative for BLV and all recipients remained serologically negative. **(Lorton et al, Theriogenology 1987;27:250.)**

Thirteen hundred and six, fresh and frozen-thawed, embryos, in approximately 20% of which the ZP was damaged, were transferred to recipients that were seronegative for BLV. The embryos were collected from dairy herds where a majority of the cattle were probably seropositive for BLV, although the exact level of infection was not known because prior health testing for this disease was not required. (No washing procedure for the embryos was described). All of the recipients remained seronegative for BLV. **(Thibier and Nibart, Theriogenology 1987;27:37-47.)**

In a field study report, 21 seronegative calves were born as a result of embryos collected from eight seropositive donors and transferred to 20 seronegative recipients. One of four calves born as a result of embryos collected from a donor that was seronegative one month before collection, but seropositive at the next test approximately nine months later, and transferred to a recipient that was seronegative 15-20 days before transfer, but seropositive after parturition, was seropositive at birth. No mention is made in the report of washing the embryos. The donors came from farms where the level of BLV infected cattle ranged from 35-45%. There is no record of the level of BLV infection in the herds of origin of the recipients. This report provides a good illustration of the importance of:

a) the proper washing of embryos between collection and transfer
b) the quarantining and retesting of seronegative donors coming from infected premises for an adequate time period prior to collection
c) the quarantining and retesting of seronegative recipients for an adequate time period prior to transfer and their isolation from positive animals throughout gestation.

(Severini et al, Atti Soc Italiana Buiatria 1984;15:481-488.)

Embryos were transferred from 28 Black Pied cows that were serologically positive for BLV to 44 seronegative recipients. The efficacy of controlling leukosis by means of embryo transfer was confirmed. **(Korolev et al, Trudy Vsesoyuznogo Instituta Eksperimental 'Noi Veterinarii 1987;64:59-62.)**

The stated purpose of the study was to "confirm the possibility of protection of calves of high breeding value of leukaemic cows against infections with EBLV using embryo transfer methods.

"From 152 donors 1390 embryos were obtained and only 676 (48.6%) were qualified as suitable for transfer. The amount and types of cellular elements in 10 consecutive dilutions of 16 tests of flushing from the uterus of infected cows were assessed. In 1 ml of uteral flushing, an average of 1.6 million various morfotic elements were found. In the first and sporadically in the second dilutions these cells were observed. From the next solutions no cellular formations were found. During histological investigations, the lymphoid cells were observed neither on the zona pellucida of embryos nor in their vicinity. Then 585 embryos from the leukaemic donors were rinsed four times and transferred resulting in 278 pregnant cows. There 274 calves were born free from leukaemia with four dying during parturition." (gel precipitation test or ELISA were tests used on calves) **(Krolinski J, et al., Medycyna Weterynaryjna 1992;48:79-81 and Krolinski J, et al., Proceed 10e Reunion A.E.T.A. - Lyon, 9-10 September, 1994:page198.)**

A case was discovered where the embryo transfer (ET) calf had been infected with bovine leukemia virus (BLV) from the recipient cow. The embryo was transferred from the BLV-uninfected donor cow to the recipient cow. However, the BLV test had not been performed on the recipient cow before ET was performed. The ET calf was raised in a calf hutch from birth to 1-month old and was given the recipient cow's colostrum and milk artificially. The ET calf was raised with the two other calves from a 1-month old to a 6-month old. The BLV test was performed on the ET calf by agar gel precipitation (AGP) and passive haemagglutination (PHA) assay when the ET calf was 6 months old. Because the ET calf was positive, the BLV test was performed on the recipient cow, the two other calves raised with the ET calf and the two dams of the two other calves. Because the recipient cow only was positive at the time of the first test, we judged that the ET calf had been infected with BLV from the recipient cow. The importance of the BLV test being carried out on the recipient cow for the prevention of enzootic bovine leukemia in a case of ET was recognised. **(Fukai K, et al., Zentralbl Veterinarmed (J Vet Med) 1999;46:511-515.)**

Bluetongue virus (BTV)

BTV did not penetrate the zona pellucida (ZP) or attach to it when day 5 to 7, ZP-I embryos (n = 120) were exposed to 10^2-10^7 pfu/ml of virus (serotype 10) for 1-24 hrs, washed and then assayed. Nor was the embryonic development of these embryos affected by exposure to the virus. **(Singh et al, Theriogenology 1982;17:437-444.)**

Thirty-six 6-7 day old bovine embryos from BTV seronegative cows were exposed to 0.75ml of a BTV (serotype 11) suspension containing $TCID_{50}$ $10^{5.6}$/0.1ml for 18-24 hours. Five embryos were prepared for electron microscopy examination (EM) without any washing: three of the five embryos had numerous particles on the surface of the zona pellucida but no evidence of particles within the embryo. After 5 washes in 0.75ml/wash, nine of 21 exposed embryos were positive in bovine turbinate cell culture based upon production of a cytopathic effect. Eight of ten embryos taken from the 5th wash after 12 hrs and examined by EM had viral particles on the surface of the zona pellucida, but no particles were observed within the embryo. **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68.)**

Twenty-one 6-7 day old bovine embryos from BTV seronegative cows were placed in a bovine turbinate cell culture containing 0.75ml of media which was seeded a few minutes later with serotype 11 BTV at a calculated ratio of one infectious virus particle for each cell (multiplicity of 1:1). After 18-24 hours in cell culture, nine unwashed embryos were processed for and examined by EM: all exposed embryos had numerous particles on the surface of the zona pellucida, but none were observed within the embryo. After 5 washes containing 0.75ml of media 11 of 12 exposed embryos caused a cytopathic effect in cell culture. After exposure for 12 hours in the 5th wash cell culture, 11 of 12 embryos also had numerous particles on the surface of zona pellucida, but none were observed within the embryo. **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68.)**

Embryos were microinjected with "50 picolitres" ($TCID_{50}$ $10^{0.33}$) of virus, washed 10x (0.75ml/wash), placed in bovine turbinate cell culture for 18-24 hrs, exposed to BTV antiserum for 1 hr and then washed 5x. Cells were then removed from each embryo that had an intact ZP. Cells were placed in BT cell culture for 7 days, followed by three blind passages of 7 days if the primary culture showed no CPE. Most embryos were also examined by EM. None of eight embryos were positive on cell culture and the one embryo examined by EM was also negative. However results are inconclusive, because less than one $TCID_{50}$ of virus was present in 50 picoliters of inoculum. **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68.)**

Exposure to BTV (serotypes 11, 17) of day 6-1/2 to 7 embryos (n - 18) from which ZP had been removed by immersion in solution at pH 2 resulted in the accumulation of BTV antigen in the blastomeres and embryonic death. The development of control embryos (n = 3) did not appear to be affected by immersion in solution at pH 2. **(Bowen et al, Am J Vet Res 1982;43:1907-1911.)**

Forty-eight, day 6-8, ZP-I embryos, collected from 17 BT-viremic (serotypes 10, 11, 13, 17, 18) dams inseminated with BTV-negative semen, were transferred to 48 BTV-seronegative recipients. Twenty-five of the 48 recipients were pregnant at 60 days at which time 11 pregnancies were terminated. There were 4 stillbirths and 10 live births. All calves and recipients remained seronegative for BTV. Calves and recipients sampled for BTV isolation were also negative. **(Bowen et al, Am J Vet Res 1983;44:1625-1628 and Thomas et al, Theriogenology 1983;19:425-431.)**

Twenty, day 6, ZP-I embryos, collected from three BTV-seronegative dams, inseminated with BTV-positive semen (serotype 17), were transferred to 16 BTV-seronegative recipients. Two of the donor heifers became infected. Eighteen of the 20 embryos were from the two viremic donors and resulted in nine of the 10 pregnancies obtained. One recipient aborted at 3 months. Nine recipients carried their calves to term. Six of the nine delivered live healthy calves, one was euthanized during dystocia. One set of twins and one single calf died during calving. None of the 16 recipients nor any of the calves developed antibodies to BTV. Nor was BTV isolated from any of the uterine flush fluids. **(Thomas et al, Theriogenology 1985;24:345-350.)**

Two superovulated seronegative cows were each inseminated three times at 12-hour intervals with virus-laden semen containing $TCID_{50}$ $10^{5.8}$/0.1ml of BTV. The cows were flushed for embryo production 6 days after the last insemination. One cow produced 7 embryos and the other one failed to provide any embryos. Attempts to demonstrate virus in or on the embryos by EM or isolation by 3 blind transfers in cell culture failed. After flushing the two cows were euthanized and many tissues, particularly from the reproductive tract, were harvested and tested for presence of virus in cell culture. The results were highlighted by the isolation of virus in cell culture from the ovary, uterine horn, cervix and blood of each animal. **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Three superovulated seronegative cows were inseminated 3 times at 12-hour intervals with semen from a seronegative bull. Immediately after the last insemination each cow was inoculated intramuscularly with 1ml of stock BTV containing $TCID_{50}$ $10^{5.8}$/0.1ml. Five days later each cow was flushed for embryos. One cow had 2 embryos and no virus could be demonstrated in these embryos by EM or in cell culture (3 blind transfers). The other 2 failed to produce embryos. Immediately after flushing, the 3 cows were euthanized and tissues harvested for virus isolation. No virus was isolated from the reproductive tract or blood of the cow that produced 2 embryos. Virus was isolated in cell culture from the blood and at least one reproductive tissue of the other 2 cows. **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Three synchronized seronegative heifers were each implanted with 2 normally-developing 6-day-old bovine embryos that had been kept in an infected cell culture for 24 hours and subsequently washed 3 times (10ml each wash). None of the heifers conceived but virus was isolated in cell culture from the vaginal swab and blood sample taken on day 7 after implantation from 1 heifer only. The progesterone levels at 4 and 7 weeks were normal. Two heifers were euthanized at 54 days after embryo transfer and virus was not demonstrated in the blood and other tissues harvested at this time. All 3 heifers seroconverted with serum neutralizing antibody titres ranging from 8 to 32 at day 35 when tested against 100 $TCID_{50}$ of BTV in the bovine turbinate cell culture system. **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Nineteen, day 10-11, hatched embryos, collected from 10 BT-viremic (serotypes 10, 11, 13, 17) dams inseminated with semen from BTV-seronegative bulls, were transferred to 19 BTV-seronegative recipients. Ten of the 19 recipients were pregnant at 60 days when pregnancy was terminated. The recipients remained BTV-seronegative throughout. **(Bowen et al, Am J Vet Res 1983;44:1625-1628.)**

Forty-two normal embryos, 9 retarded embryos and the unfertilized ova collected from infected donors were negative for BTV-antigen (serotypes 10, 11, 13, 17) by immunofluorescence. **(Bowen et al, Am J Vet Res 1983;44:1625-1628.)**

BTV was recovered from 55% of flush fluids from non-surgical flushes of viremic donors, probably as a result of blood cell contamination. **(Bowen et al, Am J Vet Res 1983;44:1625-1628.)**

A collaborative study by APHIS, ABADRL and MARC was done in 1987-88 in the USA. To avoid natural exposure to infection the animals were moved from Nebraska to Wisconsin (a vector-free area) in the vector season. Sixty heifers were infected deliberately with BTV (serotype 11) by bites of the vector and by inoculation with insect origin virus. During the 'acute' stage (12-16 days post-infection) and 'convalescent' stage (1-3 months post-infection) embryos were collected from 59 of these heifers and washed ten times as per IETS Manual recommended protocols. Embryos were also obtained from six serologically (AGID test) positive donors that were assumed to have acquired BTV infection naturally during the previous vector season; these were termed 'recovered' donors.

A total of 169 embryos was collected from the 'acute' stage donors, 141 embryos from the 'convalescent' stage donors, and 52 from the 'recovered' donors. Neither BTV nor EHDV were isolated from a total of 102 embryos (57 from 'acute', 20 from 'convalescent' and 25 from 'recovered' donors) that were tested in vitro by inoculating them onto cell cultures and embryonating chicken eggs.

A total of 248 embryos (110 from 'acute', 121 from 'convalescent' and 17 from 'recovered' donors) was transferred into susceptible recipients (no. recipients = 103?). Some recipients that returned to service evidently had further embryos transferred into them. To avoid natural exposure to infection the recipients were moved from Nebraska to Wisconsin (a vector-free area) for the summer, and they were returned in late October 1987. A total of 95 calves were born (36 from 'acute', 52 from 'convalescent' and 7 from 'recovered' donors. There was no evidence of BTV or EHDV transmission to recipients following transfer of embryos from the 'acute' and 'convalescent' stage donors, and infection was not detected in their calves. However, two recipients of embryos from one of the 'recovered' donors developed antibody to BTV sometime between five and nine months after transfer and passive antibody to BTV was also detected in their calves. Subsequent investigations yielded evidence that these two recipients had aquired BTV infection during mid- or late pregnancy as a result of natural exposure to infected insect vectors and not from the transferred embryos. It also transpired later that the 'recovered' donor of the two embryos concerned had had high EHDV antibody levels (detected and differentiated from BTV antibody by cELISA), and the response to the BTV AGID test was in fact a cross-reaction to the EHDV antibody. This donor had, therefore, recovered from an EHDV infection in the previous vector season, not a BTV infection. Thus the BTV infection that was acquired by the two pregnant recipients could not have come from the embryos. Insect vectors were found to have been active until at least a day or two after the pregnant recipients returned to Nebraska in late October so it is likely that they acquired BTV from naturally infected vectors at that time. **(Acree et al, Theriogenology 1991;36:689-698.)**

Bovine blastocysts (day 10-11) hatched from their zonae pellucidae were cultured for 24 h in the presence or absence of interferon (5000 units/ml) and then challenged with bluetongue virus (10_6 PFU) to assess the induction of an antiviral state. In contrast to its application to fetal bovine cells, where significant antiviral effects were induced, interferon treatment of embryos failed to reduce virus yield and had no effect on virus-induced cytopathology. This lack of biologic activity of interferon in bovine embryos is similar to that previously observed with undifferentiated murine embryonal carcinoma cells and is probably a manifestation of a more general mechanism regulating gene expression in the early mammalian embryo. **(Bowen, Theriogenology 1988;30:119-126.)**

Bluetongue virus (BTV) has been categorized by the OIE as a category 1 disease agent, for which proper handling between collection and transfer is thought to be sufficient to prevent transmission through embryo transfer. For bovine viral diarrhoea virus, it was shown that effectiveness of washing procedures depends on virus strains (Waldrop *et al.* 2004 *Theriogenology* **62**, 45–55). Also BTV-8 has unique characteristics in comparison with other strains (De Clercq *et al.* 2008 *Transbound. Emerg. Dis.* **55**, 352–359). The aim here was to investigate whether embryo transfer of *in vivo*-derived bovine embryos after *in vitro* exposure to BTV-8 can be performed without risk for infection of the recipients if IETS washing and trypsin treatment procedures are followed. Donor cows ($n = 2$) were synchronized and superovulated using Stimufol® (Ulg, Liége, Belgium) and subsequently inseminated. At 6.5 days post-insemination (dpi), flushed embryos ($n = 14$ and $n = 3$) were placed in 800 µL of minimal essential medium (MEM), containing $10^{4.9}$ 50% tissue culture infectious doses (TCID50) of BTV-8 (Bel 2006/2 P5, VAR, Brussels, Belgium) and incubated for 1 h at 39°C in 5% CO_2 in air (Vandaele *et al.* 2011 *Vet. Res.* **42**, 14–21). Next, embryos were washed in pairs in 5 consecutive Petri dishes containing PBS with antibiotics and 0.4% BSA, w/o Ca and Mg. Then, embryos were exposed to 2 consecutive trypsin (Invitrogen, Carlsbad, CA, 25050-014) washes of 45 s each at 39°C in 5% CO_2 in air and finally, another 5 consecutive washes in PBS with 2% FCS. Each Petri dish contained at least 2 mL of medium and was gently agitated between washes. Embryos were transferred in a maximum of 7 µL of medium and a new tip was used after every wash step. Washes 1 to 5 and washes 6 to 10 were pooled and analysed for BTV-8 (RT-qPCR). After these washes, 3 pairs of embryos ($n = 6$) were loaded in straws and transferred to 3 BTV-8 negative recipients. Two sentinel cows served as control. Cows were bled twice weekly and blood and serum samples were analysed for BTV-8 (RT-qPCR) and BTV-8 antibodies. Viral BTV-RNA was detected in all 3 recipient cows at 7 days after transfer and viraemia was confirmed by the establishment of high antibody titers at 14 days after transfer. Viral BTV-RNA was detected in washes 1 to 5 for each pair of embryos (Cp-value around 29), whereas washes 6 to 10 had Cp-values around the cut-off value (40), indicating that probably the last wash was BTV-8 negative. None of the recipients was pregnant at 28 days post-transfer. In conclusion, washing and trypsin treatment did not succeed in removing BTV-8 from *in vitro*-spiked *in vivo*-derived bovine embryos. These unexpected results stress the need for further *in vivo* research, e.g. what is the virus load *in vivo* embryos may be exposed to *in utero* during viraemia? Does BTV-8 react differently with the zona compared with other strains? Are alternative washing procedures needed to remove BTV-8 from the zona?

Vandaele L., K. De Clercq , W. Van Campe , I. De Leeuw and A. Van Soom Bluetongue Virus Infection In Cattle After Transfer Of Bovine In Vivo-Derived Embryos. Reproduction, Fertility and Development 24(1) 168-168 http://dx.doi.org/10.1071/RDv24n1Ab112

Bluetongue virus serotype 8

The aim of this study was to describe the effects of BTV infection on foetal mortality in 53 herds located in the east of France and infected by the virus between August and November 2007. In 2008, two visits were performed 50 days apart by local veterinary surgeons and pregnancy was checked by ultrasonography. BT status was found positive in 69.5 % of the cows (80 % of beef vs 67 % of dairy cows). At first visit, ultrasonography was performed in

cows which were supposed to be pregnant. At 2d visit, a positive pregnancy status has been confirmed in 78 % of the cows, but this proportion was higher in seronegative cows when compared to seropositive cows (81 % vs 76 %, p<0.02). Out of 1311 pregnant cows at first visit, only 37 presented a foetal mortality at 2nd pregnancy check (2.8 %). The rate of foetal mortality was also related to the BT seropositive status of cows (seronegative : 6/418 = 1.4 vs seropositive 31/893 = 3.5 %, p<0.05).
Ponsart C., Gatien J., Pozzi N., Humblot P., Guérin B. Relationship between bluetongue virus (BTV-8) infection and foetal mortality in cattle. Reproduction, Fertility and Development. 2010; 22(1): 253 (abstr).

The behavior of BTV-8 in cattle is different from most other serotypes not only with regards to clinical signs but certainly with respect to virus transmission (transplacental, contact). Therefore, the possibility of virus transmission by means of embryo transfer was examined by *in vitro* exposure of *in vitro* produced and *in vivo* derived bovine blastocysts to BTV-8 followed by different washing protocols, including longer exposure times (up to 120 s) to 0.25% trypsin at room temperature or at 37°C. None of the washing protocols used was successful in removing the viral genome completely from the *in vitro* produced and *in vivo* derived embryos as was demonstrated by real-time PCR. Moreover, BTV-8 virus was transmitted to recipient cows after embryo transfer of *in vivo* derived BTV8-exposed embryos, which had been subjected to routine decontamination as recommended by IETS, consisting of 5 washes in PBS followed by a double treatment of 0.25% trypsin for 45s at 37°C, and an additional 5 washes in PBS with 2% FCS. This study clearly demonstrates the necessity of vigorous application of the directives for screening of potential donors and the collected embryos, especially in regions with BTV-8, to prevent transmission of the disease.
Haegeman, A., VanDaele, L., De Leeuw, I., Oliveira, A.P., Nauwynck, H., Van Soom, A. and De Clercq, K., 2019. Failure to remove bluetongue serotype 8 virus (BTV-8) from in vitro produced and in vivo derived bovine embryos and subsequent transmission of BTV-8 to recipient cows after embryo transfer. *Frontiers in Veterinary Science*, *6*, p.432. doi: 10.3389/fvets.2019.00432

Infectious bovine rhinotracheitis/infectious pustular vaginitis virus (IBRV/IPVV/BHV-1)

Attempts to isolate IBRV from 18 ZP-I unfertilized eggs and 13 ZP-I embryos, collected from IBRV-seropositive donors for assay without further treatment, were unsuccessful. **(Singh et al, Theriogenology 1982c;18:133-140.)**

Fifty-three of 83 (63%), day 5 to 7, ZP-I embryos, exposed to 10_6 to 10_8 $TCID_{50}$/ml of IBRV (NADL strain) and then washed, retained virus, although their embryonic development was not affected. Both trypsin and IBRV-antiserum were effective in removing IBRV from the embryos. **(Singh et al, Theriogenology 1982c;18:133-140.)**

Studies were designed to test the ability of IBRV (Cooper strain) to adhere to and/or penetrate the zona pellucida of 6-8 day old bovine embryos from susceptible parents after exposure to viral culture suspensions or infected bovine turbinate (BT) cultures of equivalent viral concentrations ($TCID_{50}$ 10_5/0.1ml) for 18-24 hours. After viral exposure the embryos

were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before, in the case of EM, and after 5 washes, through medium (0.75ml) in the case of EM and cell culture **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68).**

On EM examination, prior to washing, one of eight embryos exposed to virus suspension and three of four embryos exposed in BT cell culture had viral particles associated with the ZP. After 5 washes and overnight in the 5th wash, one of seven embryos exposed to viral suspension and none of five embryos exposed in BT cell culture had viral particles associated with the ZP. In BT cell culture testing for CPE after five washes, six of 10 embryos were positive after exposure to virus suspension, and 14 of 17 were positive after exposure in BT cell culture. **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68.)**

Embryos were microinjected with 50 picoliters ($TCID_{50}$ $10_{0.5}$) of virus, washed 10x (0.75ml/wash), placed in BT cell culture for 18-24 hr, exposed to IBRV antiserum for 1 hr and then washed 5x. Cells were then removed with a micromanipulator from each embryo that had an intact ZP. Cells were placed in BT cell culture for 7 days, plus three blind passages of 7 days if the primary culture showed no CPE. Most embryos were also examined by EM. Four of four embryos were positive on cell culture and virus particles were observed by EM in three of four. **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68.)**

Sixty-three ZP-I embryos and unfertilized eggs, collected from donors shedding IBRV/IPVV (strains Colorado-1; Lethbridge 108), were assayed after trypsin treatment and found to be negative for the virus. Thirty-seven of the 63 were found in flush fluids that were positive for IBRV. **(Singh et al, Theriogenology 1983;20:169-176.)**

One hundred and eighty-five embryos were collected from 29 superovulated donors 6 to 8 days post estrus. The zona pellucida (ZP) of these embryos was either cracked, removed mechanically or removed with acidified Tyrode's solution, or left intact. Forty-eight of 103 (47%) ZP-cracked and ZP-free embryos, exposed for 24 hrs to bovine rhinotracheitis virus (IBRV) (Colorado strain), survived. No significant difference was found in the embryonic survival of the ZP-cracked embryos exposed to IBRV and control embryos not exposed to IBRV. However there was a significant ($p < 0.001$) difference in the survival of ZP-free embryos exposed to IBRV and ZP-free embryos not exposed to IBRV (30% vs 80%). **(Bielanski et al, Theriogenology 1987;28:495-501.)**

Fifty-three day 10 and 11 (ie hatched) embryos were exposed to 10^7 $TCID_{50}$ of one of four (LA, LX1161, BFN 2A, LX537) strains of BHV-1 for one (LX 1161) or two (LA, BFN 2A, LX 537) hours, washed 3X (each wash representing an approximate 100 fold dilution), and then cultured for an additional 24 to 48 hrs. Morphologically, 63.6% of the test embryos compared to 12.5% of control embryos showed degenerative changes after 24 hrs in culture. Electron microscopy of degenerating embryos showed viral nucleocapsids in most trophoblast cells and complete viral particles adjacent to the nuclear envelope and in cytoplasmic vacuoles. Infectivity studies showed a mean 10 fold increase in virus titre after 24 hrs in culture following exposure compared to zero hours following exposure. **(Bowen et al, Am J Vet Res 1985;46:1095-1097.)**

Sixty-four, day 7 and 8, ZP-I embryos were collected from IBRV/IPVV-shedding (strains Lethbridge 108, Colorado-1) donors, washed, treated with trypsin, washed again and transferred to 49 IBRV-seronegative recipients. Forty-one of these embryos were from donors that had had IBRV inoculated into the uterus on the day after estrus just prior to their second insemination. Twenty-two pregnancies resulted in 20 live calves, 5 stillbirths (2 twin), 1 induced abortion and 1 spontaneous abortion. Ten of 20 live calves and four of five stillbirths were from donors inoculated intrauterinely with IBRV, while 14 of the live calves, 4 stillbirths (2 twin) and the two abortions were from four of the five donors with IBRV in their flush fluids. All of the calves and all of the recipients remained seronegative for antibodies to IBRV, and virus isolation attempts on selected calves and recipients were all negative. **(Singh et al, Theriogenology 1983;20:169-176.)**

Thirty-eight ZP-I embryos were exposed to 10^7 $TCID_{50}$/ml of IBRV (strains Lethbridge 108, Colorado-1) in vitro, washed, treated with trypsin and transferred to twenty-two IBRV-seronegative recipients. Four pregnancies were obtained. None of the recipients receiving embryos seroconverted **(Singh and Thomas -unpublished- cited by Singh, Theriogenology 1987;27:9-20.)**

Eight ZP-I embryos were exposed to 10^7 $TCID_{50}$/ml of IBRV in vitro, washed 10x and then transferred to 4 recipients. One out of four recipients seroconverted **(Singh and Thomas -unpublished- cited by Singh, Theriogenology 1987;27:9-20.)**

IBRV has been isolated from flush fluids from 10/22 IBRV/IPVV shedding donors **(Singh et al, Theriogenology 1983;20:169-176.)**

Studies in which heifers have been infected with IBR-like strains of BHV-1 indicate that, subsequently, the virus can usually be isolated from the ovaries, with the corpus luteum the principal structure affected (Miller and Van Der Maaten, Am J Vet Res 1984;45:790-794 and Van Der Maaten and Miller, Vet Micro 1985;10:155-163), and also from the uterine body and the internal os of the cervix (Van Der Maaten and Miller, Vet Micro 1985;10:155-163.)
Twenty-two Hereford heifers were injected IM with prostaglandin F2 alpha, 11 days apart to synchronize estrous cycles. Twelve of 14 heifers that had signs of estrus were inoculated IV with 1 of 3 modified-live infectious bovine rhinotracheitis virus vaccines, and 2 were assigned to a non-vaccinated control group. Also, 6 of the 8 anestrous heifers were inoculated IV with 1 of the 3 vaccines on the fourth day after the last prostaglandin injection and the other 2 were assigned to the non-vaccinated group. Vaccine virus was isolated from the blood and nasal and vaginal secretions from the vaccinated heifers on postvaccination days 4, 7 and 9. On postvaccination day 9, all heifers were ovariectomized and ovarian tissues were processed for virus isolation and histologic examination. Vaccine virus was isolated from ovarian tissues of some heifers in each of the vaccine groups. Necrotic oophoritis characterized by multifocal areas of ovarian tissue necrosis, haemorrhage, and mononuclear lymphocytic infiltration was observed. The corpora lutea and surrounding ovarian tissues taken from vaccinated heifers in each group had varying amounts of necrotic and inflammatory change, but the changes appeared to be more severe in 1 group than in the other 2. Virus also was isolated from 2 of the controls; these heifers apparently became

infected with vaccine virus that had been excreted from the vaccinated animals **(Smith et al, Am J Vet Res 1990;51:969-972.)**

Two seronegative cows were superovulated and inseminated with semen seeded with $TCID_{50}$ $10^{4.5}$/0.1ml IBRV. One cow produced 2 non-developing, virus free embryos, the other failed to conceive. Nearly all the reproductive organs contained significant amounts of virus **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Two superovulated susceptible cows were inoculated intranasally with IBR virus ($TCID_{50}$ $10^{4.5}$/0.1ml) immediately after insemination with normal semen from bulls with proven fertility. Virus was detected in some reproductive organs and blood or spleen tissue suspensions in cell culture as detected by a specific IBR probe. Three degenerate embryos were recovered from one of the cows, but virus was not detected either by electron microscopy (1 embryo) or in cell culture by cytopathic alterations (1 embryo). Virus was, however, detected in the embryo by using an IBR probe as an indicator of replication in a cell culture suspension. Thus, caution in interpreting negative results is emphasized, if the most sensitive viral probe detection systems are not used **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Three synchronized recipient cows were implanted with six or seven-day old embryos that had been exposed in vitro to IBRV for 18-24 hours and subsequently washed in 500ml of flushing fluid. One recipient became pregnant. No virus was detected by probe in selected tissues taken at necropsy 60 days post estrus. Liver suspension tissues (1:3 dilution) of the twin 59-day old fetal tissues were tested for virus by culture and by probe. No virus or viral nucleic acid was detected. Virus was detected by IBR hybridization from several tissues of the two other recipients. Both cultures had been contaminated by fungi. Virus was also detected in cell culture from the blood of one recipient taken at day 7. Serum neutralizing antibodies were not detected in the serum samples taken on day 28 from any of the recipients at 1:4 final serum dilution. Using the Cooper strain of IBRV with endpoints of $TCID_{50}$ 10^{-6}/0.1ml in BT cell cultures, the sensitivity of the nucleic acid hybridization test (probe) was between 10^2 and 10^4 more than that of the infectivity assay in cell culture **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72.)**

Thirteen hundred and six, fresh and frozen-thawed embryos, in approximately 20% of which the ZP was damaged, were transferred to recipients that were seronegative for IBRV (Thibier and Nibart, Theriogenology 1987;27:37-47). The embryos were collected from dairy herds in the U.S.A. where approximately 95% of the cattle were seropositive for IBRV, based on 89 sera sampled from 89 donor cows. These cows yielded 750 embryos which were shipped to France and 600 of them were transferred to seronegative heifers. Two hundred and fifty calves were born. None of the recipients seroconverted, although the embryos were not trypsin-treated **(Thibier, Proc 13th Conf OIE Reg Comm for Europe,1989;pp3-36.)**

Bovine Herpes virus -1 (BHV-1)

The aim of our study was to examine whether: (1) the exposure of bovine embryos to the BHV-1 virus in vitro can compromise their further development and alter the ultrastructural

morphology of cellular organelles; (2) whether the zona pellucida (ZP) can be a barrier protecting embryos against infection; and (3) whether washing with trypsin after viral exposure can prevent virus penetration inside the embryo and subsequent virus-induced damages. The embryos were recovered from superovulated Holstein-Friesian donor cows on day 6 of the estrous cycle. Only compact morulas or early blastocysts were selected for experiments with virus incubation. We used the embryos either with intact ZP (either with or without trypsin washing) or embryos in which the ZP barrier was avoided by using the microinjection of a BHV-1 suspension under the ZP. ZP-intact embryos (n = 153) were exposed to BHV-1 at 10(6.16) TCID(50)/ml for 60 min, then washed in trypsin according to IETS guidelines and postincubated in synthetic oviduct fluid (SOF) medium for 48 h. Some of the embryos (n = 36) were microinjected with 20 pl of BHV-1 suspension under the ZP, the embryos were washed in SOF medium and cultured for 48 h. Embryo development was evaluated by morphological inspection, the presence of viral particles was determined both immunocytochemically, using fluorescent anti-IBR-FITC conjugate and by transmission electron microscopy (TEM) on the basis of the ultrastructure of the cellular organelles. It was found that BHV-1 exposure impairs embryo development to higher preimplantation stages independent of the presence of the ZP or the trypsin treatment step, as most of the embryos were arrested at the morula stage when compared with the control. Immunofluorescence analysis confirmed the presence of BHV-1 particles in about 75% of embryos that were passed through the trypsin treatment and in all the BHV-1-microinjected embryos. Ultrastructural analysis, using TEM, revealed the presence of virus-like particles inside the BHV-1-exposed embryos, where the trypsin washing step was omitted. Conversely, in trypsin-treated BHV-1-exposed embryos, TEM detected only the envelope-free virus-like particles adhered to pores of the ZP. The embryos that were microinjected with BHV-1 suspension showed the presence of BHV-1 particles, as well as ultrastructural alterations in cell organelles. Taken together these findings may suggest that BHV-1 infection compromises preimplantation development of bovine embryos in vitro and therefore the ZP may not be enough on its own to prevent virus-induced damage, unless it is not accompanied with trypsin washing.

Makarevich AV, Pivko J, Kubovicova E, Chrenek P, Slezaková M, Louda F. Development and viability of bovine preimplantation embryos after the in vitro infection with bovine herpesvirus-1 (BHV-1): immunocytochemical and ultrastructural studies. Zygote. 2007;15(4):307-15.

Although porcine-origin trypsin will effectively remove bovine herpesvirus 1 (BHV-1) associated with in vivo-derived embryos, TrypLE™, a recombinant trypsin-like protease, has not been evaluated. In Experiment 1, 17 groups of 10 in vivo-derived embryos were exposed to BHV-1, treated with TrypLE™ Express or TrypLE™ Select (10× concentration) for varying intervals, and assayed as 2 groups of 5 embryos. TrypLE™ Select treatment for 5 and 10 min (two and seven groups of five embryos, respectively) effectively inactivated BHV-1. In Experiment 2, 22 groups of 10 IVF embryos were treated and assayed. Treatment with TrypLE™ Select for 7 and 10 min (six groups of five embryos each) and with TrypLE™ Select diluted 1:2 for 10 min (seven groups of five embryos) was also effective. In Experiment 3, 17 groups of 10 IVF embryos were further evaluated with TrypLE™ Select undiluted and diluted 1:2 for 10 min. Treatment with the diluted product was effective (18 groups of five embryos), whereas the undiluted product was not completely effective (virus isolated from 2 of 16 groups). In Experiment 4, IVF embryos were treated as described in Experiment 3 and then cultured

individually or as groups of five on uterine tubal cells (UTCs) for 48 h; 60% of UTC samples associated with groups of embryos and 35% of UTC associated with individual embryo samples were positive for BHV-1. Therefore, although TrypLE™ Select appeared to have promise for the treatment of in vivo-derived embryos, it cannot be recommended for treatment of in vitro-derived embryos.

Marley MS, Givens MD, Galik PK, Riddell KP, Looney CR, Stringfellow DA. Efficacy of a recombinant trypsin product against bovine herpesvirus 1 associated with in vivo- and in vitro-derived bovine embryos. Theriogenology. 2008;69(6):746-57.

In vitro embryo production (IVP), as well as having a biotechnical importance, is a valuable tool for studies of gamete and/or embryo interaction with pathogens and xenobiotics. In consequence, it has become an excellent model not only for investigations about sanitary aspects, but also for aspects related to toxic processes. The aim of this study was to evaluate the effect of cytotoxic aqueous extract of *Ateleia glazioviana* and its interference on the interaction of bovine herpesvirus type 1 (BoHV-1) with bovine oocytes during the *In vitro* maturation (IVM) period. The statistical analysis of the experiments was made according to Student's *t*-test (P <0.05). The parameters used for this experiment were based on the morphological, physiological, and clastogenic action analysis of the bovine oocytes. The oocytes were collected from ovaries from slaughterhouse and divided into control group (G1, *n*=214), a group infected with BoHV-1 (Los Angeles sample 105.5 TCID50 mL−1(G2, *n*=210), a group exposed to the extract of *A. glazioviana*, 0.24 gmL−1; G3, *n*=228), and a group simultaneously exposed to the virus and to the extract (G4, *n*=210). For IVM, the oocytes were kept in TCM-199 supplemented with hormones and incubated at 38◦C, 5%CO2, and 95% humidity for 24 h. The oocytes in G1 showed high expansion of the cumulus cells and ooplasm uniform in appearance; oocytes in G2 showed uniform but moderate expansion of cumulus cells and retraction of ooplasm; the G3 group showed low and irregular expansion with degeneration of cumulus cells and retraction of ooplasm with a granular aspect; and oocytes in G4 showed degeneration of cumulus cells, retracted and granular ooplasm.We observed maturation rates of 81.3% in G1, 31.0% in G2, 5.7% in G3, and 1.4% in G4. As for the clastogenic action analysis, an additional group of oocytes, named *in natura* (*n*=210), was evaluated and presented 41.9% of comets class 0 (zero), 34.8% class I, 12.4% class II, 7.1% class III, and 3.8% class IV. G1 (*n*=211) presented 6.1% of comets class 0, 47.8% class I, 31.3% class II, 11.0% class III, and 3.8% class IV. Oocytes belonging to G3 (217) presented 0.5% of comets class 0, 19.8% class I, 28.1% class II, 34.1% class III, and 17.5% class IV. G2 (*n*=229) presented 4.4% of comets class 0,61.2% class I, 26.6% class II, 4.8% class III, and 3.0% class IV. Oocytes in G4 (*n*=206) presented 3.9% of comets class 0, 26.2% class I, and similar amounts of comets level II (23.8%), III (22.8%), and IV (23.3%). The statistical analysis presented a significant difference in the final results. Such results show the cytotoxic effect of *A. glazioviana* in bovine oocytes. The simultaneous exposure to the virus and the extract aggravated the effect of the virus, suggesting an increase of the pathogen within the gametic cell.

D. L. Pavão, M. M. Piccolomini, A. C. Góes, R. Harakava, M. Haraguchi, and M. Dangelo
Assessment of cytotoxicityand interference of*ateleia glazioviana* in BovineHerpesvirus type 1 (BOHV-1) interaction with *in vitro*-matured bovine oocytes
Reprod Fertil Dev 2010, 22, 295 abstr

The necessity of greater elucidation about oocyte pathogen interactions made us examine the interaction of bovine herpesvirus type 1 (BoHV-1),Los Angeles strain, with oocytes matured *in vitro* to assess the potential hazard of transmission of such infectious agent through IVF. The process of capacitation and transportation of oocytes through the oviduct depends on the quality of the female gamete. Keeping in mind that the BoHV−1 causes morphophysiological modification in oocytes, the aim of this study was to evaluate, in an experimental model of *in vitro* oviduct, whether the pathogen interferes in the transportation of oocytes. This was determined using the percentage of oocyte recovery and assessment of the alteration in the morphology of the oviduct lumen. Oviducts and oocytes were collected from bovine ovaries derived from slaughterhouse cows. The oviducts were dissected, washed, and placed in Petri dishes (100×20 mm). On the previous portions of the infundibulum, *in vitro*-matured control oocytes or oocytes exposed to the virus (105.5 TCID50 mL−1) were introduced (20 oocytes per group) and then immersed in 100mL of TCM-199 medium. Each dish was incubated at 38◦C, 5% CO2, and 95% humidity for 24 h. Then, we introduced in the infundibulum, through intramammary infusion probe, 5mL from a physiologic solution containing 1% of bovine fetal serum. Each oviduct effluent was collected separately and evaluated through a stereoscope for the recovery and counting of oocytes. Immediately after the recovery, both oviduct groups were sectioned longitudinally and observed through an optical microscope (100×) for a morphological evaluation of the luminar area. For the control group, the percentage of recovery was 14.9% (65/437), whereas in the infected group, the oocyte recovery was 23.4% (100/428). The statistical analysis was made according to Student's *t*-test (P <0.05), and presented a significant difference in the final results. The previous results show that the group of oocytes that was exposed to the virus presented a higher percentage of recovery compared with the control group. The oviducts that received the exposed oocytes presented areas where it seemed there has been cytopathic effect represented by dark and lumpy sections, in which there were round and degenerating cells mainly located in the oviducts' peripheral area. The morphophysiological modifications in contaminated oocytes and oviducts probably complicate their transportation and also interferes in the process of their liberation. It is relevant to elucidate the importance of the studies above because of their participation in the recovery of these gametic cells in bovine oviducts, as well as the transmission during IVF processes

M. M. Piccolomini, M. L. Batista, A. C. Góes, D. L. Pavão, M. F. Alves, and M. Dangelo
Assessment of the transportation of bovine oocytes experimentally exposed to Bovine Herpesvirus Type 1 (BOHV-1) during the period of maturation in *in vitro* oviducts
Reprod Fertil Dev 2010, 22, 297 abstr

This study deals with the potential for the introduction of infectious agents through the use of animal-derived products. The efficacy of a recombinant bovine trypsin (RBTr) as a replacement for porcine pancreatic trypsin and a disinfectant for bovine herpesvirus-1 (BHV-1)-infected embryos was investigated according to the sanitary guidelines of the International Embryo Transfer Society. Treatment of in vivo and in vitro fertilized embryos contaminated with BHV-1 (10(5) TCID50/mL) in the presence of RBTr (525 U/mL) for 120 s, effectively removed the infectious virus compared with untreated and washed embryos (P < 0.05). Transfer of in vivo fertilized and disinfected embryos to BHV-1 seronegative recipients (n = 24) resulted in 14 pregnancies and 11 calves born free of BHV-1. In contrast, transfer of

unwashed or undisinfected embryos to four recipients resulted in seroconversion and no pregnancies at term. It was concluded that the use of RBTr could be considered as an alternative method of rendering embryos free of BHV-1 and thus reduce the potential risk of disease transmission to embryo recipients and offspring. **Bielanski A, Algire J, Lalonde A, Garceac A. Prevention of bovine herpesvirus-1 transmission by the transfer of embryos disinfected with recombinant bovine trypsin. Theriogenology. 2013 Dec;80(9):1104-8.**

Cryopreserved bull semen contaminated with BHV-1 was used for artificial insemination (AI) of seronegative, superovulated heifers (N = 43). Embryos were collected post-mortem at 7 days post-insemination and were washed according to the International Embryo Transfer Society (IETS) guidelines. BHV-1 was detected in all samples of follicular fluid, oviductal epithelial cells, endometrium and corpora lutea tissues and a proportion of unwashed (52 of 120, 43%) and washed oocytes and embryos (7 of 113, 6%) collected from embryo donors. Of the 396 collected, unfertilized oocytes and embryos, only 29 (7%) were of ET quality. Most of the embryos and oocytes were degenerated (N = 224, 57%) or unfertilized (N = 143, 36%). The 13 heifers, which each received a single morula-stage washed embryo, maintained seronegative status, but only two (15%) became pregnant and delivered BHV-1-free calves. **Bielanski, A., Algire, J., Lalonde, A., Garceac, A. Risk of transmission of bovine herpesvirus-1 (BHV-1) by infected semen to embryo recipients and offspring. Reproduction in Domestic Animals 2014; 49: 197-201**

Highlights

•Genital organs samples from cows presented the BoHV-1.
•The virus may be present in uterine, oviduct and ovarian tissues.
•BoHV-1 was detected in 100% of uterine samples.
•The findings suggest uterus as a fetal infection source to be implicated in playing a role in abortion.

Abstract

Bovine herpesvirus 1 (BoHV-1) is a causative agent of respiratory diseases in cattle, and infection with BoHV-1 can cause reproductive failure. There are few studies regarding infections in natural conditions in the reproductive organs of bovine animals. In this context, this study investigated the presence of BoHV-1 in the uterus, oviducts, and ovarian tissues of naturally infected cows. The three genital structures were evaluated for the presence or absence of BoHV-1 by immunofluorescence assay using confocal scanning laser microscopy. Blood and genital organ samples of 75 cows unvaccinated against BoHV-1 were used. Fragments of uterus, oviduct, and ovarian tissue were processed and analyzed by confocal scanning laser microscopy. Neutralization by antibodies was observed in 54.7% (41/75) of the serum samples tested. BoHV-1 were detected in the uterus of all the seropositive cows. The oviducts contained BoHV-1 in 73.2% of the samples and the ovaries contained BoHV-1 in 58.5% of the samples from seropositive animals. The presence of the virus was not observed in any of the genital organs of seronegative animals. There was no correlation between the antibody titer and the detection of BoHV-1 in positive tissue in the different genital organs or with the number of infected structures per animal. The detection of BoHV-1 in 100% of the uterus samples from seropositive cows suggests that this organ may be a source of infection for the fetus, resulting in abortion. Further studies on the mechanism by which BoHV-1 infects the fetus via the uterine route should be performed.

Queiroz-Castro, V.L.D., da Costa, E.P., Alves, S.V.P., Guimarães, J.D., Dohanik, V.T., Santos, M.R., de Souza, L.F.L., Ribeiro, C.G., Caldas, R.T. and Silva-Júnior, A., 2019. Detection of bovine herpesvirus 1 in genital organs of naturally infected cows. *Theriogenology*, *130*, pp.125-129. https://doi.org/10.1016/j.theriogenology.2019.03.003

Bovine herpesvirus 4 (BHV-4)

Ova were collected non-surgically from superovulated donor cows at day 7. On the day of collection, 8 donors of ova for exposure to IBRV had anti-IBRV antibody in the blood, and one of the 13 donors of ova for exposure to BHV-4 had anti-BHV-4 antibody. Prior to exposure to virus, ZP-I ova were identified and washed according to International Embryo Transfer Society standards.

Ova were exposed to either 10_6 to 10_7 pfu of IBRV (Colorado strain) or 10_6 to 10_7 pfu of BHV-4 (DN599 strain) for 1 to 2 hr. Subsequently, approximately equal numbers of ova exposed to each virus were either washed 12 times and washes and ova examined for presence of infectious virus, or trypsin treated and ova examined for presence of infectious virus. Washing was in cell culture medium (minimum essential medium or Hams F10) using a separate sterile micropipette and a minimum dilution factor of 1 to 100 between washes. Trypsin treatment involved the use of 2ml of medium in twelve 35-mm tissue culture dishes. Dishes 1 to 5 contained Dulbecco's phosphate-buffered saline solution plus 0.5% bovine serum albumin, dishes 6 and 7 contained 0.25% trypsin in Hanks balanced salt solution (pH 7.6 to 7.8) and dishes 8 to 12 contained Dulbecco's phosphate-buffered saline plus 2% fetal bovine serum. Separate sterile micropipettes were used between dishes, the total time in trypsin was 120 to 150 seconds, and ambient temperature for the treatments was 25 to 27°C. Ova were washed or trypsin treated and assayed in groups of 3 to 8.

Although the 4th wash was the last positive wash, an average of 18 plaque forming units (pfu) of virus was detected from each of 6 groups (24 total ova) after exposure to IBRV and washing. No IBRV was isolated from any of 9 trypsin-treated groups (43 total ova). The 7th wash was the last positive wash for any group after exposure of BHV-4, yet an average of 2 pfu of virus was detected from each of 6 groups (29 total ova) after washing. No BHV-4 was isolated from any of 8 trypsin-treated groups (43 total ova).

The study confirmed previous reports that IBRV adheres to the bovine ZP after in vitro exposure and that trypsin is effective for insuring freedom of ZP-I ova from the virus. Adherence of BHV-4 to ZP-I bovine ova was demonstrated for the first time. Trypsin-treatment was also effective in insuring freedom from this herpesvirus after in vitro exposure **(Stringfellow et al, Theriogenology 1990;34:427-434)**.

Bovine herpesvirus 5 (BHV-5)

Bovine (Bos indicus) herpesviruses have been associated with reproductive disease. Type 1, the most studied species, is best known for its reproductive and respiratory effects. Type 5 (BoHV-5) has been detected in bull semen and aborted fetuses but not in oocytes and embryos. This study consisted of three experiments that evaluated (1) BoHV-5-infected oocytes matured in medium with fetal bovine serum (BoHV-FBS) or polyvinyl alcohol (BoHV-

PVA) and fertilized by noninfected sperm; (2) noninfected oocytes fertilized by BoHV-5-infected sperm; and (3) infection of presumptive zygotes by BoHV-5. Each treatment involved nine drops of 15 to 20 oocytes. Infection with BoHV-5 was detected by polymerase chain reaction and in situ hybridization assay, and fertilization capacity and embryonic development were assessed using in vitro culture. Experimentally induced infection was obtained in all experiments, and vertical transmission of BoHV-5 by gametes was confirmed. The cleavage rate was reduced (P=0.0201) in BoHV-FBS (80.4+/-8.9%; mean+/-SD) compared with that of noninfected oocytes (89.9+/-6.5%); neither differed from BoHV-PVA (87.3+/-7.1%), and the resulting embryo production rate was not significantly different among groups. Rates of cleavage (87.5+/-7.5% vs. 92.2+/-5.5%, control vs. infected) and development of embryos (41.7+/-9.9% vs. 44.3+/-7.7% to morula/blastocyst/expanded blastocyst [M/B/EB] and 39.6+/-10.3% vs. 40.8+/-9.2% to blastocyst/expanded blastocyst/hatching blastocyst [B/EB/HB] stages) were not compromised by infected sperm (P=0.1462, P=0.5402, and P=0.8074, respectively). However, presumptive zygotes directly infected 1 d after fertilization produced a lower number (P=0.0140 to M/B/EB and P=0.002 to B/EB/HB stages) of in vitro-produced embryos (31.6+/-4.6 vs. 25.0+/-5.5 and 31.6+/-4.6 vs. 20.2+/-5.4; control vs. infected). In conclusion, BoHV-5 infected gametes and was transmissible to the embryo during in vitro development. As zygotes infected 1 d after fertilization had compromised development, BoHV-5 has the potential to be a pathogen with economic consequences.

Silva-Frade C, Martins A Jr, Borsanelli AC, Cardoso TC. Effects of bovine Herpesvirus Type 5 on development of in vitro-produced bovine embryos. Theriogenology. 2010 Feb;73(3):324-31.

The objective of this study was to optimize an internal control to improve SYBR-Green-based qPCR to amplify/detect the BoHV-5 US9 gene in bovine embryos produced in vitro and experimentally exposed to the virus. We designed an SYBR-Green-based binding assay that is quick to perform, reliable, easily optimized and compares well with the published assay. Herein we demonstrated its general applicability to detect BoHV-5 US9 gene in bovine embryos produced in vitro experimentally exposed to BoHV-5. In order to validate the assay, three different reference genes were tested; and the histone 2a gene was shown to be the most adequate for normalizing the qPCR reaction, by considering melting and standard curves ($p < 0.05$). On the other hand, no differences were found in the development of bovine embryos in vitro whether they were exposed to BoHV-5 reference and field strains comparing to unexposed embryos. The developed qPCR assay may have important field applications as it provides an accurate BoHV-5 US9 gene detection using a proven reference gene and is considerably less expensive than the TaqMan qPCR currently employed in sanitary programs.

Cardoso TC , Silva-Frade C , Táparo CV , Okamura LH , Flores EF. Validation of a reference control for an SYBR-Green fluorescence assay-based real-time PCR for detection of bovine *herpesvirus* 5 in experimentally exposed bovine embryos." *Molecular and cellular probes* 27.5 (2013): 237-242.

Bovine viral diarrhea virus (BVDV)

Ninety-six, day 5 to 7, ZP-I embryos, exposed to 10^4 - 10^5 $TCID_{50}$/ml BVDV (NADL strain) in vitro for 24 hrs at 37°C, then washed and assayed for infectivity by CPE in FBK cells and plaque assay in passaged FBK cells under an overlay containing MEM, 0.8% agar and 10% FBS, were found to be negative. The development of these embryos proceeded normally compared with controls **(Singh et al, Theriogenology1982;17:437-444).**

Twenty-one unfertilized ZP-I eggs and 8 ZP-I embryos, collected from 13 BVDV seropositive donors, were also found to be negative for infectivity using the above assay **(Singh et al, Theriogenology1982;17:437-444).**

Studies were designed to test the ability of a cytopathic strain (Singer) of BVDV to adhere to and/or penetrate the zona pellucida of 6-8 day old bovine embryos from susceptible parents after exposure to viral culture suspensions ($TCID_{50}$ 10^5/0.1ml) or infected bovine turbinate (BT) cultures of equivalent viral concentrations for 18-24 hours. After viral exposure, the embryos were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before (EM only) and after 5 washes (0.75ml) through cell culture medium **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68).**

On EM examination, prior to washing, four of 16 embryos exposed to virus suspension and two of 11 embryos exposed in BT cell culture had viral particles associated with the ZP. After 5 washes and overnight in the 5th wash, one of 11 embryos exposed to viral suspension and one of four embryos exposed in infected BT cell culture had viral particles associated with the ZP. In BT cell culture testing for CPE, after 5 washes, one of the 28 embryos was positive after exposure to virus suspension, and six of 20 embryos were positive after exposure to infected BT cell cultures **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68)**.

Seven embryos were microinjected with ? 50 picoliters ($TCID_{50}$ $10^{0.15}$) of virus, washed 10x (0.75ml/wash), placed in BT cell culture for 18-24 hrs, exposed to BVDV antiserum for 1 hr and then washed 5x. Cells were then removed with a micromanipulator from each embryo that had an intact ZP. Cells were placed in BT cell culture for 7 days, followed by three blind passages of 7 days if the primary culture showed no CPE. Some embryos were also examined by EM for viral particles. None of the embryos were positive on cell culture and none of five embryos examined by EM showed viral particles. However, the results must be viewed as inconclusive since less than one $TCID_{50}$ was in the ± 50 picoliters inoculum **(Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68).**

Fifty-two day 6.5 and 7.5 embryos were divided into 15 pools representing four groups (i) viable embryos with intact ZP (n = 10), (ii) viable embryos without ZP (n = 10), (iii) dead embryos with intact ZP (n = 14) and (iv) dead embryos without ZP (n = 16) for exposure to 10^4 TCID50/ml of a cytopathic (Singer) strain of BVD. There were at least three embryos/replicate. Following exposure, embryos were rinsed 3x by successive passages through 2ml of PBS with goat serum and then sonicated in 2ml of the same for virus isolation in bovine turbinate cells. No uptake of BVD was evident in any of the embryos on the first washes. A reduction in virus titre was noted when the virus was incubated for 24 hours at 37°C in PBS with goat serum or MEM with goat serum **(Potter et al, Am J Vet Res 1984;45:1778-1780).**

The in vitro effect of bovine viral diarrhoea virus (BVDV) on the survival of day 7 to day 7.5 bovine embryos collected from superovulated donors was studied. Fifty-four experimental embryos with the zona pellucida (ZP) intact, damaged or removed were exposed to 1 x 10^4 $TCID_{50}$/ml of the NADL cytopathic strain of BVDV at 37°C for for 24 hrs and compared to 36 control embryos that were cultured for 24hr. Seven embryos with the ZP removed were similarly exposed for 48 hrs and compared to five control embryos. The overall survival rate was 68% for embryos exposed to BVDV for 24 hrs and 77% for embryos not exposed ($p > 0.05$). Extended exposure of the embryos, with the ZP removed, to virus for 48 hrs did not affect their survival rate compared to controls. Damage to the ZP by cracking or total removal of the ZP by micromanipulation or acidic Tyrode's solution had no effect on subsequent embryonic survival in the presence of BVDV. It was concluded that exposure to BVDV in vitro is not cytopathic for morula and blastocyst stage bovine embryos over a 48 hr period, even when they are not protected by the ZP **(Bielanski and Hare, Vet Res Comm 1988;12:19-24).**

Seven day-old embryos from BVDV-seronegative heifers were co-cultured with a bovine embryo trophoblast (BET) cell line for 48h. and those progressing to expanded blastocysts were used as follows: a) uninoculated control group (n = 15), b) inoculated with cytopathic BVDV strain NADL (n = 15), c) inoculated with non-cytopathic (NCP) BVDV strain SD-1 (n = 15) and d) inoculated with NCP BVDV strain LR-91 (n = 15). Following blastocyst expansion and approx 12h. before hatching the wells containing the embryos were inoculated with 25µl virus stock containing $10^4 CCID_{50}$/ml. After hatching the diameter of the expanding blastocysts was measured daily for 14d. Cytopathic effect (CPE) was evident by day 2 in all hatched embryos in group b), but no CPE was observed in groups c) and d). Rates of blastocyst expansion in groups c) and d) were not different from those in group a). At hatching blastocyst diameter averaged 150µm but by day 14 post-hatching it had increased to 350µm. BVDV-specific antigen was detected by immunofluorescence at 14d post-hatching. However, despite the replication of NCP virus in the hatched blastocysts, no CPE was evident and expansion was unaltered. **(Brock and Stringfellow, Theriogenology 1993;39:196abstr).**

Twenty-two embryos, (8, ZP-I; 14 ZP-free), were collected on day 13 from 5 donors that had had 2ml of BVDV (Singer strain, 10^3 $TCID_{50}$/ml) inoculated into the right uterine horn on day 10 post estrum. Normal, hatched embryos were recovered from the uninfected left uterine horns; normal hatched embryos and degenerating ZP-I embryos were recovered from the infected horns. Electron microscopic (EM) examination of the embryos revealed BVD virus-like particles under the ZP of degenerating embryos; similar examination of embryos from the uninfected horns did not reveal virus-like particles. Virus isolation was negative on all uterine flushings from uninfected horns, but positive on all uterine flushings from infected horns **(Archbald et al, Theriogenology 1979;11:81-89).**

Two superovulated cows were inseminated with semen seeded with $TCID_{50}$ $10^{4.8}$/0.1ml BVDV (Singer strain) three times at 8 hour intervals after the detection of oestrus. One of the two donors inseminated with infected semen produced two degenerating embryos 6 days later and were placed on cell culture for 24 hours. No embryos were recovered from the

other cow. Microscopic lesions found in reproductive tissues from these cows included only mild or moderate diffuse purulent vaginitis. No lesions were present in the uteri. One embryo was processed for EM examination and cells from the other collected for culture following exposure of the intact embryo to BVD antiserum and 10 washes. During removal of blastomere cells by micromanipulation, the embryo ruptured and was placed on culture cells. No cytopathic effect was observed nor was viral nucleic acid detected in primary or first transfer cell cultures. EM examination of the intact embryo revealed no identifiable viral particles. The BVDV probe was used on primary cell cultures of tissues from this cow because of fungal contamination. No virus was detected by the probe technique on those cultures. Tissues from the second cow inseminated with semen containing BVDV were shown to contain virus through positive probe hybridization on cultures of cervix, uterine tube, and regional lymph node. Using the Singer strain of BVDV with endpoints of $TCID_{50}$ 10^{-6}/0.1ml in BT cell cultures, the sensitivity of the nucleic acid hybridization test (probe) was at least 10^2 logs more than that of the infectivity assay in cell culture **(Schlafer et al, Dtsch tierarztl Wschr 1990;97:68-72).**

A persistently infected Holstein heifer aged approximately 16 months was superovulated, inseminated with BVDV-negative semen and flushed for embryos by standard whole uterine body flush using Dulbecco's PBS containing BVDV-negative fetal bovine serum. Three unfertilized ova were obtained. The titre of BVDV in the 2 litres of flush fluid was 10^2 $CCID_{50}$/ml which corresponds to aprroximately 10^5 $CCID_{50}$/ml within the uterine environment. Titres of BVDV in other samples from this animal were: serum 10^6 $CCID_{50}$/ml, urine 10^4 $CCID_{50}$/ml, vaginal mucus 10^6 $CCID_{50}$/ml and faeces 10^4 $CCID_{50}$/g **(Brock et al, J Vet Diagn Invest 1991;3:99-100).**

A persistently infected heifer was superovulated, inseminated and flushed for embryos at 7 days. Six embryos, including one viable one were collected. BVDV was isolated from the fluids of the uterus. The one viable embryo was washed 10x, treated with trypsin then transferred to a heifer that was immune to BVDV. After a normal pregnancy a calf was born. The calf was not persistently infected with BVDV and it developed antibodies to BVDV before the age of 2 months, indicating a natural infection with BVDV and a normal immunity to the virus **(Wentink et al, Vet Rec 1991;129:449-450).**

Two persistently infected heifers were superovulated, inseminated with semen certified free from BVDV, and flushed for embryos at 5••• or 8 days. BVDV was found in the flushing medium from both heifers but not in the fluids from the 10th wash. Eight eggs/embryos were collected and after washing 10x six embryos were transferred to six heifers. Four heifers were pregnant at 35 days and two calved. Neither of the calves had BVDV or antibodies **(Bak et al, Vet Rec 1992;131:37).**

Virological and serological investigations for the detection of BVD virus and corresponding neutralizing antibodies were carried out in nearly 100 bull calves randomly selected following embryo transfer. The results revealed four calves persistently infected with BVDV in three different cattle breeding herds. Follow-up tests in one of these herds detected four additional calves persistently infected and also derived from embryo transfers. Furthermore, two contact calves were found exhibiting transient viraemia. The age of the persistently

infected animals when detected was eight days to five months. None of them showed any clinical signs or growth retardation during the whole of the observation period of five to ten months. As far as possible, virological and serological tests were performed in donor and recipient animals related to the persistently infected calves but BVDV was not isolated from any that were sero positive.
The hygienic risks of embryo transfer especially in relation to BVDV infections were discussed. Bulls and their possible involvement in the transmission of BVDV via embryo transfers drew particular attention. The role of donors and recipients was also considered. Finally control measures were proposed in order to reduce BVDV infection risks arising from embryo transfers. **(Liess et al, Dtsch tierarztl Wschr 1987;94:506-508).**

Four large US artificial insemination (AI) centres implemented a program of surveillance of resident and entering bulls for persistent bovine pestivirus (BVDV) infection. Twelve cases were identified among 1538 bulls. Ten of these were ET bulls. Several clinical abnormalities were evident among the persistently infected bulls, including acute and chronic mucosal disease. Semen produced by such bulls consistently contained BVDV, and such contamination was not always accompanied by diminished seminal quality. These animals were detected by virus isolation tests performed on blood specimens, but not by serological tests. Virological surveillance of breeding herds, AI and embryo transfer centres, and the cattle trade for persistently infected animals is necessary to prevent the spread of this virus by modern cattle breeding practices **(Howard et al, JAVMA 1990;196:1951-1955).**

Bos indicus heifers (n=169) and cows (n = 38) were artificially inseminated and the 51d and 210d pregnancy rates of those seropositive to BVDV at AI were compared with those that were seronegative. By day 51 after AI 70% and 32% of cows and heifers respectively of those previously seronegative had seroconverted, and between 51d and 210d 17% and 3% respectively had seroconverted. The 51d pregnancy rate of cows that were seropositive at AI was similar to that of cows which became infected and seroconverted around the time of AI. However, the pregnancy rate of the seropositive heifers (44%; n = 54) was significantly (P = 0.04) greater than the rate of the heifers which became infected and seroconverted around the time of AI (24%; n = 37). Between day 51 and day 210 only one cow aborted and this animal had been infected around the time of AI. However, of the seroconverting heifers 4 of 9 (44%) aborted versus 3 of 21 (14%) of the seropositive ones. These findings indicate that at least in heifers BVDV infection around the time of AI (or ET) may significantly reduce pregnancy rate and increase abortion rate **(McGowan et al, Theriogenology 1993;39:443-449).**

"Bovine viral diarrhea virus (BVDV) was isolated from 137 out of 639 commercial batches of bovine foetal serum. Virus isolation was made by inoculation of the serum in bovine embryo lung (BEL) cell culture. The isolates were identified by indirect immunofluorescence. Batches were considered negative for virus if no immunofluorescence was detected after three serial passages of the inoculated cells." **(Abraham, Israel J Vet Med 1993;48:120).**

Twelve cows and 13 heifers, all seronegative to BVDV, were programmed for superovulation, AI and embryo recovery. Nine days before AI half were given an intranasal inoculum of non-cytopathic BVDV while the remainder were left uninfected. BVD-free semen was used for

the AIs and the embryos were recovered non-surgically 7 d. after AI. One of the inoculated cows failed to become infected with BVDV so there were 12 animals in the infected group and 12 in the control group. Mean numbers of CLs, embryos/ova recovered and transferable embryos were 11.4, 4.6 and 2.7 respectively in the control group, and 3.0, 1.0 and 0.7 respectively in the infected group. It would appear that if active BVDV infection takes place just prior to ovulation there can be a dramatic reduction in the superovulatory response **(Kafi, et al, Theriogenology 1994;48:985-996).**

To assess the possibility of detecting BVDV in single oocytes and blastomeres 'pinched' from early embryos PCR primers specific for cellular actin transcripts have been used successfully to amplify RNA from the equivalent of 0.1 cells. With these levels of sensitivity, it is now possible to assess the BVDV infectivity of emrbyos from viraemic animals **(Brownlie, Ann Rep: Institute for Anim Health 1994;pp72-73).**

Experience gained in Drenthe province confirmed that calves born of carrier cows could themselves carry the virus. Three blind passages were needed to ensure that fetal calf serum (used in embryo transfer) was free from BVD pestivirus. Virus culture was needed to prove that serologically negative cattle were free from the virus **(Dijkstra et al. Tijdschrift-voor-Diergeneeskunde 1994;119:18).**

This study was done to examine the reproductive efficiency of embryo transfer donors that were persistently infected with bovine viral diarrhea virus (BVDV) and to determine the potential for vertical or horizontal transmission of BVDV during embryo transfer from persistently infected donors. The reproductive inefficiency of 7 different persistently infected donors was evident by consistent failure at superovulation and/or fertilization. Washing of embryos according to the recommendations of the IETS prevented adherence of BVDV to embryos and to unfertile and degenerated ova, as determined by virus isolation and PCR assay. In addition, a normal BVDV antibody seronegative and BVDV-negative calf was born following transfer from a PI donor to a seronegative recipient. **(Brock et al. Theriogenology 1997;47:837-844).**

Twenty-five Friesian heifers (n=12, negative controls; and n=13, artificially infected via intranasal inoculation of 2 ml of serum with 5.5 x10^5 $TCID_{50}$/ml of noncytopathic pestivirus 9 days prior to artificial insemination) were used in the study. All animals were treated for superovulation with standard, twice-daily injections of FSH-P and then were inseminated twice beginning 12 h after onset of estrus. Three of 13 pestivirus-infected heifers displayed signs of estrus while 10 of 12 control heifers showed estrus. Mean ova/embryos from controls was 5.75+2.31 (4.00+0.72 transferrable). Mean ova/embryos from infected heifers was 0.60+0.34 (0.23+0.22 transferrable). Ultrasonographic examination at 12 h after the first AI revealed that 82% and 17% of presumptive follicles had ovulated in the control and infected groups respectively. Conclusions included that pestivirus infection during the preovulatory could adversely affect ovulation and lead to reduced number of palpable Cls and number and quality of embryos recovered. **(Kafi et al. Theriogenology 1997;48:985-986).**

Ovaries of 5 BVDV-sero-negative and -virus-negative pubertal heifers were monitored ultrasonographically for 4 consecutive estrous cycles, noting position and size of follicles (>5mm) and luteal structures. Also, peripheral blood progesterone and estradiol levels were measured daily. The heifers were infected via intranasal inoculation with noncytopathic BVDV following ovulation in the second estrous cycle. After acute BVDV infection maximum diameter and rate of growth of dominant ovulatory and anovulatory follicles were reduced. Also, the number of subordinate follicles associated with both the anovulatory and ovulatory dominant follicles was reduced. There were no significant differences in other parameters, including peripheral progesterone and estradiol levels. Thus, ovarian follicular growth following acute infection with BVDV differed from growth prior to infection. **(Grooms DL et al. Theriogenology 1998;49:595-605).**

The objective of the study was to determine which ovarian cells were infected with BVDV after acute infection. Twelve heifers were acutely infected with noncytopathic BVDV. Ovaries were removed between 4 and 60 d after infection. Viral antigen was detected in macrophage-like cells and stromal cells in the ovarian cortex and oophoritis was observed from 6 to 60 days after infection. **(Grooms et al, J Vet Diagn Invest 1998;10:125-129)**

The purpose of the study was to determine if BVDV could be detected in the ovary after vaccination with modified-live-BVDV vaccine. In 2 trials, 2 heifers and 4 mature cows were vaccinated with modified-live vaccines and ovaries removed between 7 and 30 d after vaccination. Cytopathic BVDV was isolated from ovaries removed on days 8, 10 and 12. Bovine viral diarrhea viral antigen was detected between days 10 and 30 using immunohistochemistry. **(Grooms et al. J Vet Diagn Invest 1998;10:130-134)**

Objective of the study was to investigate the incidence of BVDV RNA in embryos/ova from persistently infected heifers. Friesian heifers (26-month-old and a 22-month-old) persistently infected with BVDV were used as were two negative control heifers. Animals were superstimulated, artificially inseminated, and embryos/ova collected nonsurgically. Embryos/ova were frozen at -80$_{o}$ C until PCR assay was conducted. Eight “normal” and 9 degenerated ova were recovered from the infected heifers. Twelve “normal” and one degenerated ovum were collected from the control heifers. A 299 bp RT-PCR product was detected from 3 degenerated ova and 2 “normal” embryos from infected heifers and from all positive controls for the assay. All other ova/embryos from infected and uninfected heifers, third washing solutions, and serum from control heifers were negative. When a second round of PCR was performed to obtain sufficient amplified products for restriction enzyme digestion, reamplified products were observed in the 2 “normal” embryos. Remaining samples (3 degenerated ova) appeared as “smears on the gel”. The RT-PCR fragments obtained from the 2 embryos were subjected to restriction endonuclease analysis, producing fragments of the expected sizes. Thus of 17 samples from the two persistently infected heifers, BVDV RNA was detected from only two “normal” embryos (a compact morula and an early blastocyst). They concluded that their results suggest that BVDV RNA was not detectable in almost all embryos/ova and that such embryos/ova remained uninfected. They indicated that positive samples could have been due to associated virus or actual infection, but that their study could not discriminate between the two. **(Tsuboi and Imada Vet Rec 1998;42:114-115)**

Batches of fetal bovine serum (FBS) harvested from abattoirs in Illinois, Minnesota and South Dakota were examined for BVDV and anti-BVDV antibody. Noncytopathic or cytopathic BVDV was isolated from 203 of 1000 lots tested. Analysis of viral isolates identified 115 type 1, 65 type 2 and 23 mixed (2 or more) isolates that were not typed. BVDV- neutralizing antibody was detected in 113 lots of FBS. Differential viral neutralization indicated that type 1 BVDV induced the antibody detected in 48 lots and type 2 BVDV induced the antibody detected in 16 lots of FBS **(Bolin SR and Ridpath JF, J Vet Diagn Invest 1998;10:135-139)**.

In a study designed to examine the effects of BVDV infection on estradiol, progesterone and PGF2α, 7 BVDV-free cows were challenged with non-cytopathic BVDV (strain Pe 515: 5 $x10^6$ $TCID_{50}$) with the peak of viremia occurring during early luteal phase of synchronized estrous cycles. Seven sham infected cows were also synchronized.
Between Day 4 and Day 9 post estrus, the BVDV-infected cows had significantly lower plasma estradiol levels than controls ($P<0.01$). However, the BVDV infection did not alter rectal temperatures, plasma progesterone concentrations or PGF2 α secretion at 17, 18 and 19 days after estrus. They concluded that data highlighted a potential causal link between BVDV viremia, endocrine dysfunction and poor fertility in the cow. **(Fray MD, et al., Theriogenology 1999;51:1533-1546)**

The tissue distribution and cellular localisation of bovine virus diarrhoea virus (BVDV) was investigated in the uterus, placentomes, intercotyledonary foetal membranes and foetal organs of three persistently infected (PI) pregnant heifers. The uterus and ovaries of a non-pregnant PI heifer were also included in the study. Cryostat sections were examined using immunohistochemical techniques and monoclonal antibodies against BVDV. A double immunofluorescence technique was used to identify BVDV positive cells that also showed staining for either the leukocyte common antigen CD45 or the cytoskeletal filament vimentin. BVDV antigen was detected in all the organs examined, and was present in both epithelial and non-epithelial cells. In all organs many of the virus-positive cells also showed reactivity for vimentin. In the foetal liver and spleen a small, scattered population of virus-positive cells showed reactivity for CD45. A few cells showed reactivity both for BVDV antigen and for CD45 in the placentomes and intercotyledonary foetal membranes. In contrast to earlier reports, only scattered cells in the foetal part of the placentomes, the cotyledons, showed reactivity for BVDV antigen. However, in the chorion of the intercotyledonary foetal membranes, a larger proportion of the trophoblast cells showed reactivity for BVDV, especially the binuclear trophoblast cells. In the uterus, pregnancy appeared to favour virus replication, as the section from the pregnant heifers showed much stronger staining and a higher proportion of viral antigen-positive cells than sections from the non-pregnant PI heifer. **(Fredriksen B et al, Vet Microbiol 1999;64:109-122)**

During the investigation of an outbreak of BVD in a dairy herd, a 17-month-old heifer of high genetic merit was found to be persistently infected with BVDV. The heifer was subjected to an embryo transfer program in an effort to conserve her genetics and to evaluate the effectiveness of washing procedures recommended by the IETS. After estrus induction, the heifer was treated for superovulation, artificially inseminated (BVDV-free semen), and embryos collected on Day 7 by nonsurgically flushing each uterine horn separately with 210

milliliters of Dulbecco medium containing sterile pluronic surfactant. Embryos were held until transfer in BVDV free medium and washed ten times (Dulbecco medium) according to the IETS protocol.
Bovine viral diarrhea virus was isolated from the flush fluid but not from the tenth wash. Despite a poor superovulatory response (one palpable CL), two grade 1 (IETS) blastocysts were recovered and transferred nonsurgically. The recipients were BVDV-antibody positive at the time of transfer. One recipient was found to be pregnant at 42 days and eventually gave birth to a bull calf that was free of BVDV and anti-BVDV antibody (pre-colostral sample). The donor heifer that was healthy at the time of embryo collection developed mucosal disease and died eight-months-later at 26 months of age. **(Smith AK et al, Vet Rec 2000;146:49-50)**

The report involved a Swedish recipient herd for frozen-thawed bovine embryos (established in 1997). All embryos transferred in the herd originate in the European Union, USA or Canada. Recipients are assembled at 13-18 months of age from certified BVDV free dairy herds. In addition, before entering the herd, each animal is tested twice for BVDV and anti-BVDV-antibody. Virus had not been isolated from any of the animals and only one had antibody on arrival. In May 1998, 15 recipients were tested as required prior to movement. One heifer (number 73) had seroconverted. In subsequent tests, three more antibody positive animals (out of 46) were found (numbers 9, 17, and 36). Four months earlier, heifers 9, 17 and 73 had received in vivo-derived embryos from the same mating and freezing. Heifers 9 and 73 did not conceive and number 17 aborted after 2 months. No gross pathology was noted in the fetus, but virus isolation was not attempted. Heifer 36 was pregnant, but she was found to have seroconverted before receipt of an embryo based on evaluation of a stored serum sample. All possible routes of infection were evaluated, and biosecurity precautions were confirmed to have been implemented correctly. The embryo is believed to have been contaminated by fetal bovine serum or have been from a persistently infected donor. The sire was confirmed to not be persistently infected. It is hypothesized that the fourth heifer (number 36) was infected by horizontal transmission. **(Lindberg A et al, 14th International Congress on Animal Reproduction, 2-6 July 2000, Volume I, p250 abstr.)**

This experiment was designed to determine if BVDV could compromise preovulatory follicle development in the cow. Combined CIDR and prostaglandin therapy was used to synchronize estrus in 13 Friesian cows. Nine days before the anticipated estrus (estrus = day 0), 6 cows were exposed to a noncytopathic isolate of BVDV (Pe515: 2.5 x 10^6 $TCID_{50}$/nostril). The other 7 cows served as negative controls. Follicular development was monitored by transrectal ultrasonography between Days 9 and 10. The BVDV exposure was followed by a significant leukopenia between days -5 and 0, and a viremia between days -4 and 1. Pyrexia was not observed. Clinical parameters for control cows were normal. The BVDV did not prevent ovulation, but it significantly extended the interval between CIDR withdrawal and ovulation and delayed the fall of plasma oestradiol at the end of the follicular phase. Presence of the BVDV did not alter the peak plasma oestradiol level recorded during the follicular phase. They concluded that BVDV could delay ovulation and prolong follicular phase oestradiol secretion, providing one method by which the virus compromises fertility in the cow. **(Fray MD et al, J Reprod Fertil 1999;abstr series 24:p6)**

Standard washing procedures that are recommended by the IETS for use on in vivo-derived embryos were confirmed to be effective for removal of a cytopathic strain (NADL) of BVDV after artificial exposure to the virus (Singh et al. Theriogenology 1982;17:437-444). However, the washing procedures had not been evaluated using other BVDV isolates including representative strains of noncytopathic virus. Thus, the objective of this research was to test washing procedures using an in vitro-in vitro approach with noncytopathic BVDV strains.
Over a 6 month period, embryos were collected nonsurgically from 32, BVDV-seronegative, superovulated cows. A total of 65 ZP-I morulae and blastocysts (MB) and 28 ZP-I, nonfertile and degenerated ova (NFD) were exposed to viral strain SD-1 (noncytopathic, genotype 1 BVDV). Also, a total of 64 ZP-I MB and 28 ZP-I NFD were exposed to viral strain CD-87 (noncytopathic, genotype 2 BVDV). After collection, all MB and NFD were washed and exposed for 1 h to either SD-1 or CD-87 (10^4 to 10^6 cell culture infective doses [50%] per ml). Following exposure, groups of e to 10 MB or NFD were washed using IETS guidelines except that 12 washes were used. Then groups of embryos were sonicated and sonicate fluids assayed for presence of BVDV using virus isolation (VI) and immunoperoxidase assay as well as reverse transcriptase nested polymerase chain reaction (RT-nPCR). The percentages of groups positive by either VI or RT-nPCR are shown in the following table.

	SD-1 (noncytopathic genotype 1 BVDV)		CD-87 (noncytopathic genotype 2, BVDV)	
	MB	NFD	MB	NFD
VI positive	25 % (2/8)	33 % (1/3)	0 % (0/8)	0 % (0/5)
RT-nPCR positive	25 % (2/8)	67 % (2/3)	0 % (0/8)	0 % (0/5)

Results of this study indicate that IETS procedures for washing will be more effective for some isolates of BVDV than for others. This also was described in a recent report of a study in which multiple isolates of BVDV were artificially introduced during IVF embryo production (Givens et al. Theriogenology 2002:54:1093-1107). It is possible that the use of trypsin treatment would ensure freedom of in vivo-derived embryos from isolates that remain associated despite washing. Further, it remains to be determined if an intrauterine infective dose of a high affinity isolate of BVDV might ever be associated with individual washed embryos. **(Waldrop JG et al. Theriogenology 2002;57:575 abstr.)**

In this letter to the Veterinary Record, authors reported the “first definitive detection” of a bovine viral diarrhea virus (BVDV) genotype 2 strain in British cattle. The type 2 viral genome was detected in a blood sample from a 9-month-old bull, which had presented with chronic diarrhea and weight loss. The bull was described as “likely to have been persistently infected”, as it died before this diagnosis could be confirmed. There was no history of disease signs compatible with BVDV on the farm before or after this case. The bull in question was the result of embryo transfer, having been imported as an embryo from North America in December 2000. Phylogenetic analysis of the viral genome revealed it to be very similar to a low-pathogenic strain from Nebraska, USA. Further analysis of other cattle present at the same time revealed than a 12-month-old animal was also infected with the virus. It is emphasized that they had not confirmed the source of the virus but considered the most likely origin was to be via the ET process. They commented that fetal bovine serum

used in embryo transfer might be a source. **(Drew TW et al. Veterinary Record 2002;151:551)**

Bovine viral diarrhoea virus (BVDV) is a major pathogen of cattle and is responsible for considerable reproductive loss. In this study, the in vivo responses in six multiparous cows were investigated after a non-cytopathogenic BVDV challenge (strain Pe 515; 5 x 10(6) tissue culture infective dose 50) given 9 days before a synchronized ovulation. Six similar cows challenged with non-infectious culture medium served as controls. The experimental noncytopathogenic BVDV infection was followed by a viraemia and leucopenia at days 5-9 after challenge, but no other clinical signs of infection were detected. However, the BVDV infection altered endocrine function. Mean LH pulse frequency immediately before CIDR withdrawal was lower (P < or = 0.05) in the BVDV-infected (2.17 +/- 0.34 pulses per 8 h) compared with the sham-infected (4.83 +/- 1.04 pulses per 8 h) animals. At day 3 after CIDR withdrawal, plasma oestradiol concentrations remained high (P < 0.05) in the infected cows [2.19 +/- 0.51 pg ml(-1)] compared with the sham-infected controls (0.72 +/- 0.29 pg ml(-1)). However, there was no difference in the peak oestradiol concentration [BVDV: 2.31 +/- 0.29 versus sham: 2.34 +/- 0.41 pg ml(-1)]. In addition, non-cytopathogenic BVDV significantly (P < 0.05) increased the duration of the interval between ovulation and onset of the postovulatory progesterone increase [values 1.0 ng ml(-1)] (BVDV: 3.0 +/- 0.26 versus sham: 4.0 +/- 0.26 days). The viral infection also significantly (P < 0.01) decreased mean plasma progesterone concentrations between day 3 and day 11 after ovulation [BVDV: 2.59 +/- 0.32 versus sham: 4.13 +/- 0.27 ng ml(-1)]. These data show that non-cytopathogenic BVDV viraemias during the follicular phase can modulate the secretion of gonadotrophins and sex steroids, in particular progesterone, during a synchronized oestrous cycle. Therefore, viraemias during the follicular phase may reduce the fertility of cattle by disrupting the capacity of the ovulatory follicle to form a competent corpus luteum, thereby compromising early embryo development and maternal recognition of pregnancy. **(Fray MD et al. Reproduction 2002;123:281-289)**

Two experiments (Experiment I, n=12 Holstein-Friesian heifers; Experiment II, n=8 Jersey cows) were conducted to investigate the pathogenesis of bovine pestivirus-induced ovarian dysfunction in cattle. In both experiments, the cattle were superovulated with twice daily injections of a porcine pituitary extract preparation of follicle stimulating hormone (FSH-P),for 4 days commencing on Day 10+/-2 after a presynchronised oestrus. The heifers received a total dose of 30 mg and the cows 32 mg of FSH-P. Prostaglandin F(2alpha) [PGF(2alpha)] was administered 48 h after commencement of superovulation and all cattle were artificially inseminated (AI) between 48 and 66h after PGF(2alpha) treatment. In both experiments, bovine pestivirus seronegative cattle (Experiment I, n=6; Experiment II, n=4) were inoculated intranasally with an Australian strain of non-cytopathogenic bovine pestivirus (bovine viral diarrhoea virus Type 1) 9 days prior to AI. Bovine pestivirus infection was confirmed by seroconversion and/or virus isolation in all of the inoculated cattle, consistent with a viremia occurring approximately between Day 5 prior to AI and the day of AI. Ovarian function was monitored in both experiments by daily transrectal ultrasonography and strategic blood sampling to determine progesterone, oestradiol-17beta, luteinising hormone (LH) and cortisol profiles. Non-surgical ova/embryo recovery was performed on Day 7 after AI. In Experiment II, half the cattle were slaughtered on Day 2 and the remainder

on Day 8 after AI, and the ovaries submitted for gross and histopathological examination including immunohistochemistry to demonstrate the presence of bovine pestivirus antigen. In both studies, comparisons were made between infected and confirmed uninfected (control) animals. Overall, the bovine pestivirus infected cattle had significantly lower ($P<0.05$) ova/embryo recovery rates compared to the control cattle. There was evidence of either an absence (partial or complete) of a preovulatory LH surge or delay in timing of the LH peak in the majority (90%) of infected heifers and cows, and histologically, there was evidence of non-suppurative oophoritis with necrosis of granulosa cells and the oocyte in follicles from the infected cows. By contrast, only 20% of the control heifers and cows had evidence of absence of a pre-ovulatory LH surge. These experiments collectively demonstrate that bovine pestivirus infection during the period of final growth of preovulatory follicles may result in varying degrees of necrosis of the granulosa cells with subsequent negative effects on oestradiol-17beta secretion which in turn negatively affects the magnitude and/or timing of the preovulatory LH surge. **(McGowan MR et al. Theriogenology 2003;1051-1056)**

Washing procedures (without trypsin treatment) recommended by the International Embryo Transfer Society (IETS) for use on in vivo-derived embryos effectively removed a cytopathic strain (NADL) of bovine viral diarrhea virus (BVDV) after artificial exposure. However, these washing procedures have not been evaluated using other isolates of BVDV, including representative non-cytopathic strains. Thus, the objective of this study was to evaluate the efficacy of the IETS procedures following artificial exposure of in vivo-derived bovine embryos to two different strains and biotypes of BVDV. One hundred and twenty-nine zona pellucida-intact (ZP-I) morulae and blastocysts (MB) and 56 non-fertile and degenerated (NFD) ova were collected 7 days following exposure to bulls from 32, BVDV-negative, superovulated cows. After collection, all MB and NFD ova were washed according to IETS standards. Subsequently, half of the MB and NFD ova were exposed for 1h to approximately 10_6 cell culture infective doses (50% endpoint) per milliliter of viral strain SD-1, and the other half were exposed to the same concentration of CD-87. After exposure, groups of >3 and <10 MB or NFD ova were washed using methods that met or exceeded IETS standards. Then, the washed groups were sonicated, and sonicate fluids were assayed for presence of virus using virus isolation and a reverse transcription nested polymerase chain reaction. No virus was detected in any group of MB or NFD ova that had been exposed to the CD-87 isolate. However, virus was detected in association with 50% of the groups of MB and 33% of the groups of NFD ova that had been exposed to the SD-1 isolate. Therefore, standard embryo-washing procedures recommended by the IETS are more effective for removal of some isolates of BVDV than for others. It remains to be determined if the quantity of a high-affinity isolate of BVDV associated with individual washed embryos would infect recipients via the intrauterine route. Further, it should be determined if an alternative embryo processing procedure, washing and trypsin treatment, would be more effective for removal of high-affinity isolates. (**Waldrop JG, et al. Theriogenology 2004;62:45-55.)**

Early research indicated that bovine viral diarrhea virus (BVDV) would not adhere to zona pellucida-intact (ZP-I), in vivo-derived bovine embryos. However, in a recent study, viral association of BVDV and in vivo-derived embryos was demonstrated. These findings raised questions regarding the infectivity of the embryo-associated virus. The objectives of this

study were to evaluate the infectivity of BVDV associated with in vivo-derived bovine embryos through utilization of primary cultures of uterine tubal cells (UTC) as an in vitro model of the uterine environment and to determine if washing procedures, including trypsin treatment, were adequate to remove virus from in vivo-derived embryos. One hundred and nine ZP-I morulae and blastocysts (MB) and 77 non-fertile and degenerated (NFD) ova were collected on day 7 from 34, BVDV-negative, superovulated cows. After collection, all MB and NFD ova were washed according to International Embryo Transfer Society (IETS) standards and exposed for 2h to approximately 10^6 cell culture infective doses (50% endpoint) per milliliter of viral strain SD-1. Following exposure, some groups of <10 MB or NFD ova were washed in accordance with IETS standards. In addition, an equivalent number of MB and NFD ova were subjected to IETS standards for trypsin treatment. Subsequently, NFD ova were immediately sonicated and sonicate fluids were assayed for presence of virus, while individual and groups of MB were placed in microdrops containing primary cultures of UTCs and incubated. After 3 days, embryos, media, and UTCs were harvested from each microdrop and assayed for BVDV. Virus was detected in the sonicate fluids of 56 and 43% of the groups of NFD ova that were washed and trypsin-treated, respectively. After 3 days of microdrop culture, virus was not detected in media or sonicate fluids from any individual or groups of MB, regardless of treatment. However, virus was detected in a proportion of UTC that were co-cultured with washed groups of MB (30%), washed individual MB (9%) and trypsin treated individual MB (9%), but no virus was detected in the UTC associated with groups of trypsin-treated embryos. In conclusion, virus associated with developing embryos was infective for permissive cells. Further, the quantity of virus associated with a proportion of individual embryos (both washed and trypsin treated) was sufficient to infect the UTC. In light of these results, an attempt should be made to determine if the quantity of a high-affinity isolate of BVDV associated with an individual embryo would infect recipients via the intrauterine route. (**Waldrop JG, et al. Theriogenology 2004;62:387-397.)**

The objective of this study was to determine if the quantity of a high affinity isolate of BVDV that remains associated with single washed or trypsin-treated embryos is sufficient to cause infection in vivo. Twenty ZP-I, Day 7 morulae and blastocysts (MB) were collected from superovulated cows. After collection, all MB were washed according to IETS standards, and all except 4 (negative controls) were exposed for 2 hours to approximately 10^6 $CCID_{50}$/ml of viral strain SD-1. Following exposure, one-half of the MB were washed and one-half were trysin-treated according to IETS standards. All MB were then individually sonicated, and sonicate fluids were injected intravenously into seronegative calves. Blood was collected from each calf on Days 0, 3, 6, 9, 12, 15, 20, 25 and 30, and sera were assayed for BVDV and anti-BVDV antibodies. All cattle used in the study were determined to be virus- and antibody-negative 30 days prior to the day of intravenous inoculation of sonicate fluids into the calves. Viremia was not detected in any calf following injection, possible due to intermittent sampling and/or small amount of embryo associated virus present. However, seroconversion of 38 and 13 % of the calves occurred following injection of sonicate fluids from washed and trypsin-treated embryos, respectively. Findings demonstrated that the quantity of a high affinity isolate of BVDV associated with single washed or trypsin-treated embryos is sufficient to be infective in vivo as evidenced by seroconversion. These results emphasize the need for studies to determine if the virus associated with exposed individual

embryos constitutes an infective dose when placed into the uterus. (**Waldrop JG, et al. Reproduction, Fertility and Development 2004;16:215)**

During routine genotyping of BVDV isolates in the United Kingdom, a type 2 isolate was identified. This isolate was characterized and possible sources of this type 2 virus (which is rare in UK) were discussed. This paper represents an elaboration of an earlier preliminary report (Drew TW, et al. Vet Record 2002;151:551) which seemed to incriminate embryo transfer as the likely source of introduction of the virus. Unfortunately, the earlier report is not referenced in this paper. In the current report, it is clarified that there were multiple possible sources for introduction of the virus (including importation of post natal animals from another country) and that it is unlikely that the embryos themselves were infected. **(Wakeley PR et al. Veterinary Microbiology 2004;102:19-24.)**

The objective of this study was to investigate whether the use of imported embryos for transfer is a risk factor for BVDV infection in Swedish cattle herds. Eight-hundred herds where ET had been performed between 1 January 1995 and 30 June 2002 where included in the study. In case herds, persistently infected animals had been identified during the study period (n=267). The association between exposure to imported embryos and BVDV status was investigated using logistic regression. Additional covariates were herd size, herd type, prevalence of BVDV in the area and number of years outside of the eradication scheme. There were significant effects of herd size, prevalence and number of years the herd had been outside the scheme, all proxies for well-known risk factors to BVDV. The effect of being exposed to imported embryos was not significant. **(Lindberg A. Proceedings of the Xth International Symposium for Veterinary Epidemiology and Economics, Vina del Mar, Chile. November 2003:17-21.)**

Two recent studies demonstrated that a high-affinity isolate of BVDV (SD-1), remained associated with a small percentage of in vivo-derived bovine embryos following artificial exposure to the virus and either washing or trypsin treatment. Further, the embryo-associated virus was infective in an in vitro environment. Therefore, the objective of this study was to determine if the quantity of a high-affinity isolate of BVDV associated with single-washed or trypsin-treated embryos could cause infection in vivo. Twenty zona-pellucida-intact morulae and blastocysts (MB) were collected on day 7 from superovulated cows. After collection, all MB were washed according to International Embryo Transfer Society (IETS) standards, and all but 4 MB (negative controls) were exposed for 2h to 10(5)-10(6) cell culture infective doses (50% endpoint) per milliliter (CCID (50)/mL) of viral strain SD-1. Following exposure, according to IETS standards, one half of the MB were washed and one half were trypsin treated. All MB were then individually sonicated, and sonicate fluids were injected intravenously into calves on day 0. Blood was drawn to monitor for viremia and (or) seroconversion. Seroconversion of calves injected with sonicate fluids from washed and trypsin-treated embryos occurred 38% and 13% of the time, respectively. Therefore, the quantity of a high-affinity isolate of BVDV associated with single-washed or trypsin-treated embryos was infective in vivo. **(Waldrop JG, et al. Theriogenology 2005;65:594-605.)**

Because of its broad distribution among populations of cattle and its association with materials of animal origin used in embryo production, bovine viral diarrhea virus (BVDV) is a

potential problem in applications of embryo technologies. While some isolates of BVDV are known to associate with both *in vivo*-derived and *in vitro*-produced bovine embryos, it has yet to be determined if the quantity of virus associated with exposed zona pellucida-intact embryos is sufficient to infect susceptible recipient cows via the intrauterine route. Techniques to detect and quantify BVDV associated with single transferable embryos are important to determine the risk of transmitting BVDV via embryo transfer. The objectives of this study were to define reproducible techniques to detect and quantify BVDV associated with single or small groups of bovine embryos contained in small aliquots of medium using virus isolation (VI) or real time quantitative polymerase chain reaction (Q-PCR) assays. *In vivo*-derived and *in vitro*-produced embryos were exposed for 2 h to approximately 10_6-cell culture infective doses (50% endpoint) per mililiter of a high affinity strain of BVDV, SD-1, and then washed according to IETS guidelines. Embryos were assayed in groups of five or two embryos, or single. There were 5 replicates of the group of five embryos, 4 of the group of two embryos, and 3 of the single embryos for the *in vivo*-derived embryos undergoing VI; 5, 4, and 2 replicates, respectively, undergoing Q-PCR, and 2, 5, and 2 replicates, respectively, for the *in vitro*-produced embryo groups undergoing VI and Q-PCR. Those to be assayed byVI were sonicated and the sonicate fluids were layered onto Madin Darby Bovine Kidney (MDBK) cells and passaged to allow for viral replication; an immunoperoxidase monolayer assay was then used for viral detection. A Roche® RNA/DNA extraction kit (Roche Diagnostic Systems, Inc., Somerville, NJ, USA) was used to extract RNA from virally exposed embryos, and extracted samples were assayed in duplicate Q-PCR reactions consisting of 100µL. The primers used were L1 and U3 which are specific for conserved areas of the 5 prime nontranslated regions of the viral genome of BVDV. The PCR product was detected using hybridization probes s1 and s2 as in Struder *et al*. 2002
Biologicals 40, 289–296. *In vivo*-derived groups of five or two embryos, or single embryos, were positive for BVDV 100, 50, and 30% of the time, respectively, when VI was used and 100, 75 and 100%, respectively, when Q-PCR was used. The virus was detected in all of the *in vitro*-produced embryo groups of five, or two embryos, or single embryos, 100% of the time using VI, and in 100, 80, and 100% respectively, using Q-PCR. The virus isolation technique is highly sensitive but the need to destroy embryos by sonication to identify any embryo-associated virus precludes its use for embryos intended for transfer. Techniques for Q-PCR were sufficiently sensitive to detect and quantify 10 copies of RNA in a sample and to detect BVDV associated with single embryos. (**Waldrop et al, Theriogenology 2006;18:214-215 (abstr.))**

The objectives of this study were to develop techniques to detect BVDV associated with single or small groups of bovine embryos contained in small aliquots of medium using either virus isolation (VI) or real time quantitative polymerase chain reaction (RT-QPCR) assays. In vivo-derived and in vitro-produced bovine embryos at 7 d post-fertilization were exposed to SD-1, a high affinity strain of BVDV, for 2 h and then processed according to the International Embryo Transfer Society (IETS) guidelines prior to testing. Groups of five or two in vivo-derived embryos, and single in vivo-derived embryos, were VI positive for BVDV 100, 50, and 33% of the time, and were RT-QPCR positive 100, 75, and 42% of the time, respectively. The virus was detected by the VI technique in all of the groups of five or two in vitro-produced embryos and in all of the single in vitro-produced embryos, and it was detected in 100, 80, and 50%, using RT-QPCR. Techniques for RT-QPCR were sufficiently sensitive to detect 10

copies of viral RNA in a sample and to detect BVDV associated with single embryos. Application of this new technology, RT-QPCR, will facilitate additional studies to further assess the risk of transmission of BVDV through embryo transfer. (**Gard JA, et al. Theriogenology, 2007; 67:1415-1423.)**

Quantitation of bovine viral diarrhea virus (BVDV) associated with individual transferable embryos is prerequisite to a thorough assessment of the risk for transmission of BVDV via embryo transfer. One objective of this study was to determine the proportion of *in vivo*-derived bovine embryos that remained virus-positive after artificial exposure to a high-affinity strain of BVDV and thorough washing. A second objective was to determine the quantity of virus associated with these individual embryos. A total of 87 zona pellucida-intact, Day 7, *in vivo*-derived bovine embryos were exposed to a type 1 noncytopathic strain of BVDV (SD-1) and washed according to International Embryo Transfer Society recommendations. Subsequently, individual embryos were sonicated, and the RNA was extracted from the sonicate fluids and stored at -80°C until assayed using a real-time quantitative polymerase chain reaction (QPCR). Twenty-six percent (23/87) of the embryos contained virus. The average quantity of virus associated with individual embryos after viral exposure and washing was 1.12 viral copies per 5 µL (SD = 1.57 copies 5 per μL^{-1}; SEM = 0.33 copies 5 per μL^{-1}). Assessment of data using tolerance intervals (P = 0.05) indicates that 90% of contaminated embryos will be associated with ≤4.64 viral copies per 5 µL, whereas 99% of contaminated embryos will be associated with ≤6.62 copies per 5 µL. Obviously, only extremely small quantities of virus were associated with less than one-third of the embryos tested. Based on previous research, it is presumed that this virus is associated with the outer layers of the zona pellucida. A logical next step in the risk assessment would be to determine if these quantities of zona-associated virus are sufficient to infect naïve recipients and/or embryonic cells after embryos are transferred. Further, similar efforts should be made to estimate the quantity of virus associated with *in vitro*-derived, zona pellucida-intact, bovine embryos after exposure to the same high-affinity strain of virus and washing. (**Gard JA, et al. Reproduction, Fertility and Development, 2007;19:233 (abstr))**

Bovine diarrhea virus (BVDV) causes a variety of economically important enteric and infertility problems. For that reason, several countries have eradicated the disease and some others have schemes in progress to achieve freedom from it. Although there is a considerable amount of information about the risk of BVDV transmission through contaminated semen used for AI, there is no available evidence to indicate whether the resulting embryos, when used for embryo transfer (ET), can lead to the transmission of BVDV to recipients and offspring. For this experiment, semen from a bull persistently infected with BVDV (10^5 $TCID_{50}$/mL of NY strain) was used for insemination (2 times at estrus) of BVDV-seronegative, superovulated heifers (n = 27).All heifers seroconverted to BVDV within 10 days post-insemination. Embryos and unfertilized oocytes were collected nonsurgically (n = 92) or postmortem (n = 52) 7 days post-insemination and were either washed according to IETS recommendations (without trypsin treatment) or left unwashed. In total, out of 144 unfertilized oocytes and embryos collected, 23 (16%) were of ET quality. Most of the embryos were degenerated or unfertilized. On 17 occasions, 1 or 2 washed embryos were transferred to BVDV-seronegative recipients. After ET, all pregnant and nonpregnant recipients remained free of BVDV and antibodies. In total, 6 heifers became

pregnant and 5 calves free of BVDV and BVDV antibodies, including 2 sets of twins, have been born to date. Post-insemination, BVDV was detected in 29% (12/41) of unwashed and 10% (4/40) of washed embryos, 100% (4/4) of follicular fluid samples, oviductal epithelial cells, endometrium, and corpora lutea tissues as determined by the virus isolation test. Results herein suggest that BVDV can be transmitted by AI, resulting in the production of some proportion of contaminated embryos. However, it appears that such embryos, when washed according to the IETS guidelines, do not cause BVDV transmission to recipients or their offspring.

Bielanski A, Lalonde A and Algire J. Transmission of noncytopathic bovine diarrhea virus by infected semen to embryo donors and by embryos to embryo transfer recipients and offspring. Reproduction, Fertility and Development. 2010; 22(1): 251 (abstr).

The aim of the present study was to evaluate the development and ultrastructure of preimplantation bovine embryos that were exposed to bovine viral diarrhea virus (BVDV) in vitro. The embryos were recovered from superovulated and fertilized Holstein-Friesian donor cows on day 6 of the estrous cycle. Compact morulae were microinjected with 20 pl of BVDV suspension (10(5.16) TCID(50)/ml viral stock diluted (1:4) under the zona pellucida (ZP), then washed in SOF medium and cultured for 24-48 h. Embryos were evaluated for developmental stages and then processed immunocytochemically for the presence of viral particles, using fluorescent anti-BVDV-FITC conjugate. Ultrastructure of cellular organelles was analysed by transmission electron microscopy (TEM).After microinjection of BVDV under the ZP, significantly more ($p<0.001$) embryos (83.33%) were arrested at the morula stage compared with the intact control (30.33%). Immunocytochemical analysis localized the BVDV-FITC signal inside the microinjected embryos. TEM revealed: (i) the presence of virus-like particles in the dilated endoplasmic reticulum and in cytoplasmic vacuoles of the trophoblast and embryoblast cells; (ii) the loss of microarchitecture: and (iii) abnormal disintegrated nuclei, which lacked reticular structure and the heterochromatin area. In all, the embryo nuclear structure was altered and the microarchitecture of the nucleolus had disappeared when compared with the nuclei from control embryos. Dilatation of the intercellular space and the loss of the intercellular gap junctions were often observed in bovine BVDV-exposed embryos. These findings provide evidence for the adverse effect of BVDV virus on the development of bovine embryos, which is related to irreversible changes in the ultrastructure of cell organelles.

Kubovicová E, Makarevich AV, Pivko J, Chrenek P, Grafenau P, Ríha L, Sirotkin AV, Louda F. Alteration in ultrastructural morphology of bovine embryos following subzonal microinjection of bovine viral diarrhea virus (BVDV). Zygote. 2008;16(3):187-93.

The objective was to determine the average amount of bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced bovine embryos following recommended processing procedures for embryos. In vivo-derived and in vitro-produced bovine embryos at 7d post-fertilization were exposed (for 2h) to 2 x 10(5-7) cell culture infective dose (CCID(50))/mL of SD-1 (a noncytopathic, Type 1a strain of BVDV), and then washed according to International Embryo Transfer Society (IETS) guidelines prior to testing. Of the 87 in vivo-derived embryos tested, 27% were positive for virus by quantitative polymerase chain reaction (qPCR). The range in amount of virus associated with 99% of the contaminated embryos was <or=6.62+/-1.57 copies/5 microL; 90% of the contaminated

embryos had <or=4.64+/-1.57 viral copies/5 microL of embryo-associated virus, using tolerance intervals (P<0.05). The SEM was 0.33 and the mean of averages was 1.12/5 microL. Of the 87 in vitro-produced embryos, 42% were positive for virus. The range in amount of virus associated with 99% of the contaminated embryos was <or=3.44+/-0.89 copies/5 microL; 90% of the contaminated embryos had <or=2.40+/-0.89 viral copies/5 microL of embryo-associated virus using tolerance intervals (P<0.05; S.E.M. was 0.14 and the mean of averages was 0.55/5 microL). Therefore, although many embryos were positive for virus, there were limited numbers of copies, thereby posing doubt regarding their potential for contamination following embryo transfer.

Gard JA, Givens MD, Marley MS, Galik PK, Riddell KP, Stringfellow DA, Zhang Y, Edmondson MA. Bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced preimplantation bovine embryos following artificial exposure. Theriogenology. 2009;71(8):1238-44.

Bovine viral diarrhea virus (BVDV) has been shown to be associated with single transferable in vivo-derived bovine embryos despite washing and trypsin treatment. Hence, the primary objective was to evaluate the potential of BVDV to be transmitted via the intrauterine route at the time of embryo transfer. In vivo-derived bovine embryos (n=10) were nonsurgically collected from a single Bos taurus donor cow negative for BVDV. After collection and washing, embryos were placed into transfer media containing BVDV (SD-1; Type 1a). Each of the 10 embryos was individually loaded into an 0.25-mL straw, which was then nonsurgically transferred into the uterus of 1 of the 10 seronegative recipients on Day 0. The total quantity of virus transferred into the uterus of each of the 10 Bos tarus recipients was 878 cell culture infective doses to the 50% end point (CCID(50))/mL. Additionally, control heifers received
1.5 x 10(6) CCID(50) BVDV/.5 mL without an embryo (positive) or heat-inactivated BVDV (negative). The positive control heifer and all 10 recipients of virus-exposed embryos exhibited viremia by Day 6 and seroconverted by Day 15 after transfer. The negative control heifer did not exhibit a viremia or seroconvert. At 30 d after embryo transfer, 6 of 10 heifers in the treatment group were pregnant; however, 30 d later, only one was still pregnant. This fetus was nonviable and was positive for BVDV. In conclusion, the quantity of BVDV associated with bovine embryos after in vitro exposure can result in viremia and seroconversion of seronegative recipients after transfer into the uterus during diestrus.

**Gard JA, Givens MD, Marley MS, Galik PK, Riddell KP, Edmondson MA, Rodning SP.
Intrauterine inoculation of seronegative heifers with bovine viral diarrhea virus concurrent with transfer of in vivo-derived bovine embryos.
Theriogenology 2010 May;73(8):1009-17. Epub 2010 Feb 2**

Bovine diarrhea virus (BVDV) causes a variety of economically important enteric and infertility problems in cattle. For that reason, several countries have eradicated the disease, and some others have schemes in progress to achieve freedom. Although there is a considerable amount of information about the risk of BVDV transmission through contaminated semen used for artificial insemination (AI), there is no evidence to indicate whether the resulting embryos, when used for embryo transfer, can lead to the transmission of BVDV to recipients or offspring. For this experiment, semen from a bull persistently infected with BVDV (10(5) 50% tissue culture infective doses/mL NY strain) was used for

insemination (two times at estrus) of BVDV-seronegative, superovulated cows (N = 35). Embryos were collected 7 days after insemination and subsequently were washed according to the International Embryo Transfer Society recommendations or left unwashed. Out of 302 collected oocytes and embryos, 173 (57%) were fertilized and the remaining 129 (43%) had degenerated. Infectious BVDV was detected in 24% (17/71) of unwashed and 10% (8/77) of washed embryos, and in all (N = 11) follicular fluid samples, oviductal epithelial cells, endometrium, and corpora lutea tissues as determined by the virus isolation test. After transfer of 39 washed embryos to 27 BVDV-seronegative recipients, 12 (44%) cows became pregnant and 17 calves free of BVDV and BVDV antibodies, including five sets of twins, were born. After embryo transfer, all pregnant and nonpregnant recipients remained free of BVDV and antibodies. In conclusion, results herein suggest that BVDV can be transmitted by AI resulting in the production of some proportion of contaminated embryos. However, it appears that such embryos, when washed according to International Embryo Transfer Society and the World Organization for Animal Health guidelines do not cause BVDV transmission to recipients or their offspring.
Bielanski A, Algire J, Lalonde A, Garceac A. Embryos produced from fertilization with bovine viral diarrhea virus (BVDV)-infected semen and the risk of disease transmission to embryo transfer (ET) recipients and offspring. Theriogenology. 2013 Sep 15;80(5):451-5.

Parvovirus (BPV)

ZP-free morulae exposed to BPV in culture developed normally compared to control morulae. Electron microscopic studies showed no evidence of viral replication 24 and 40 h. after exposure to BPV. **Bowen et al, Theriogenology 1978;9:88 (abstr**).

Akabane virus (AV)

Eighty, day 5 to 7, ZP-I embryos, exposed to 10_4 to 10_6 pfu/ml of AV (Plum Island) for 1 to 24 h. and then washed 10 times, were negative when assayed using a plaque assay in Vero cells under an overlay of MEM, 0.8% agar and 4% FBS (**Singh et al, Theriogenology 1982;17:437-444**).

Exposure to AV had no apparent deleterious effects on in vitro embryonic development compared to controls (**Singh et al, Theriogenology 1982;17:437-444**).

Foot and mouth disease virus (FMDV)

One hundred and sixty-nine, day 6 to 8, ZP-I embryos were exposed to 10_6 pfu/ml FMDV (Type 0_1 strain) for 4-18 hrs, washed 10 times and then assayed by plaque assay and steer tongue inoculation. All were negative for FMDV. Embryonic development in vitro was not affected by exposure to FMDV. Forty-two, day 12, ZP-free (hatched) embryos were exposed to approximately 10_6 pfu/ml FMDV for 2 hrs, washed and then assayed. Thirty-five per cent were positive for virus after washing (**Singh et al, Theriogenology 1986;26:587-593**).

Studies to determine if ZP-I embryos collected from eight FMDV infected donors carried infectious virus after washing gave negative results based on 48 embryos tested in tissue

culture and by steer tongue inoculation. In a second experiment donors were infected with FMDV and allowed to recover. Forty-two embryos were collected from these donors 21 days post FMDV-infection. All of the embryos were negative for FMDV in both tissue culture and steer tongue inoculation tests (**McVicar et al, Theriogenology 1986;26:595-601**).

FMDV was recovered from uterine flush fluids from 12 out of 16 FMDV viremic donors (**McVicar et al, Theriogenology 1986;26:595-601**).

Four hundred and thirty-six embryos and/or unfertilized ova were collected at slaughter from 30 superovulated cattle. These donor cattle were infected i/v 22h prior to embryo collection. All but two of the donors had a greater than 10_2 pfu/ml viremia at the time of slaughter. One of the animals from which FMDV was not isolated from the blood had a temperature of 105.5F when killed and FMDV was isolated from the flush fluid sediment of this donor. Low titre FMDV was also isolated from the flush fluid sediment of 6 other donors, and from the flush fluid supernate and sediment of one donor. Two hundred and four rejected washed embryos and unfertilized ova were sonicated and injected IDL into steers. The steers remained clinically normal and seronegative for FMD. Thirty two embryos/ova with cracks in their zona pellucida were assayed in cell culture and no FMDV was found. One hundred and six fresh embryos and 43 frozen embryos were transferred into 80 and 31 FMD seronegative recipients, respectively. All recipients remained clinically normal and seronegative for FMD. Twenty-two of the recipients were pregnant 4 months after implantation of the embryos. From these there were born 15 normal calves, 5 sets of twins delivered prematurely which died, and 2 fetuses which died at about 5 months gestation and were aborted. Sera from the normal calves and one set of premature twins were negative for FMD antibody. Sera were not obtained from the other sets of twins (**Mebus and Singh, Proc 92nd Ann Mtg USAHA 1988;pp183-185 and Mebus and Singh, Theriogenology 1991;35:435-441**).

In summary, of the above experiments, 695 bovine embryos have been used. One hundred and sixty-nine were exposed in vitro and tested both by in vitro methods and by IDL inoculation. Five hundred and twenty six embryos were collected from FMDV infected donors (viraemic or recovered) but 51 of these had to be discarded without testing due to liquid nitrogen tank failure. Of the remainder, 90 were tested for FMDV in vitro and by IDL inoculation, 32 were tested in vitro alone, 204 were tested by IDL inoculation alone and 149 were implanted into recipients. No FMDV was isolated and all test animals remained clinically negative and seronegative for FMDV antibody (**Mebus and Singh, Proc 92nd Ann Mtg USAHA 1988;pp183-185 and Mebus and Singh, Theriogenology 1991;35:435-441**).

In addition to the experiments using deliberately infected cattle, a field trial in an endemic FMD area of Argentina has been reported. Two hundred and fifty three embryos were collected non-surgically from 48 FMDV seropositive (convalescent) cows and then washed 10 times. Embryos (171), flushing and washing fluids were tested for presence of FMDV by IDL inoculation and/or cell cultures, all with negative results. The remaining 82 embryos were cryopreserved and 42 of them were transferred to seronegative cattle in a FMD-free area of the country. Fourteen live and 3 dead calves were born. Both the calves and the

recipient cows remained serologically negative and clinically free of FMDV (**Villar et al, Revista de Medicina Veterinaria 1990;71:268-276**).

From 24 superovulation attempts 94 ZP-I embryos/unfertilized ova were obtained by non-surgical flushing. The embryos/ova were exposed to high titres of FMDV ($10^{7.5}$ $TCID_{50}$/ml) for 16 hours in vitro and washed as per IETS Manual protocols. They were then either tested directly for FMDV (79 embryos) or cultured for 24 hours and then tested (15 embryos). Attempts were also made to isolate virus from the embryo washings. The virus was isolated from the first embryo washing fluid but not from any others of the washings or from the washed embryos. Exposure to FMDV appeared to have no effect on embryonic development in the cultured group (**Caamano et al, Revista de Medicina Veterinaria 1993;74:350-353**).

Vesicular stomatitis virus (VSV)

Vesicular stomatitis virus New Jersey serotype (VSV-NJ) adhered to 14 of 20 zona pellucida intact (ZP-I), day 6-8, bovine embryos exposed in vitro. The VSV-NJ-exposed ZP-I bovine embryos were washed by a single or multiple-pipette procedure. The multiple-pipette washing procedure was more efficient in removing unattached virus than the single pipette procedure, but neither washing procedure was effective in consistently removing attached virus from ZP-I embryos. The virus plaque assay with Vero-MARU cells was more sensitive than was the suckling mouse intracerebral inoculation procedure for detection of VSV-NJ from the sonic extracts of bovine embryos. A maximum of 15 infective VSV-NJ particles were detected adhering to one virus-exposed, washed ZP-I bovine embryo (**Lauerman et al, J Clin Micro 1986;24:380-383**).

Ninety-six, day 6-8, ZP-I embryos were exposed to 10^{5}-10^{7} pfu/ml of VSV (Indiana) and then washed 10x. Thirty per cent of the embryos were subsequently found to be carrying the virus when assayed. Exposure to the virus had no detectable effect on development and there was no evidence of viral replication. Twenty-three, day 6-8, ZP-I embryos were exposed to 10^{7} pfu of VSV and then treated with 0.25% trypsin at pH 7.6-7.8 for 60-90 sec. None of these embryos were positive for VSV on assay (**Singh and Thomas -unpublished- cited by Singh, Theriogenology 1987;27:9-20**).

Fourteen, day 7, ZP-I bovine embryos were exposed to $10^{6\text{-}7}$ pfu per ml of VSV-Indiana, washed 12 times, sonicated immediately in 1ml of tissue culture medium, and inoculated into monolayers of Vero cells for plaque assay. Virus from plaques was identified by neutralization with specific antiserum. Plaques were identified from the sonicate fluids of 4 of 8 single embryos and 1 of 2 groups of 3 embryos. An additional 11 groups of 3-6 day 7 ZP-I embryos (n = 46) were also exposed to $10^{6\text{-}7}$ pfu of VSV-Indiana. After exposure, embryo groups were washed 5x in Ca^{++} and Mg^{++} free PBS plus 0.4% BSA, 2x in 0.25% trypsin (90-180 seconds in trypsin), and 5x in PBS plus 2% FBS. Immediately after treatment embryos were sonicated and assayed for virus. VSV-Indiana was detected in 4 of the 11 trypsin-treated embryo groups (**Stringfellow et al, Am J Vet Res 1989;50:990-992**).27

Studies were designed to test the ability of vesicular stomatitis virus (VSV) New Jersey strain to adhere to and/or penetrate the zona pellucida of 6-8 day old bovine embryos from

susceptible parents after exposure to viral culture suspensions ($TCID_{50}$ 10^5/0.1ml) or infected bovine turbinate (BT) cultures of equivalent viral concentrations for 18-24 hours. After viral exposure, the embryos were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before (EM only) and after 5 washes (0.75ml) through cell culture medium (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

Bovine blastocysts (day 10 to 11) hatched from their zonae pellucidae were cultured for 24 h in the presence or absence of interferon 5000 units/ml and then challenged with vesicular stomatitis virus (10^6 pfu) to assess the induction of an antiviral state. In contrast to its application to fetal bovine cells, where significant antiviral effects were produced, interferon treatment of embryos failed to reduce virus yield and had no effect on virus-induced cytopathology. This lack of biologic activity of interferon in bovine embryos is similar to that previously observed with undifferentiated murine embryonal carcinoma cells and is probably a manifestation of a more general mechanism regulating gene expression in the early mammalian embryo (**Bowen, Theriogenology 1988;30:119-126**).

Rinderpest virus (RPV)

Zona-pellucida intact bovine embryos were exposed to 10^{6-7} RPV in vitro , washed 10x and assayed in vitro. It was found that virus adhered to 1-2% of the embryos (**Mebus, Proc 92nd Ann Mtg USAHA 1988;p246**).

One hundred and seven bovine embryos/eggs were collected from 10 rinderpest viremic donors and assayed in cell culture and in steers. No rinderpest virus was recovered in culture and the test animals remained seronegative for rinderpest (**Mebus, Proc 91st Ann Mtg USAHA 1987;p10**).

Seventeen ZP-I bovine embryos removed from nine rinderpest donors were washed 10x and transferred to 15 seronegative recipients. Six pregnancies resulted and none of the recipients seroconverted. Another 51 degenerated embryos and unfertilized eggs were washed, sonicated and injected subcutaneously into steers. None of these steers seroconverted (**Mebus and Singh, Proc 92nd Ann Mtg USAHA 1988;pp 183-185 and Mebus and Singh, Theriogenology 1991;35:435-441**).

Pseudorabies virus (PrV)

Studies were designed to test the ability of pseudorabies virus (PrV) (Shope strain) to adhere to and/or penetrate the zona pellucida of 6-8 day old bovine embryos from susceptible parents after exposure to viral culture suspensions ($TCID_{50}$ $10^{6.0}$/0.1ml) or infected bovine turbinate (BT) cultures of equivalent viral concentrations for 18-24 hours. After viral exposure, the embryos were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before (EM only) and after 5 washes (0.75ml) through culture medium (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On EM examination, prior to washing, none of 13 embryos exposed to virus suspension and two of three embryos exposed in BT cell culture had virus particles associated with the ZP.

After 5 washes and overnight in the 5th wash, none of 11 embryos exposed to virus suspension and one of five embryos exposed in BT cell culture had virus particles associated with the ZP (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On testing for CPE in BT cell culture, after 5 washes, three of 22 embryos exposed to virus suspension and 10 of 11 embryos exposed in BT cell culture were positive (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

Five embryos were microinjected with ± 50 picoliters (5 $TCID_{50}$) of virus and then washed 5x. The embryos were then placed in BT cell culture for 18-24 hours. A few cells were then removed from each embryo that had an intact ZP and placed in a BT cell culture for 7 days followed by three blind passages of 7 days if the primary culture showed no CPE. Some embryos were also examined by EM for viral particles. Four embryos were positive on cell culture and one of four embryos was positive on EM (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

Parainfluenza-3 virus (PI_3V)

Studies were designed to test the ability of parainfluenza 3 virus (PI_3V) (Reissinger SF_4 strain) to adhere to and/or penetrate the zona pellucida of 6-8 day old bovine embryos from susceptible parents after exposure to viral culture suspensions ($TCID_{50}$ $10^{5.5}$/0.1ml) or infected bovine turbinate (BT) cultures of equivalent viral concentrations for 18-24 hours. After viral exposure, the embryos were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before (only EM) and after 5 washes (0.75ml) through culture medium (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On EM examination, prior to washing, neither the one embryo exposed to virus suspension, nor the one embryo exposed in BT cell culture showed virus particles associated with the ZP. After 5 washes, none of eight embryos exposed to virus suspension and none of six embryos exposed in BT cell culture had virus particles associated with the ZP (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On testing for CPE in cell culture, after 5 washes, none of 12 embryos exposed to virus suspension and none of six embryos exposed in BT cell culture were positive (Gillespie et al, 1990). Four embryos were microinjected with ± 50 picoliters ($TCID_{50}$ $10^{0.5}$) of virus, washed 10x (0.75ml/wash), placed in BT cell culture for 18-24 hrs, exposed to PI_3V antiserum for 1 hr and then washed 5x. Cells were then removed with a micromanipulator from each embryo that had an intact ZP. Cells were placed in BT cell culture for 7 days, followed by three blind passages of 7 days if the primary culture showed no CPE. Embryos were also examined by EM. Results were negative but must be considered inconclusive because less than one $TCID_{50}$ of virus was present in the ± 50 picoliters inoculated (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

Bovine enterovirus (BEV)

Studies were designed to test the ability of bovine enterovirus (BEV) (serotype 6) to adhere to and/or penetrate the zona pellucida of 6 to 8 day old bovine embryos from susceptible parents after exposure to viral culture suspensions ($TCID_{50}$ $10_{6.0}$/0.1ml) or infected bovine turbinate (BT) cultures of equivalent viral concentrations for 18-24 hours. After viral exposure. the embryos were tested for virus in BT cell culture and by visualization using electron microscopy (EM) before (EM only) and after 5 washes (0.75ml) through culture medium (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On EM examination, prior to washing, one embryo exposed to virus suspension had no virus particles associated with the ZP. After 5 washes, neither the embryo exposed to virus suspension, nor the embryo exposed in BT cell culture had virus particles associated with the ZP (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

On testing for CPE in BT cell culture, after 5 washes, none of four embryos exposed to virus suspension and none of four embryos exposed in BT cell culture was positive (**Gillespie et al, Dtsch tierarztl Wschr 1990;97:65-68**).

Brucella abortus (B. abortus)

Twenty cows from which *B. abortus* had been isolated were flushed at various times ranging from between 25 days to more than 26 months post-partum and without regard to the stage of the oestrous cycle. Cultures of the uterine flushings of all 20 cows were negative on culture (**Stringfellow et al, Theriogenology 1982;18:733-743**).

Subsequently, 15 of the above 20 cows, ranging from 5 to more than 27 months post partum, were superovulated, inseminated and flushed on day 6 to 9 of the oestrous cycle. Cultures of the uterine flushings were all negative (**Stringfellow et al, Theriogenology 1982;18:733-743**).

Two experiments were conducted to test for the recovery of brucella organisms from uterine flushings and harvested embryos of sero-positive embryo donor females. In Experiment I, 16 sero-positive cows were superovulated with FSH treatments and artificially inseminated at 12, 24 and 36 hours following the onset of estrus with brucella-free semen. At 48 hours after the onset of estrus, one half the potential donor females were administered an intrauterine inoculation of 3.3 to 4.6 x 10_4 *Brucella abortus* (strain 2308) organisms while the remainder received a control inoculation. In Experiment II, the same 16 cows were similarly administered superovulatory treatments and inseminated following estrus. The uterine inoculation was increased to 1.5 to 2.5 x 10_8 organisms administered 48 hours following estrus. Samples of recovered flushing medium and homogenized embryo residues were placed into a validated in vitro culture system to detect the presence of brucella bacteria. Uterine flushings and embryos recovered from 31 females exhibiting estrus following FSH treatments were free from either field strain or the inoculation *B. abortus* (strain 2308) contamination. The flushings obtained from a single female, which did not respond with estrus following FSH treatment but was inoculated at appointment, did contain *B. abortus* which was identified as the inoculated strain 2308 and not field strain organisms. These results indicate that brucella contamination of flushing media and

harvested embryos will not be likely when collecting embryos from seropositive donor females. They offer further encouragement for the use of embryo transplantation as a method to produce brucella-free offspring from infected cows (**Voelkel et al, Theriogenology 1983;19:355-366**).

Five pregnant cows (4 S19 vaccinated, 1 non-vaccinated) were inoculated with 1.0 x 10^9 cfu *B. abortus* (strain 2308) midgestation. All five aborted. Post abortion uterine flushings from 3/5 animals were positive for *B. abortus*; from one animal until 41 days post abortion, one until 36 days post abortion and one until 17d post-abortion. The 2 animals with positive flushings at 5-6 weeks post abortion had negative flushings during the estrus just prior to that positive flush (**Stringfellow et al, Theriogenology 1983;20:77-83**).

Fifty-four day 6 through day 10 (estrus = day 0) embryos were collected nonsurgically from 13 superovulated, brucellosis-free mixed breed cows. Forty-eight excellent and good zona pellucida-intact (ZP-I), three zona pellucida-defective (ZP-D), and three zona pellucida-free (ZP-F) embryos were incubated in media containing *Brucella abortus* (2 x 10^5 to 1.8 x 10^{14} organisms/ml). Subsequently, embryos were washed ten times in groups of one, two, three or four. Embryos and serial washes were cultured, but brucellae were not isolated from any ZP-I embryo or from any washing beyond the sixth serial wash. Brucellae were not isolated from the three ZP-F embryos but were detected in the eighth wash for one and in the tenth wash for the others. Brucellae were isolated from one of three ZP-D embryos. Results show that ZP-I embryos can be effectively washed free of *B. abortus* (**Stringfellow et al, Theriogenology 1984;21:1005-1012**).

Forty-eight day 5 and 6 embryos were divided into four groups: group 1 (n = 13) was exposed to 1.6 x 10^2 brucella organisms/ml; group 2 (n = 12) to 1.6 x 10^4 organisms/ml; group 3 (n = 5) to 1.6 x 10^6 organisms/ml, and group 4 (n = 18) was the control. Embryos were cultured for 24 hrs at 37°C in phosphate buffer supplemented with 10% FCS (inactivated and without antibodies to *B. abortus*), but without the addition of antibiotics. After culture embryos were washed by passing them either through ten successive washes with 5 min in each wash and changing the pipette at each wash, or through three successive washes using a micro-pipette and transferring the minimum of wash fluid followed by grinding. No brucella organisms were isolated a) from embryos, b) beyond the seventh, or eighth in the case of the highest concentration of exposure, washing using the ten wash method, or c) from the final wash using the three wash method. The brucella organisms had a deleterious effect, particularly at the higher concentrations, on embryonic development (**Mallek et al, Bull Acad Vet France 1984;57:479-490**).

Studies have been conducted to evaluate the effects of embryo cryopreservation procedures on the survivability of *B. abortus* in embryo support medium. There was no statistical difference between means calculated at 2 weeks and 6 months within any treatment. Freezing and thawing resulted in a 64% reduction in the number of viable *Brucella*. The addition of antibiotics resulted in a 99.9% reduction in viability of the organism. Glycerol (1.4M) protected the organism during freezing and thawing in the absence of antibiotics, but did not interfere with the high percent reduction seen when antibiotics were present. Dimethylsulfoxide (DMSO, 1.5M), however, not only protected the organism during freezing

and thawing but also appeared to negate the deleterious effects of the antibiotics (**Stringfellow et al, Theriogenology 1986;26:553-559**).

Sixteen open, nulliparous, Holstein heifers were artificially exposed via the ocular route to *Brucella abortus.* Infection was verified in each animal by positive blood cultures. Cervical mucus was collected twice weekly beginning 15 days postexposure and until serum antibody levels had stabilized and blood samples were culture-negative. Brucellae were isolated from only one cervical mucus sample collected from one heifer out of a total 256 samples from 16 heifers, indicating that virgin heifers recently exposed to *B. abortus* are not likely to shed significant numbers of brucellae from the uterus nor are they likely to shed for a significant length of time (**Sparling and Stringfellow, Theriogenology 1986;25:721-732**).

Uterine flushings were collected three times at pre-determined intervals from 11 mixed-beef cows and cultured for *Brucella abortus*. Prior to sampling, all cows had aborted fetuses from which brucellae had been isolated. Initial collections were made between 21 and 34 days following abortion. The second flushing was conducted at the onset of injections used for inducing superovulation and the third flushing was conducted 6 to 8 days after the ensuing estrus. The latter two flushes were conducted between 60 and 120 days following abortion. Brucellae were isolated from uterine flushings collected from 6 of the 11 cows on the initial round of sampling. Cultures of all subsequent uterine flushings collected before and after injections for superovulation were negative. It was concluded that the superovulatory treatment is not likely to reactivate the release of brucellae into the uterine lumen during the period when embryos are normally collected (**Stringfellow et al, Theriogenology 1985;23:701-710**).

In a field study, seven donor cows, with history of being chronically seropositive to the plate serum agglutination, acidified plate antigen, card and rivanol tests (antigens donated by the USDA) and from four herds, were superovulated with a standard FSH-P prostaglandin treatment. During estrus cows were inseminated with imported frozen semen. Ova were collected 7-8 days after estrus by a non-surgical technique. Embryos classified as transferrable were placed in fresh PBS medium and maintained at 30 ± 5°C in an incubator. Embryos were not washed further. Thirty-nine brucellosis seronegative recipients received a single embryo, non-surgically, using a Cassou insemination gun. Twenty-two of these recipients became pregnant (56.4%). For the serological studies it was possible to collect blood repeatedly from 14 recipients and from their offspring after birth. The samples were processed and serum tested for the presence of antibodies to *Brucella abortus*. All samples from both recipients and offspring were found to be negative (**Del Campo et al, Theriogenology 1987;27:221 abstr**).

One hundred and ninety-two ova were collected from 12 mixed-breed beef and dairy cows that had been artifically infected with *Brucella abortus* 1 Strain 2308. A minimum of 60d after abortion (6 acutely infected) or parturition (6 chronically infected) was allowed before treatments began. Each donor cow was treated for superovulation, and nonsurgical collections were performed on one, two or three occasions. A total of 27 collections on Days 6 through 12 (estrus = Day 0) resulted in the recovery of an average of 7.1 ova (4.4 fertilized and 2.7 nonfertilized). Ova were washed ten times in groups of not more than 10 and

cultured for the isolation of *B. abortus.* A portion of the recovery medium, including sedimented uterine debris from each collection, was also cultured for isolation of the organism. *Brucella abortus* was not detected in any sample of the recovery medium or in any group of ova (**Stringfellow et al, Theriogenology 1988;29:1105-1112**).

Nine, *Brucella abortus* culture positive 2 year old cows were used to test the hypothesis that embryos and ova collected from such cows are not infected. They were selected from a group of cows that had been challenged with 1 x 10_7 CFU *B. abortus* S2308 when 2 and 5 mo pregnant. Superovulation was induced at varying times (57-145 d) post-partum or post-abortion with FSH. The cows were artificially inseminated with *B. abortus*-negative semen. Superovulations and nonsurgical embryo collections were attempted twice for each cow (at 93-96 d through to 181-185 d post partum/abortion). Jugular blood, udder secretions, cervical swabs, uterine collections, embryos and ova were cultured bacteriologically from the nine cows simultaneously at nonsurgical embryo collections, and *B. abortus* was isolated only from the udder secretions of seven cows. *Brucella abortus* was not isolated from 15 uterine collections, 21 embryos, or 18 ova from the culture-positive cows. It was concluded that *B. abortus* was not present at the detection limits of the culture method employed, which supports the view that embryos and ova collected from donor cows at 100 days or greater postpartum or post-abortion are not likely to harbor *Brucella* (**Barrios et al, Theriogenolgy 1988;29:353-361**).

Forty-two ova were collected from seven flushes of a *B. abortus* seropositive cow. Of these, 36 were unfertilized oocytes and only 6 were transferable embryos. Three of the six embryos were transferred to brucellosis seronegative recipients and developed into viable offspring. The remaining three embryos were frozen and later thawed and transferred. Only one recipient became pregnant and gave birth to a live calf. The six offspring of this seropositive cow (four obtained by embryo transfer and two by natural breeding) were tested for brucellosis at 4 and 11 months of age and had no positive reactions. A large number of unfertilized ova were also recovered from this cow, and a careful microscopic examination of these ova showed that the zona pellucida exhibited irregularities on the surface. We have observed a similar phenomenon in the zona of ova collected from other brucellosis seropositive cows. After ovulation ova to be used for embryo transfer are exposed to the maternal environment for about 6 days before collection. During this period, some damage to the zona may occur in brucellosis seropositive cows. However, more studies are needed to prove this hypothesis.
In conclusion, the case reported here indicates that the offspring (either natural or those obtained by embryo transfer) of a brucellosis seropositive cow are not potential transmitters of the disease. However, veterinary practitioners involved in embryo transfer must be aware that brucellosis seropositive animals may have low fertilization rates. This problem may be attributed to a hostile oviductal and/or uterine environment which may alter the integrity of the zona, or the fertilization processes of the male gamete (**Del Campo et al, Theriogenology 1989;31:1249-1251**).

The aim of this study was to investigate antibodies against brucellosis, leptospirosis, bovine rhinotracheitis (IBR/ BoHV-1), bovine viral diarrhea (BVD) and neosporosis through immunodiagnostic. These agents may directly or indirectly compromise the female bovine

reproductive tract as well as embryonic and fetal development, therefore we also tried to establish a probable relation between those antibody with the utilization rates of fixed-time embryo transfer (FTET) protocols, with pregnancy rate per embryo transfer (ET) and the occurrence of miscarriages. Embryos were obtained by *in vitro* fertilization of the ovum and sperm from the Gyr dairy breed, which were transferred to 235 zebu-crossbred cows under FTET protocols. The blood samples to obtain serum were collected by venous puncture from coccygeal vein, only a few days of the completion of ET. For the immunodiagnostic research of brucellosis, leptospirosis, IBR/BoHV-1, BVD and neosporosis, the complement fixation assay, the microscopic agglutination test, virus neutralization, and linked immunosorbent assay to enzymes in conjunction with the indirect immunofluorescence were used. The utilization rates of the FTET protocols related to pregnancy and abortion in D16, D35, and D75 were 67.23%, 34,18% and 20,37% respectively. The immunodiagnostic results related to seropositive animals were obtained for brucellosis (7/235 = 2.89%), leptospirosis (128/235 = 54.47%), IBR (103/235 = 43.83%), BVD (174/235 = 74.04%), and neosporosis (150/235 = 63.82%). In seropositive recipients, there was an observed influence ($P < 0.05$) of leptospirosis and neosporosis on the rate of protocol utilization of brucellosis on both the pregnancy and abortion rate, and of BVD and IBR on abortion rate. (**JV Diniz, JC Ochoaa, LM Montoyaa,c, R Satrapab, LH Okudad, EM Pitucod,RR Marcelinob, E Obaa. Immune-serological identification of infectious agents with influence on bovine embryo transfer in the north of Brazil. *Arch Med Vet* 48, 145-152 (2016)**.

Chlamydophila abortus (formerly: Chlamydia psittaci)

Embryos were recovered 3-. to 5 days after the start of estrus from eight heifers that received chlamydia contaminated semen 1-. days after the start of estrus. Four heifers yielded normal 8-16 cell embryos. One heifer yielded an apparently degenerating 8-cell embryo. One heifer yielded an embryo with a cracked ZP and three blastomeres comparable in size to those of an 8-cell embryo. Two heifers yielded unfertilized eggs. Chlamydial inclusions were not observed in the embryos (**Bowen et al, J Infect Dis 1978;138:95-98**).

Histophilus somnusformerly Haemophilus somnus (H. Somnus)

Fifty-eight ZP-I and 9 ZP-F, day 6-9 embryos were collected and exposed to *H. somnus* for 18 hrs and subsequently washed 10 or 15 times. After exposure, 20 of the ZP-I embryos were held in antibiotic containing medium (100 units penicillin base, 100 mcg streptomycin base, 0.25 mcg fungizone/ml) for 2 hrs at 25°C prior to washing 10-15 times. Attempts were made to isolate *H. somnus* from all embryos after washing. The organism was isolated from 10 of 42 ZP-I, 0 of 9 ZP-F and 0 of 32 antibiotic treated embryos. The organism was found to the last wash in embryos not pretreated with antibiotic. All embryos had degenerated or started to degenerate by the end of the 18 hour exposure period (**Thomson et al, Am J Vet Res 1988;49:63-66**).

Twenty-three superovulated heifers were artifically inseminated 12 and 24 hrs after standing estrus using high-quality, *Haemophilus*-free semen from a single ejaculate of one bull. Treatment heifers (n = 12) were exposed by intrauterine infusion 12 hrs after the second insemination to approximately 1.5 x 10^9 *H.somnus* organisms (Iowa strain 1229) suspended

in 10 ml of sterile 0.85% phosphate buffered saline (PBS). Control heifers (n = 11) were inseminated and then infused with sterile PBS. Embryos were recovered 8 d after the second insemination using non-surgical technique and evaluated microscopically and graded on their estimated survivability. Representative embryos were also examined for in vitro culture survival time, histopathological changes, vital stain uptake and bacterial contamination. Following embryo recovery, uterine flush solution was centrifuged at 10,000 x G. Sediment was submitted for bacteriologic examination and supernatant preserved for quantitation of *H.somnus* immunoglobulins. Results to date indicate that *H.somnus* had a detrimental effect on early bovine embryos. *H.somnus* was recovered from the tissues of one treated animal. Significantly more ($p < 0.005$) degenerated embryos were recovered from *H.somnus*-infected heifers than from control heifers. Embryos from *H.somnus*-infected heifers survived in culture media for a significantly ($p < 0.005$) shorter time than embryos from control heifers (**Kaneene et al, Theriogenology 1986;26:189-198**).

It is unclear whether this paper is about infections of recipient cattle with *Histophilus somnus* prior to or after transfer of embryos. I was unable to retrieve it. (**Genovez ME, et al. December 6, 2005. Napgama 2003;6:15-18).**

Escherichia coli (E.coli)

ZP-I embryos (degenerated ones collected non-surgically on day 7) were washed 10 x in PBS plus antibiotics (100 iu/ml penicillin G and 100µg/ml streptomycin) and incubated at 38.5ºC for 24h and then incubated for a further 18h in PBS without antibiotics. These embryos (n = 82) were randomly assigned to 29 groups (1 to 8 per group) and placed in 2 ml PBS containing *E.coli* (serogroup 09:K99) at concentrations ranging from 10_1 to 10_9 CFU/ml and they were incubated for periods of 1 to 18h. Following their bacterial exposure 13 groups of embryos were washed 10 x (IETS protocol), 6 groups were treated with trypsin for approximately 90 seconds (IETS protocol) and 10 groups were incubated for 2 h with 50µg/ml gentamycin. After the 10 x washing treatment *E.coli* was recovered from embryos that had been exposed to bacterial suspensions greater than 10_5 CFU/ml for over 1h and from those exposed to lower concentrations for longer periods. *E.coli* was also recovered from the groups of embryos that had been treated with trypsin, ie those exposed to 10_5 and 10_9 CFU/ml for 1h and 18h. However, no bacteria were recovered from any of the groups of embryos that had been treated with gentamycin prior to washing. This work indicates that appropriate antibiotics plus the 10 x washing protocol are effective for removal of *E.coli* from embryos, but trypsin and washing without antibiotics are not effective (**Otoi et al, J Vet Med 1993;55:1053-1055**).

No abstract found
M M Piccolomini et al., - Evaluation of morphology changes during the development of bovine embryos fertilized in vitro with semen experimentally contaminated with Escherichia coli. *Acta Scientiae Veterinariae*, 38 (Supl 2) s762, 2010 (Abstract 140).

Ureaplasma diversum (U.diversum)

Twenty one morulae were recovered from three superovulated, mature, Holstein cows six or seven days postestrus. The embryos were divided into three groups (A,B,C) and incubated for 16 hours at 37°C in humidified air with 10% CO_2. Group A was incubated in embryo culture medium alone, Group B was incubated in culture medium with sterile ureaplasma broth added and Group C was incubated in culture medium containing 1.7 x 10_6 colony forming units *Ureaplasma diversum* strain 2312. After incubation, the morulae were washed 2x (no dilution factor given) and examined using an electron microscope. Structures morphologically identical to *U. diversum* were present on the outer surface of the zonae pellucidae of all the morulae exposed to the organism and none were present on the unexposed control embryos. No other morphological differences were observed in either the ureaplasma-exposed embryos or the two groups of control embryos (**Britton et al, Can J Vet Res 1987;51:198-203**).

U. diversum was isolated from three of the five embryos incubated in culture medium with sterile ureaplasma broth added. These three embryos were recovered from one donor cow which cultured positive for *U. diversum* from the vulva and flush fluid. This finding suggests that the contaminating organisms entered the embryo culture wells either in the embryo collection medium or attached to the embryos (**Britton et al, Can J Vet Res 1987;51:198-203**).

It was concluded that *U. diversum* strain 2312 attached to the outer surface of the bovine zona pellucida. *U. diversum* 2312 was not pathogenic for bovine morulae and embryos may be contaminated with ureaplasmas when flushed from a cow with vulvar colonization (**Britton et al, Can J Vet Res 1987;51:198-203**).

Sixteen hour cultures of *U. diversum* strain 2312 were incubated with either specific antiserum or nonimmune serum, followed by exposure to protein A gold and negative staining. The ureaplasmas which were incubated with specific antiserum were labelled with gold particles while those ureaplasmas which were incubated with nonimmune serum were not labelled. Twenty-three unhatched, day 7 bovine embryos were then incubated in either embryo culture medium (ECM) alone, ECM with sterile ureaplasma broth added or ECM with 1.7 x 10_6 colony forming units of *U. diversum* strain 2312 per embryo. After 16 hours, the embryos were washed twice and incubated with either specific antiserum or nonimmune serum. The embryos were then incubated with medium containing protein A gold and examined by electron microscopy. No ureaplasmas were identified on the zona pellucida of the control embryos. Ureaplasmas were identified on the outer surface of the zona pellucida of 13 of the 17 embryos which had been exposed to the organism. Of these, the embryos which were incubated with specific antiserum had labelled ureaplasmas, while the embryos which were incubated with nonimmune serum had unlabelled ureaplasmas on the zona pellucida. It was concluded that the protein A gold method was a suitable technique for the identification of ureaplasmas in EM preparations. The presence of ureaplasmas on the outer surface of the bovine zona pellucida following in vitro exposure to the organism was confirmed (**Britton et al, Can J Vet Res 1989;53:172-175**).

Twenty-six bovine embryos and ova were exposed to *U.diversum* strain 2312 in vitro for 16 hours and subsequently washed ten times. Fifteen of the embryos and their wash fluids were cultured for ureaplasmas. Of the remaining 11 embryos, six were incubated with rabbit anti-ureaplasma immunoglobulin (RAI) and five were incubated with serum from naive rabbits (NRS), after which all were incubated with protein A gold and prepared for electron microscopy. On ultrastructural examination, ureaplasmas were observed on the outer surface of the zona pellucida of all 11 embryos. The ureaplasmas on the six embryos incubated with RAI were labelled with gold particles, while those on the five embryos incubated with NRS were not labelled. Ureaplasmas were recovered from all 15 of the cultured embryos and all of the first and second wash fluids, as well as intermittently from the third, fourth, sixth, seventh, eighth, and ninth wash, but not from the fifth or tenth wash. It was concluded that viable ureaplasma adhered to the zona pellucida during in vitro exposure of bovine embryos and were not removed by ten washes (**Britton et al, Theriogenology 1988;30:997-1003**).

Two bovine embryo recovery results are outlined from different herds. Both cases involve significant late gestational loss from embryos relating back to a single donor. *Ureaplasma diversum* was confirmed in 3 of 4 cases submitted for postmortem examination. Natural infection originating from the donor and transmitted to the recipient has not previously been documented.
Crane, M.B. and Hughes, C.A., 2018. Can Ureaplasma diversum be transmitted from donor to recipient through the embryo? Two case reports outlining U. diversum losses in bovine embryo pregnancies. The Canadian Veterinary Journal, 59(1), p.43.

Leptospira spp.

A total of 30 Holstein heifers were artificially infected with a culture of *Leptospira borgpetersenii* serovar *hardjobovis* via one or more routes (uterine, cervical, supraconjunctival, intranasal) and oviductal and uterine fluids recovered post mortem or in vivo following superovulation with FSH. All routes of administration were effective in establishing *Leptospira* infection in the reproductive tract and leptospires were identified in the oviductal and uterine fluids of all 30 heifers by microscopy. The incidence of infection was confirmed by positive identification of serum antibodies by the microscopic agglutination test (MAT). Twenty-one samples of the embryos (n=59) recovered were cultured using bacteriological procedures and all tested negative for the infectious microorganism. However, using polymerase chain reaction (PCR) assay, showed that 29% (7/24) of morulae and balstocyst stage embryos, and one out of 29 oocytes tested positive for the presence of leptospiral DNA. A single oocyte or embryo collected from the infected heifers was inoculated intravenously into 26 test heifers. None of the test heifers developed antibody titers to *Leptospira*. It was concluded that, despite the presence of the leptospires in the reproductive tract of donor animals and the association of leptospiral DNA with uterine stage embryos, the transmission of the disease is unlikely to occur by transfer of in vivo produced embryos in the bovine. **(Bielanski A, Surujballi O, Golsteyn TE, et al., Anim Reprod Sci 1998;54:65-73.)**

No abstract found

A C Goes et al., Detection of Leptospira interrogans.... in bovine oocytes after maturation period.... *Acta Scientiae Veterinariae*, 38 (Supl 2) s754, 2010 (Abstract 124)

Mycobacterium bovis

In this "In vivo-In vitro" study, an attempt was made to isolate *Mycobacterium bovis* from the reproductive tract, embryos and ova of 12 heifers that had been experimentally infected with *M bovis* (field strain 90/510). Approximately 4.5 months after inoculation (via trachea) heifers were superovulated and inseminated with semen from a tuberculosis free bull. Seven days later, heifers were slaughtered and examined for lesions of TB. All had lesions in the thoracic cavity, but none had lesions in the abdominal lymph nodes or reproductive tract. Uterine horns and tubes were flushed with PBS + BSA (no antibiotics) and ovarian follicles were aspirated. In total, 56 embryos or unfertilized eggs and 29 COCs were collected. Ova/embryos and COCs from each animal were washed separately (10x) in PBS +BSA as recommended by IETS and then cultured (in groups of 2 to 5) in Lowenstein Jensen and Midlebrook 7H11 media to try to isolate *M bovis*. Also, follicular fluid and and uterine/oviductal washes were centrifuged and pellets cultured for organism. After 6 months, all cultures remained negative for *M bovis*. **(Bielanski A, Hutchings D, and Turcotte C, Theriogenology 1999;51:270 abstr.)**

Mycoplasma spp.

Incubation of day 7 bovine embryos with 10_4 or 10_6 CFU/ml of *Mycoplasma bovis (M. bovis)* or microinjection of *M.bovis* into the cells of day 7 embryos did not influence embryonic development. *M.bovis* was recovered from all embryos washed 10 times by a standard pipetting method or vortexed and pipetted 10 times. *M.bovis* was also recovered from zonae pellucidae removed and washed from microinjected embryos. Neither treatment with trypsin nor exposure of embryos to combinations of penicillin, streptomycin, lincomycin and spectinomycin, or gentamicin, tylosin, lincomycin and spectinomycin inactivated *M.bovis* (**Bielanski et al, J IVF Emb Trans 1989;6:236-241**).

In vitro exposure of bovine embryos to *Mycoplasma bovis* and *Mycoplasma bovigenitalium* were conducted to determine if these organisms adhered to the zona pellucida-intact (ZP-I) bovine embryo, and standard procedures for washing and treating embryos were evaluated to determine their effectiveness for removing or killing mycoplasmas. *Mycoplasma bovis* and *Mycoplasma bovigenitalium* were isolated from 19 of 19 and 24 of 24 ZP-I embryos, respectively, after in vitro exposure and subsequent washing, thus demonstrating adherence of the two species of *Mycoplasma* to the ZP. Additionally, *M.bovis* was isolated from 20 of 20 and 23 of 23 embryos, while *M.bovigenitalium* was isolated from 25 of 25 and 22 of 22 embryos after antibiotic and trypsin treatment, respectively. It was concluded that neither of the standard procedures currently used for cleansing embryos should be relied upon for insuring freedom from mycoplasmas (**Riddell et al, Theriogenology 1989;32:633-641**).

The efficacy of various antimicrobials for removal/destruction of *Mycoplasma bovis* adherent to ZP-I bovine embryos was studied. Day 7 embryos were exposed to approximately 10_7CFU *M.bovis* (ATCC PG45) in PBS + 2% FBS for 1 hour at 37°C, then treated with kanamycin (200µg/ml), tetracycline (10µg/ml), tylosin (200µg/ml) or a synthesized halamine (3-chloro-4, 4-dimethyl-2-oxazolidinine) (5ppm). For the antibiotic treatments embryos were washed 10 x in PBS + 2% FBS containing the given antibiotic then incubated at 37°C for 4 hours in PBS + 2% FBS containing the same antibiotic. Halamine treatment consisted of prewashing 4 x in PBS + 0.4% BSA for 5 minutes, treatment in 0.01N sod. thiosulphate in PBS + 0.4% BSA and post washing 4 x in PBS + 0.4% BSA. After these treatments embryos were cultured in modified Frye's broth for 7 d to isolate *Mycoplasma spp.* Untreated exposed embryos were cultured for mycoplasmas to validate the culture system. Developmental potential of embryos after treatment with tylosin was assessed by comparing the time for treated and untreated embryos to hatch when cultured in Ham's F10 + 10% FBS + EGF. *M.bovis* was isolated from 27/27 (100%) embryos treated with tetracycline,7/18 (39%) embryos treated with halamine, 6/18 (33%) embryos treated with kanamycin and 0/53 (0%) embryos treated with tylosin. Times to hatching for 18, 8 and 1 tylosin treated embryos were 48, 72 and >72h respectively, while times for 15 and 8 control embryos were 48 and 72h respectively. The level of tylosin that was used here to ensure 100% freedom from this isolate of *Mycoplasma* is twice the level recommended for cell culture, but it did not appear to affect development of embryos (**Riddell et al, Theriogenology 1993;39:297 abstr**).

The efficacy of various antibiotics for eliminating *M.bovis* from ZP-I, in vivo derived (7d.) bovine embryos was evaluated using a total of 367 embryos/ova from 32 superovulated cows. After their recovery, embryos were washed 10 x in PBS + 2% FBS + 100 iu penicillin, 100µg streptomycin and 0.25µg amphotericin B per ml (PBS + P/S/A), then incubated at 37ºC for a minimum of 1 h. in PBS + 2% FBS without antibiotics. Groups of 20 embryos were put in 2ml PBS + 20% FBS containing approx 10_7 CFU/ml *M.bovis* and incubated for 1 h. following which they were treated with tetracycline (50µg/ml), gentamycin (100µg/ml), kanamycin (1000µg/ml) or tylosin (200µg/ml). For the antibiotic treatments, groups of 10 or fewer embryos (a total of 274) were washed 10 x in PBS + 2% FBS + the particular antibiotic and then incubated for 4 h. in the same medium. Control embryos were washed and incubated in PBS + P/S/A which in previous work had been shown ineffective for destroying the *M.bovis* isolate. *M.bovis* was isolated from all groups of control embryos, from all groups treated with tetracycline and from 72% of those treated with gentamycin. However, the treatments with kanamycin and tylosin were wholly effective in eliminating *M.bovis* from the embryos. To assess embryotoxicity of the rather high levels of kanamycin and tylosin used in this study, groups of good and excellent quality embryos (a total of 93 embryos) that had been exposed to the same antibiotic concentrations at 37ºC for 4h in PBS + 2% FBS, were further cultured to hatching in Ham's F10 nutrient medium to assess their developmental potential. Compared to controls, the two antibiotics had no apparent detrimental effects on times to hatching in vitro. It was concluded that the kanamycin and the tylosin treatment protocols were effective for eliminating *M.bovis* and were without observable embryotoxic effects (**Riddell et al, J Assist Reprod Genetics 1993;10:488-491**).

Mycoplasma bovis (Mbo) has been isolated from genital tracts of bulls, and it can survive in processed semen. Experimental studies have shown that Mbo inoculation into the uterus or insemination with Mbo-infected semen can cause bursitis, salpingitis, abortion, and infertility. The control of Mbo is very difficult because of latent carrier animals, increasing resistance to antibiotics, and unavailability of effective vaccines. The aim of this study was to follow the passage of Mbo infection from naturally contaminated semen to transferable embryos during bovine *in vitro* embryo production (IVP). (Unless otherwise stated, all chemicals used were purchased from Sigma-Aldrich.) Two batches of slaughterhouse-derived oocytes were matured in tissue culture medium 199 (TCM-199) with glutamax-I (Gibco™; Invitrogen Corporation) supplemented with 0.25 mM sodium pyruvate, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 2 ng mL^{-1} FSH (Puregon, Organon), 1 µg mL^{-1} β-oestradiol (E-2257), and 10% heat-inactivated fetal bovine serum (FBS; Gibco™) for 24 h at 38.5°C in maximal humidity in 5% CO_2 in air. Matured oocytes were fertilized for 20 h in IVF-TL medium supplemented with 10 µg mL^{-1} of heparin and 2 mM of PHE at 38.5°C in maximal humidity in 5% CO_2 in air, using spermatozoa per mL as a final concentration. The batches of oocytes were divided between uninfected IVP bull ($N = 205$) and naturally Mbo-infected AI bull ($N = 690$). Zygotes were cultured in G1/G2 media (Vitrolife) supplemented with bovine serum albumin, fatty acid free (4 mg mL^{-1}), at 38.5°C in maximal humidity in 5% O_2, 5% CO_2, and 90% N_2. Blastocysts were collected for Mbo cultures on Days 7 and 8 (IVF = Day 0). Samples of washed semen, fertilization medium, cumulus cells, culture medium, all wash media, and transferable embryos (with and without zona pellucidae) were collected for Mbo cultures. Half of the embryos were treated with trypsin according to IETS standards after the collections. The Mbo cultures were performed in accordance with procedures previously described by Bölske (1988 Zentralbl. Bakteriol. Mikrobiol. Hyg. A **69**, 331-340), followed by detection with real-time PCR. Infection with Mbo does not seem to have negative effects on fertilization (cleavage rates: 77.1% and 89.0% for IVP and Mbo AI bulls, respectively) or embryo development rates (blastocyst rate: 26.3% and 32.5% for IVP and Mbo AI bulls, respectively). Following Mbo cultures, only washed semen was found to be Mbo positive via real-time PCR. We conclude that *M. bovis* is not likely transmitted in bovine IVP when using naturally infected semen.

Peippo, J., Vähänikkilä, N., Mutikainen, M., Lindeberg, H., Pohjanvirta, T., Simonen, H., Pelkonen, S. and Autio, T., 2020. 110 Absence of transmission of Mycoplasma bovis via naturally contaminated semen during in vitro fertilization. *Reproduction, Fertility and Development*, *32*(2), pp.182-182. https://doi.org/10.1071/RDv32n2Ab110

Mycobacterium avium subsp. paratuberculosis

To test the efficacy of the 10-step wash procedure for removing *M.paratuberculosis* from bovine ova, washed ZP-I ova (unfertilized) were incubated in Dulbecco's phosphate-buffered saline (DPBS), supplemented with 2% fetal bovine serum (FBS) containing concentrations of 10_4, 10_3, 10_2, 10_1, 10_0 colony forming units of *M.paratuberculosis* (strain 19698)/ml for 12 hours at 22°C. Ten ZP-I ova were removed from each concentration and washed through 10 changes of DPBS supplemented with 15% FBS (1:100 dilution). Medium from each wash step was inoculated onto slants of Herrold's yolk medium. The ova were included with the medium from the 10th wash step. *M.paratuberculosis* was isolated from 1 of 10 tenth-wash steps at the 10_4 concentration and 5 of 10 tenth-wash steps at the 10_3 concentration, although medium for the seventh, eighth and ninth-wash steps for these concentrations were negative. These observations suggest that *M.paratuberculosis* adhered to the ova

throughout the 10-step wash procedure at concentrations of 10^4 and 10^3 (**Rohde et al, Am J Vet Res 1990;51:708-710**).

Four cows known to be clinically, serologically and bacteriologically positive with *M. paratuberculosis* were subjected to uterine lavage using a conventional embryo recovery technique with precautions to avoid fecal and aerosol contamination. Aliquots of the flush fluids were cultured for up to 16 weeks. Colonies of *M. paratuberculosis* were isolated at 12 weeks from one cow and at 16 weeks from two others. If this organism were to adhere to embryos obtained from superovulated, infected donors the potential for transmission may exist (Rohde and Shulaw, JAVMA 1990;197:1482-1483).
M.paratuberculosis was isolated from fetal tissues of 5 of 58 subclinically infected cows. There was no correlation with the dam's serological status but all 5 culture-positive fetuses were from cows classed as heavy faecal shedders (Sweeney et al, Am J Vet Res 1992;53:477-480).
Paratuberculosis is a chronic and progressive disease of the intestine in ruminants caused by Mycobacterium avium subsp. paratuberculosis (Map). The bacterium is transmitted to young animals, becomes manifest in adulthood and leads to economic losses. The aim of this study is to investigate if cows shedding Map possess oocytes and embryos that are carriers of the bacterium. New genetical material can enter the dairy farm using embryo transfer but the question as to whether this technique is safe with respect to transmission of paratuberculosis has yet to be addressed. We selected and bought 16 cows, all proven to be moderate shedders of the bacterium in the faeces immediately prior to the experiment, but none were clinically sick. One sample of uterine content was collected from each animal by flushing the uterus on the day of heat, and five samples of homogenised uterine tissue were collected on the eighth day of the same cycle by biopsy. In addition, 217 cumulus-oocyte complexes (COCs), ranging from 3 to 35 COCs per animal, were collected using ultrasound guided transvaginal puncture of the ovarian follicles (OPU). On the seventh day of the subsequent cycle, 31 embryos were obtained using the classic technique of super ovulation induction, artificial insemination (AI), followed by flushing of the uterus. These embryos have been washed and trypsinised. Fourteen of the 16 cows were treated again for super ovulation in the subsequent cycle and 19 foetuses were collected by opening of the uterus after euthanasia on Days 35-49 of the cycle. All samples were cultured for presence of Map and checked every 2 months during 1 year for bacterial growth. None of the samples showed growth of Map after 12 months of culture. Pathological examination of the cows revealed different degrees of severity of pathological alterations of the intestinal tract and mesenteric lymph nodes. However, the results suggest that neither in vivo embryo's nor oocytes are carriers of the bacteria and do not form an extra risk at transfer. However, due to the limited size of the experiment (sample size of 16 cows), a certain margin for error remains. (Kruip TA et al. Theriogenology 2003;59:1651-1660)
This represents the first report of intrauterine transmission of *Mycobacterium paratuberculosis* from a recipient cow to a fetus resulting from embryo transfer. The report does not provide novel information on transmission of Johne's Disease, but it does emphasize the importance of selecting recipients that are Johne's-free! **(Manning EJB, et al. The Bovine Practitioner 2003;37:20-22.)**

Over a 5-year interval, experiments were conducted to determine if Mycobacterium avium ssp. paratuberculosis (Map) is associated with in vivo and in vitro fertilized (IVF) embryos and whether it can be transmitted by embryo transfer. The present studies included: collection of embryos from five asymptomatic, naturally infected donors and transfer to

uninfected recipients; collection of oocytes from two naturally infected donors with overt clinical signs; exposure of in vivo and IVF embryos to Map and transfer to uninfected recipients; and the inoculation (transfer) of ‘‘clean’’ IVF embryos to the uterine lumen of infected cows. The presence of Map was confirmed in the uterine horns of all asymptomatic, infected donors. None of the tested embryos, which were not used for embryo transfer, or unfertilized ova (two per batch), were positive for Map, as determined by culture (n = 19) or by PCR (n = 13). However, all in vivo fertilized embryos exposed to Map in vitro (and subsequently sequentially washed) tested positive for Map, by both culture (12 batches) and PCR (15 batches), whereas IVF embryos treated in the same manner tested positive on culture (51%, 18/35 batches) and by PCR (28%, 20/71 batches). Transferring both in vivo embryos and IVF embryos potentially contaminated with Map into 28 recipients resulted in 13 pregnancies and eight calves born without evidence of disease transmission to either the recipients or the offspring over the following 5-year period. In samples collected from one of the clinically infected animals, two of seven (28%) cumulus oocyte complexes (COC) and follicular fluid tested positive by PCR and 10/10 cumulus oocyte complexes on culture for Map. From the second clinically infected cow, three of five batches of IVF embryos (n = 20) were positive on PCR and two of four batches containing unfertilized oocytes and embryos were positive on culture. Only 10% of embryos reached the morula and blastocyst stage 10 days after fertilization. In conclusion, Map is unlikely to be transmitted by embryo transfer when the embryos have been washed as recommended by the International Embryo Transfer Society. (**Bielanski et al, Theriogenology 2006;66:260-266.)**

Neospora caninum

The objective of this study was to evaluate efficacy of embryo transfer into seronegative recipients using the procedures proposed by the IETS for preventing vertical transmission of *Neopsora caninum* in cattle. *Neospora caninum* serologic status of donors (n=22 from 9 dairy herds) and recipients (n=87 cows and heifers) was determined before collection and transfer of embryos. Viable embryos were washed and treated with trypsin. Recipients in experimental groups A (n=50) and B (n=29) were seronegative and received embryos from seropositive and seronegative donors, respectively. Antibody titers were determined monthly during pregnancy in recipients and in calf blood samples collected at birth. Tissues collected from stillborn calves and aborted fetuses were analyzed histologically and by immunohistochemical (IHC) methods. Seventy-six calves and 11 fetuses and stillborn calves were examined. All calves from group A (seropositive donors) and B (seronegative donors) were seronegative (n=70) or lacked evidence of infection by use of tissue analysis (n=9). In group C (seropositive donors receiving embryos from negative donors) 5 of 6 calves were seropositive at birth, and IHC results were positive for 1 of 2 calves. Vertical transmission rate was significantly lower in groups A and B (0%) than in group C (75%). Conclusions were that embryo transfer into seronegative recipients, using the procedure proposed by IETS, is an effective way to prevent vertical transmission of *Neopsora caninum*, and results provide support for pre-transfer testing of all recipients. **(Baillargeon P, et al. JAVMA 2001;218:1803-1806.)**

Abortions are reported in a herd of Normandy and Holstein cows. Serology reveals the presence of *Neospora caninum*. Genealogy studies carried out on the herd, where embryo

transfer is used, show that the transmission of the parasite occurs via the placenta. The 7 day embryo does not seem to transmit the parasite, but the surrogate mother could infect the implanted embryo. The origin of the introduction of the parasite in the herd (farm dogs, feed, cows) remains unknown. **(Journel C, et al. Le Pointe Veterinaire 1999;30:49-56.)**

(Note: This report does not directly address epidemiology of embryo transfer, but it is relevant to exposure-transmission timing) *Neosporum caninum* is an important cause of abortion in cattle. It is transmitted vertically or horizontally, and infection might result in abortion or the birth of a live, clinically normal but infected calf at term. Only a proportion of infected cattle abort and the pathogenesis of abortion in not understood. Groups of cattle were infected with 10_7 *Neospora caninum* tachyzoites intravenously at different stages of gestation. Intravenous inoculation was chosen to reproduce the putative hematogenous spread of the parasite following either recrudescence of endogenous infection or *de novo* infection. In all cattle, infection was accompanied by high γ-interferon and lymphoproliferative responses , and a biased IgG_2 response indicating that *Neopsora caninum* infection is accopmanied by a profound Th1 helper T cell-like response. Infection at 10 weeks gestation resulted in foetopathy and resorption of foetal tissues 3 weeks after infection in 5 out of 6 cows. Infection at 30 weekss gestation resulted in the birth of asymptomatic, congenitally-infected calves at full term in all 6 cows, whereas the 6 cows infected before artificial insemination gave birth to live, uninfected calves. These results suggest the reason some cows abort is related to the time during gestation when they become infected or an existing infection recrudeses. (The article raises the question of whether cows protect fetuses from infection or vice versa). **(Williams DJL et al. Parasitology 2000;121:347-358.)**

A donor cow from a herd with a high incidence of *Neospora caninum* infection was identified. She had previously given birth to three heifer calves that were seropositive and had aborted a 5-month fetus that had evidence of a typical non-suppurative encephalitis consistent with infection with *N caninum*. Four seronegative recipients were identified from the same herd. The donor cows was superovulated, and four A-grade embryos were collected and transferred to the negative recipients. It was noted that embryos were only washed 3 times. Three recipients were diagnosed pregnant at 42 days after embryo transfer. However, one recipient aborted an 8-month fetus (likely caused by BVDV). This recipient remained seronegative for *N caninum*. The other recipients were bleed at 3 and seven months of gestation and after the birth of their calves. The remained seronegative as were there calves. **(Landmann JK et al. Aust Vet J 2002;80:502-503.)**

This study was carried out to determine the prevalence of neosporosis in an area of intensive dairy production, in Portugal. Sera samples were obtained in a random basis from 114 cows in 49 herds (group A), and from 1237 cows in 36 herds with a history of abortion outbreaks (group B). All sera samples were tested for neosporosis by direct agglutination test (DAT). Additionally, attempts to isolate Neospora caninum in 42 aborted bovine fetuses from 38 dairy herds (group C) were carried out, utilizing a bioassay with immuno-depressed Swiss Webster mice. Parasitological confirmation was done by indirect fluorescent antibody test (IFAT). The prevalence of neosporosis in the group A was 28%. Group B had a significantly ($P < 0.001$) higher prevalence (46%) and Neospora caninum was isolated in 36%

of the aborted fetuses (group C). These results indicate that neosporosis, a disease only recently (2001) diagnosed in Portugal, has a high prevalence in the country, particularly in populations with a story of abortion. Thus, neosporosis should systematically be considered in the differential diagnosis of abortion. In the context of embryo transfers, the importance of selecting Neospora-free embryo recipients is discussed, as well as the pertinence of assessing the Neospora status of traded and imported cattle. (**Canada N, et al. Theriogenology. 2004;62:1229-1235.)**

OBJECTIVE: To investigate the potential of different Neospora caninum tachyzoite doses to infect heifers (experiment 1) and cows (experiment 2) when administered in utero by artificial insemination via contaminated semen. METHODS: In experiment 1, five groups of 5, 7, 8, 9, and 5 cyclic heifers were hormonally synchronized and artificially inseminated with semen containing 0 (A, controls), 10(2) (B), 5 x 10(3) (C), 5 x 10(4) (D), and 5 x 10(5) (E) live N. caninum NC-1 isolate-tachyzoites, respectively. Experimental infection was followed for 100 days. Parasitaemia and specific serum IgG, and interferon-gamma (IFN-gamma) responses were studied. In experiment 2, four groups of 9, 10, 9, and 9 adult multiparous cows with confirmed infertility problems of diverse aethiology were hormonally synchronized and artificially inseminated with semen containing 0 (a, controls), 10(2) (b), 5 x 10(3) (c), and 5 x 10(5) (d) live N. caninum NC-1 isolate-tachyzoites, respectively. Experimental infection was followed for 63 days. Parasitaemia and specific serum IgG responses were studied. RESULTS: In experiment 1, parasitaemia was detected in 1, 2, and 3 heifers from groups B, C, and D, respectively, between 9 and 23 days after insemination. Persistent specific serum antibody responses were detected in 2 and 3 heifers from groups D and E, respectively. Transient specific serum antibody responses were detected in 2, 1 and 1 heifers from groups C, D, and E, respectively. In addition, 1 heifer from group B showed a serum-specific antibody level higher than cut off value at 21 days post-insemination. Heifers seroconverted between 23 and 47 days after insemination. Specific IFN-gamma levels were detected in 1, 4, 6, and 3 heifers from groups B, C, D, and E, respectively, between 9 and 55 days after insemination. Pregnancy rate in the control group (60%) was higher than those observed in inoculated heifers (0-42.9%). Pregnancy rates in inoculated heifers were lower when the tachyzoite dose was increased (B 42.9%, C 12.5%, D 11.1%, and E 0%). In experiment 2, no Neospora DNA in blood nor specific serum IgG to N. caninum were detected in any of the cows studied, except in one cow inoculated with 5 x 10(5) tachyzoites (group d) which showed a relative index x100 (RIPC) values of 9.4, 18.9, and 18.1 at 42, 56, and 63 days after insemination, respectively. CONCLUSIONS: This study provides evidence that the intrauterine infection via contaminated semen using 5 x 10(4) and 5 x 10(5) tachyzoites caused persistent serum-specific antibody responses in some heifers. On the basis of serological data, a dose-response effect was also observed. In addition, N. caninum would be a probable cause of early foetal death in inoculated heifers. In contrast, results obtained in a similar experiment with cows showing confirmed infertility indicate that higher doses, such as of 5 x 10(5) tachyzoites, were necessary to induce seroconversion in at least one animal. **(Serrano-Martínez E, et al., Theriogenology. 2007 Mar 1;67(4):729-37.)**

The aim of this research was to determine the influence of Neospora caninum (NC) infection on reproductive parameters of Holstein dairy cows. The study was performed at 32 farms in the south of Brazil, latitude 27°30'S to 31°S and longitude 51°30'W to 55°30 W Farms

included in this study met rigorous vaccination schedule for infectious bovine rhinotracheitis, bovine viral diarrhea virus, leptospirosis, and brucellosis. Blood samples collected from the dairy cows were submitted to serologic testing by an indirect fluorescent antibody test (IFAT) and were distributed into 2 groups: seropositive (GP; n = 160) and seronegative (GN; n = 558). The dogs (n = 51) of the farms were tested by IFAT. After abortion, fetuses were frozen at -80°C and submitted to PCR detection of NC in tissues. Seropositive cows that aborted were also tested by IFAT. Pregnancies of GP and GN cows were mon- itored by periodic ultrasound exams. After parturition or abortion all cows in GP and GN groups were submitted to gynecological exams. Cows that developed disease (clinical mastitis, metabolic disease, or laminitis) after parturition or abortion were excluded from the analysis to prevent any distortion of data. Farms were divided into 3 categories according to production technology, specifically, average milk production per cow per lactation: high (>25 kg), medium (15-25 kg), and low (< 15 kg). Data was analyzed by ANOVA using SAS® software (SAS Institute, Cary, NC, USA) at a level of 5% significance and Student's t-test for average reproductive parameters. The alternative hypothesis of the study was that at least 1 reproductive parameter was influenced in GP after abortion compared with GN cows that aborted. The null hypothesis was that there was no influence of NC antibody titer on reproductive parameters. The prevalence of antibody for NC in dairy cows was 24.48%. There was no prevalence difference among production systems (P > 0.05). The prevalence in dogs was f
48.6% and did not differ among production systems. Fifty-nine (41.37%) NC-seropositive cows aborted. This differs significantly from the seronegative cow abortion rate (n = 17; 2.6%; P < 0.001). Occurrence of retained fetal membranes (RFM), endometritis, absence of uterine infection after abortion, and days between abortion and first estrus can be observed in Table 1. Abortion occurred between the fifth and eighth month of pregnancy. Occurrence of RFM was more frequent in cows that were seropositive. The period between abortion and first estrus was 79.06 v. 65.8 days for seropositive and seronegative groups, respectively, which differed significantly (P < 0.001). The results suggest that the higher occurrence of RFM after abortion in dairy NC-seropositive cows affects uterine involution, resulting in a delay of ovarian activity.

Pessoa GA, Silva CAM, Rubin MIB, Vogel FSF, Trentin JM, Dalcin VC, Araujo LO, Da Rosa DC, Leonardi CEP. Effect of Neospora caninum infection on reproductive parameters of dairy cows. Reproduction, Fertility and Development. 2010; 22(1): 252 abstr.

Neospora caninum, an intracellular protozoon, causes encephalomyelitis in dogs (Bjerkas I et al. 1984 Zentralblat fur Parasitenkunde 70, 271-274). For the past decade, neosporosis has been a main cause of abortion in dairy cattle worldwide (Anderson M et al. 2000 Anim. Reprod. Sci. 60-61, 417-431; Dubey JP 2003 Korean J. Parasitology 41, 1-16). Vertical transmission has been indicated as an important way of spreading neosporosis (Hall CA et al. 2005 Vet. Parasitology 31, 231-41); thus, we investigated whether the protozoon could be transferred by embryo production techniques. Blood samples were collected from 92 dairy cows with history of reproductive failure and abortion within the previous 90 days at 7 dairy farms in Tizayuca, Mexico. For serology evaluation, a commercial indirect ELISA kit (Civtest Bovis Neospora, Laboratories Hipra S.A, Girona, Spain), yielded 46.74% (43/92) positive results, 46.74% (43/92) negative results, and 6.52% (6/92) suspicious to N. caninum infection. Thirteen positive cows were chosen for uterine flush (UF), ovum pickup (OPU), and

a blood sample collection. Lymphocytes from blood and cells within the UF and OPU collection fluids were collected after centrifugation and DNA was extracted. All samples were tested for the presence of N. caninum by PCR, using primers and protocols that amplified a 275-bp fragment of the genomic region (5-GGGTGAACCGAGGGAGTTG-3 and 5-CCTCCCAATGCGAACGAAA-3). The N. caninum vaccine (Bovilis® NeoGuard, Intervet, Santiago Tianguistenco, Mexico) was used as a positive control and water as a negative control. Uterine flush could not be obtained from 1 cow. From 13 cows seropositive to N. caninum, only 38% were positive to PCR from blood lymphocytes. In contrast, PCR amplification was obtained from OPU cell sediment in 92.31% (12/13) and in 33.33% (4/12) of UF. Of these 12 OPU- and 4 UF-positive samples, only 5 and 3 of their corresponding blood lymphocytes were positive. Our results using uterine and follicular fluid were contradictory to those published by Moskwa et al. (2008 Vet. Parasitology 158, 370-375) where oocytes and embryos were evaluated. These results indicate that N. caninum is present in the ovary and uterine lumen of the cows, suggesting a possible risk of neospora transmission during oocyte and embryo collection and transfer techniques.g

Marques AF, Ortiz CG, Lima MR, Zanella EL, Rangel L, Morales E, and Gutierrez CG. Detection by polymerase chain reaction of *Neospora caninum* from ovum pickup and uterine flushing fluids from dairy cattle in Mexico. Reproduction, Fertility and Development. 2010; 22(1): 252 (abstr.).

Neosporosis is considered to be one of the main causes of abortions in bovines. In this study we evaluated the congenital transmission and occurrence of abortions by Neospora caninum in an embryo transfer center in Neropolis, Goiás. Serological samples from 101 recipients, 61 donors, and 90 calves were analyzed. Among these animals, 32.67% of the recipients, 22.22% of the donors, and 6.66% of the calves were positive for N. caninum. The rate of vertical transmission was 24%. There was a statistically significant difference between antibody titer of the recipient cows and the serological status of the calves. There was no statistically significant relationship between N. caninum serological status and reproductive rates such as estrum repetition and embryo production. Eight abortions occurred, six (75%) in positive females and two (25%) in negative females. These results indicate that neosporosis may be an important cause of failing reproduction and that in embryo transfer centers recipients should be previously examined, and those that are seronegative for Neospora should be chosen to reduce abortion and the birth of seropositive calves.

de Oliveira VS, Alvarez-Garcia G, Ortega-Mora LM, Borges LM, da Silva AC. Abortions in bovines and Neospora caninum transmission in an embryo transfer center. Vet Parasitol. 2010 Oct 29;173(3-4):206-10. Epub 2010 Jun 26.

Neospora caninum, an intracellular protozoon, causes encephalomyelitis in dogs (Bjerkas I *et al.* 1984 Zentralblat fur Parasitenkunde **70**, 271–274).For the past decade, neosporosis has been a main cause of abortion in dairy cattle worldwide (AndersonM*et al.* 2000Anim. Reprod. Sci. **60–61**, 417–431; Dubey JP 2003Korean J. Parasitology **41**, 1–16).Vertical transmission has been indicated as an importantway of spreading neosporosis (HallCA *et al.* 2005Vet. Parasitology **31**, 231–41); thus, we investigated whether the protozoon could be transferred by embryo production techniques. Blood samples were collected from 92 dairy cows with history of reproductive failure and abortion within the previous 90 days at 7 dairy farms inTizayuca, Mexico. For serology evaluation, a commercial indirect ELISA kit (Civtest Bovis Neospora, Laboratories Hipra S.A, Girona, Spain), yielded 46.74% (43/92) positive

results, 46.74% (43/92) negative results, and 6.52% (6/92) suspicious to *N. caninum* infection. Thirteen positive cows were chosen for uterine flush (UF), ovum pickup (OPU), and a blood sample collection. Lymphocytes from blood and cells within the UF and OPU collection
fluids were collected after centrifugation and DNA was extracted. All samples were tested for the presence of *N. caninum* by PCR, using primers and protocols that amplified a 275-bp fragment of the genomic region (5-GGGTGAACCGAGGGAGTTG–3 and 5-CCTCCCAATGCGAACGAAA–3). The *N. caninum* vaccine (Bovilis® NeoGuard, Intervet, Santiago Tianguistenco, Mexico) was used as a positive control and water as a negative control. Uterine flush could not be obtained from 1 cow. From 13 cows seropositive to *N. caninum*, only 38% were positive to PCR from blood lymphocytes. In contrast, PCR amplification was obtained from OPU cell sediment in 92.31% (12/13) and in 33.33% (4/12) of UF. Of these 12 OPU- and 4 UF-positive samples, only 5 and 3 of their corresponding blood lymphocytes were positive. Our results using uterine and follicular fluid were contradictory to those published by Moskwa *et al.* (2008 Vet. Parasitology **158**, 370–375) where oocytes and embryos were evaluated. These results indicate that *N. caninum* is present in the ovary and uterine lumen of the cows, suggesting a possible risk of neospora transmission during oocyte and embryo collection and transfer techniques.

Marques A. F., C. G. Ortiz, M. R. Lima, E. L. Zanella, L. Rangel, E. Morales, and C. G. Gutierrez, Detection By Polymerase Chain Reaction Of *Neospora Caninum* From Ovum Pickup and Uterine Flushing Fluids From DairyCattle In Mexico
Reprod Fertil Dev 22 (2010), p252 abstract

Bovine spongiform encephalopathy (BSE) agent

Two hundred cows, all suspect BSE cases, were brought from the field to the laboratory where they were checked for suitability as donors. They were then superovulated and flushed non-surgically to collect embryos at 7 days. Semen from 8 BSE-positive bulls was used for approximately half the inseminations, and semen (collected pre-1981) from 5 healthy bulls was used for the remainder. Deteriorating condition of the donors prevented flushing in some cases, but most were flushed one or more times at monthly intervals (some up to 6 or 7 times) until it became necessary to euthanatize them. The average number of flushings per cow as 2.9. From a total of 575 flushings 2675 embryos/ova were collected and frozen, and 47.8% of these were of transfer quality (grade 1 or 2). Prior to freezing all embryos/ova were checked for intact ZP and washed 10 x (as per IETS Manual). Thirty three of the donors were found BSE negative post mortem so their embryos (287) were not used. Unfertilized and poor quality embryos from BSE +ve donors and samples of the uterine flush-fluid are being bioassayed in mice (intracerebral injection of 20 sonicated embryos per mouse) for presence of the BSE pathogen.
Of the good quality embryos, 587 have been transferred non-surgically into recipients. These recipients were drawn from 352 Hereford x Fresian heifers that had been imported by air from New Zealand and are kept isolated from all contact with British cattle and sheep on an upland farm in northern England (240 miles from the Laboratory). ETs took place in 3 sessions: July - August 1991; July - August 1992 and November - December 1992. Some recipients that returned to oestrus after ET had further ETs within the same or (in 1992) subsequent session. A total of 347 recipients had one or more ETs. Pregnancy rate (by

ultrasonography at 45d) averaged 52.3% for the 587 transfers, but the rate for first ET (60.2%) was better ($p < 0.001$) than for second or subsequent transfers (42.3%). A total of 218 recipients have calved so far (June 1993). All recipients and all ET progeny are to be monitored clinically for BSE for 7 years from date of transfer. Survivors and any that die during the monitoring period will be examined post mortem for histopathological lesions of BSE.
Mouse inoculations with embryos/ova and uterine flushings have been completed and approximately 1100 mice have been used. Mice are monitored for 650 - 700 d. post-inoculation, and the survivors plus those dying during the monitoring period are examined post mortem for histopathological lesions of BSE. No evidence of BSE transmission to mice has been found so far but final results will not be available until about 1996 (**Wrathall et al, Theriogenology 1994;41:337 abstr**).

Data on embryo transfer (ET) and the control of spongiform encephalopathy (SE) in sheep and cattle is being investigated in the USA and the UK. In regard to sheep there are conflicting results. Washed embryos from experimentally infected sheep in the USA have not transmitted scrapie to recipients though no account was taken of the genetic susceptibility of the embryos or recipient. In contrast in the UK using Sip and PrP genotyped experimentally-infected sheep and unwashed embryos, scrapie occurred in the homozygous susceptible (sAsA) offspring within 979 days. It is concluded that the question as to whether ET can be used to control natural scrapie in sheep is thus unresolved. Further, studies to investigate the effect of washing embryos are in progress. In the UK 1000 embryos have been collected from confirmed BSE cows and some have been transferred into 347 heifers imported from New Zealand as calves and subsequently held under quaratine conditions. Some embryos and uterine flushings have been inoculated into susceptible mice. No disease has resulted but the experiment will not be completed until 2001 (**Bradley, Livest Prod Sci 1994;38:51-59**).

This work was part of an ongoing project to assess the risk of transmitting BSE by embryo transfer. All the donor cows and some of the bulls used for artificial insemination were clinical BSE cases. Embryos and fluids were collected by nonsurgical uterine flushing seven days after AI. Further collections were at monthly intervals until the donors had to be killed. Nontransferrable embryos and ova with intact ZP and free from adherent material were washed ten times (IETS guidelines) then frozen using glycerol cryoprotectant. After thawing they were sorted into groups of similar donor/bull status, sonicated, and injected intracerebrally into weanling mice (20 embryos in 0.02 ml suspension per mouse). Embryos of status +/+ were from BSE+ve cows given semen from BSE+ve bulls while those of status +/- were from +ve cows given semen from -ve bulls. Status of BSE+ve cows and bulls was confirmed postmortem. Flush fluid samples, with the sediment, were frozen (-20^0C). After thawing, samples from 40 cows (successive flush fluids from a cow were pooled if of same donor/bull status) were injected into groups of mice by the combined intracerebral (0.02ml) and intraperitoneal (0.10ml) routes.
BSE-susceptible strains of mice (Sinc s7 RIII and Sinc s7 C57BL) were used, with groups of approx. 25 mice (same strain) for each individual inoculum. In bioassays of BSE-infected brain samples these, two mouse strains have incubation periods of 316-327 and 407-438 days, respectively. Mice were kept up to 700 days post-injection (d.p.i.), then killed. Their

brains, including those of mice culled for inter-current illness, were examined histologically for spongiform encephalopathy (SE).

Table 1. Results for the embryos (E) and the flush fluids from donor cows (C).

Type &No.	Donor Status	Mice used	Result +ve	±ve	-ve	Nil result	Interval (d.p.i.) To brain exam. <399	400-499	500-599	>599
E=500	+/-	25	0	0	22	3	0	3	5	14
E=520	+/+	26	0	0	26	0	8	1	3	14
C=23	+/-	578	0	1	535	42	49	53	104	329
C=17	+/+	422	0	0	410	12	19	23	58	310

All but one mouse (an RIII mouse given flush fluid of status +/-) were negative for SE. The survival time (447 d.p.i.) of this non-negative mouse was inconsistent with even a hypothetically very low infective dose of BSE which this would represent if it was a single positive case in a batch of 25 injected mice. The spongiform changes in its brain may have been due to senility.
The authors concluded that neither the embryos nor the flush fluids contained detectable BSE infectivity in the mouse bioassay. However, since it is possible that mice are less sensitive to BSE than cattle, viable embryos from BSE+ve donors were transferred in 1991-1992 into heifers imported from New Zealand. To date there has been no evidence of BSE in these heifers or in their embryo transfer offspring, but the project is not scheduled to finish until the year 2000. (**Wrathall et al, Theriogenolgy 1997;47:384 abstr**).

Semen from 13 bulls, eight with clinical bovine spongiform encephalopathy (BSE), was used to artificially inseminate (AI) 167 cows with clinical BSE, and their resultant embryos were collected non-surgically seven days after AI. The viable and non-viable embryos with intact zonae pellucidae were washed 10 times (as recommended by the International Embryo Transfer Society) then frozen. Later, 587 of the viable embryos were transferred singly into 347 recipient heifers imported from New Zealand, and 266 live offspring were born of which 54.1 per cent had a BSE-positive sire and a BSE-positive dam. The recipients were monitored for clinical signs of BSE for seven years after the transfer, and the offspring were monitored for seven years after birth. Twenty-seven of the recipients and 20 offspring died while being monitored but none showed signs of BSE. Their brains, and the brains of the recipients and offspring killed after seven years, were examined for BSE by histopathology, PrP immunohistochemistry, and by electron microscopy for scrapie-associated fibrils. They were all negative. In addition, 1020 non-viable embryos were sonicated and injected intracerebrally into susceptible mice (20 embryos per mouse) which were monitored for up to 700 days, after which their brains were examined for spongiform lesions. They were all negative. It is concluded that embryos are unlikely to carry BSE infectivity even if they have been collected at the end-stage of the disease, when the risk of maternal transmission is believed to be highest. (**Wrathall AE et al. Vet Rec 2002;150:365-378.)**

Miscellaneous or multiple microorganisms

(*Anaplasma marginale*) "A group of cows affected by anaplasmosis in an embryo transfer company in Mexico appeared to be less fertile. Since the underlying mechanism was unclear

and no reports were found concerning the effects of anaplasmosis on fertility, this retrospective study was undertaken in order to determine which stages of a routine embryo transfer program were altered in cows that were positive to *Anplasma marginale*. Records from three years of commercial embryo transfer in Tamaulipas (northeastern Mexico) were analyzed. Simmental cows (5% were simmental-zebu) were subjected to superovulation and nonsurgical embryo collection and transfer. Data recorded from 401 embryo collections included total number of ovulations (ova/eggs recovered) as well as the proportion of unfertilized ova, degenerate and viable (transferred) embryos. When available pregnancy rates in recipients were also recorded. Each donor cow was considered positive or negative to anaplasmosis, based on observation of *A. marginale* in blood smears prepared on the day of embryo collection; recipient females were not examined. Variables from positive cows were compared to those from negative cows. Of the 41 cows that were positive to anaplasmosis, 21.9% did not respond to hormonal treatment (no estrus or embryo recovery), compared to 8.6% of the 360 negative cows ($P<0.005$). Positive cows showed a significantly reduced ovulation rate ($P<0.005$); in these cows, the proportion of unfertilized ova increased while that of viable embryos decreased ($P<0.05$); the proportion of grade 1 embryos was not altered. Thus, it appears that reproductive function was affected by anaplasmosis, because fewer cows responded to hormonal treatment, and those responding produced fewer ovulations, fertilized ova and viable embryos. The mechanism may be related to periods of fever during hormonal treatment, ovulation or early embryonic development. Body temperature was recorded in 31 donor cows and tended to be higher in positive cows ($P=0.09$). Neverless positive cows did not exhibit the increased rate of embryo degeneration that would be expected after an increased temperature. Furthermore, 743 viable embryos that were transferred from cows in both groups produced similar pregnancy rates ($P>0.1$), suggesting that viability after transfer was not impaired. In conclusion, ovarian response (ovulation rate) and fertilization rate appear to be affected by anaplasmosis (**Dominguez et al., Proc 13th ICAR, Sydney, Australia 1996;2:13-11**).

The prevalence of serum antibody to enzootic bovine leukosis (EBL), bluetongue (BT), bovine herpesvirus-1 (BHV 1), and bovine virus diarrhea (BVD) viruses in bovine embryo donors (D) and recipients (R) from Minas Gerais State, Brazil was investigated. Of 451 sera (130 D plus 321 R) tested for antibodies against EBL virus 104 (23.1%) were positive. Of 410 sera (130 D plus 280 R) tested for antibody to BT, BHV 1 and BVD viruses the respective numbers of positive sera were 313 (76.3%), 209 (51.0%) and 153 (37.3%). Donors had significantly (Chi-square test, $p<0.05$) higher prevalence rates of antibody than recipients to EBL and BVD viruses. The donors were 60 *Bos indicus* and 70 *Bos taurus* purebred cows. Antibody to EBL virus was significantly less common among *Bos indicus* (**Castro et al., Trop Anim Hlth Prod 1992;24:173-176**).

Small ruminants

Small ruminant lentiviruses

Reproductive biotechnologies are essential to improve the gene pool in small ruminants. Although embryo transfer (ET) and artificial insemination (AI) greatly reduce the risk of

pathogen transmission, few studies have been performed to quantify this risk. The aim of this review is to contribute to the elements needed to evaluate the risk of lentivirus transmission in small ruminants (SRLV) during ET, from embryos produced in vitro or in vivo, and with the use of the semen destined for AI. The purpose is to consider the genetic possibilities of producing uninfected embryos from infected females and males or bearers of the SRLV genome. We have reviewed various studies that evaluate the risk of SRLV transmission through genital tissues, fluids, cells, and flushing media from female and male animals. We have only included studies that apply the recommendations of the International Embryo Transfer Society, to obtain SRLV-free offspring from infected female animals using ET, and the justification for using healthy male animals, free from lentivirus, as semen donors for AI. As such, ET and AI will be used as routine reproductive techniques, with the application of the recommendations of the International Embryo Transfer Society and World Organization for Animal Health. **Cortez-Romero C, Pellerin JL, Ali-Al-Ahmad MZ, Chebloune Y, Gallegos-Sánchez J, Lamara A, Pépin M, Fieni F. The risk of small ruminant lentivirus (SRLV) transmission with reproductive biotechnologies: State-of-the-art review.Theriogenology. 2013 Jan 1;79(1):1-9. doi: 10.1016/j.theriogenology.2012.09.021**

Sheep

Border disease virus (BDV) and Bovine viral diarrhoea virus (BVDV)

Forty-nine, day 5 and 6, ZP-I sheep embryos were washed three times and then exposed to 44 x 10_2 PFU of BVDV (Singer) at 35°C and 7.5% CO_2 in air for 24, 48 or 72 hours. Following incubation embryos were transferred to medium + 10% anti-BVDV serum at 35°C for 30 min to inactivate any free or unabsorbed virus. The embryos were then washed twice and transferred, one embryo per well, to 96 well microtiter plates with monolayers of bovine testicular (BTes) cells. Fifteen control embryos were similarly cultured without virus. All 64 embryos developed `in vitro' with no difference noted between inoculated and control embryos. No cytopathogenic effects developed in the supporting BTes cells as a result of co-cultivation (**Evermann et al, 24th Ann Proc Amer Assn Vet Lab Diag, 1981;pp407-426**).

Thirty-five, day 5 and 6, ZP-I embryos were cultured at 35°C on BTes cells until they had hatched. Within 24 hours of complete hatching, 23 blastocysts were exposed to 44 x 10_3 PFU of BVDV (Singer strain) at 35°C and 7.5% CO_2 in air for 24 hrs. Twelve hatched blastocysts were similarly cultured without virus. Following incubation embryos were transferred to medium + 10% anti-BVDV serum and incubated at 35°C for 30 min. They were then washed twice and transferred, one embryo per well, to 96 well microtiter plates with monolayers of BTes cells. All 35 embryos continued to develop in size with no difference between inoculated and control embryos. No cytopathogenic effects developed in the supporting BTes cells as a result of co-cultivation (**Evermann et al, 24th Ann Proc Amer Assn Vet Lab Diag, 1981;pp407-426**).

Pregnant ewes were infected on day 54 ± 2 of gestation with BDV. Testis and ovary were taken from a) 6 newborn lambs with clinical signs of Border disease, b) 3 normal newborn lambs, c) foetuses of 85 days gestation and d) a 5-month-old lamb severely affected at birth. BD antigen was detected by immunofluorescence in the supporting cells and gonocytes of foetal and newborn testes, and in cells within seminiferous tubules close to the basement

membrane in the older lamb. In the ovary fluorescence was observed in scattered large cells considered to be primary oocytes (**Gardiner, J Comp Path 1980;90:513-518**).

Bluetongue virus (BTV)

Fourteen, day 4 or 5 ZP-I embryos, collected from BTV-infected (100,000 $CEIVLD_{50}$ of biting gnat source BTV serotype 11) ewes that were superovulated and bred naturally by uninfected rams, were washed 10 x and transferred to 8 uninfected recipients (6 double transfers, 2 single transfers). Three pregnancies resulted which delivered 1 stillborn lamb, 1 single liveborn lamb and twin liveborn lambs. None of the recipients developed antibodies to BTV and they were also negative for BTV isolation 1 month post partum. The lambs were negative for antibodies to BTV precolostrally and at 15 and 30 days post partum. They were also negative at 30 days post partum for BTV isolation (cell culture and sheep inoculation) (**Hare et al, Am J Vet Res 1988;49:468-472**).

Thirty-five, day 4 or 5, embryos and 4 unfertilized eggs, collected from BTV-infected (100,000 $CEIVLD_{50}$ of biting gnat source BTV serotype 11) ewes that were superovulated and bred naturally and artificially by BTV-infected (similar dose and serotype) rams were washed 10 x and transferred to 19 uninfected recipients (18 double transfers, 1 triple transfer). Thirteen lambs were born. Recipients remained seronegative for antibodies to BTV throughout gestation, and attempts to isolate BTV from recipients and lambs at 30 days post partum were unsuccessful (**Hare et al, Am J Vet Res 1988;49:468-472**).

Thirteen ZP-I embryos that had been exposed to 10^4 pfu/ml of BTV (type 10) for 8 hours were washed 4x (each wash dilution factor 1 in 60) and transferred to 3 BTV-uninfected recipients (5 to the oviduct, 8 to the uterine horn). Nine of the 13 recipients seroconverted, four with the embryo transferred to the oviduct, five with the embryo in the uterus (**Evermann et al, 24th Ann Proc Amer Assn Vet Lab Diag, 1981;pp407-426**).
Twenty ZP-I embryos collected from 8 viremic donors were transferred to 15 BTV uninfected recipients (6 to oviduct, 9 to uterine horn). (It is not stated if the embryos were washed). Viremia and seroconversion occurred in 2/15 recipients, one of which gave birth to a seronegative lamb from which BTV could not be isolated (**Gilbert et al, Theriogenology 1987;27:527-540**).

Three experiments were done to assess use of embryo transfer in sheep as a means of controlling bluetongue transmission. The first experiment involved in vitro exposure of clean embryos to bluetongue virus (BTV) while three subsequent experiments involved the collection of embryos from BTV-infected donor ewes and their transfer to disease-free recipients.
In experiment I, 22 embryos/ova were exposed to BTV type 11 (BTV-11) for 1 h, washed 10, times in PBS and assayed in pairs for BTV. All 11 samples were positive for BTV in the 11-d-old embryonated chicken egg (ECE) assay system and 5/11 samples were positive in baby hamster kidney-21 (BHK-21) cells.
In experiment II, 5 donors were infected with BTV type 10 (BTV-10). All embryos from these donors were washed 10 times prior to assay or transfer. Thirty-three embryos/ova were

assayed in groups of 2 or 3 and none yielded virus in ECE. Two BTV-seronegative recipients each received 6 embryos and a total of 3 lambs free of BTV antibodies were delivered.
In experiments III and IV, a total of 9 donors were infected with BTV-11. All embryos were washed 10 times prior to assay or transfer. Seventy-four embryos/ova were assayed in groups of 2 or 3 and none yielded virus in ECE, while for each experiment, 6 embryos were transferred into 2 BTV-seronegative recipients. The four recipients and their 3 lambs and 2 aborted fetuses were also seronegative for BTV. (**Singh et al, Theriogenology 1997;47:1205-1214**)

Bluetongue (BT), a disease that affects mainly sheep, causes economic losses owing to not only its deleterious effects on animals but also its associated impact on the restriction of movement of livestock and livestock germplasm. The causative agent, bluetongue virus (BTV), can occur in the semen of rams and bulls at the time of peak viraemia and be transferred to a developing foetus. The risk of the transmission of BTV by bovine embryos is negligible if the embryos are washed according to the International Embryo Transfer Society (IETS) protocol. Two experiments were undertaken to determine whether this holds for ovine embryos that had been exposed to BTV. Firstly, the oestrus cycles of 12 ewes were synchronised and the 59 embryos that were obtained were exposed *in vitro* to BTV-2 and BTV-4 at a dilution of 1 x 102.88 and 1 x 103.5 respectively. In the second experiment, embryos were recovered from sheep at the peak of viraemia. A total of 96 embryos were collected from BTV-infected sheep 21 days after infection. In both experiments half the embryos were washed and treated with trypsin according to the IETS protocol while the remaining embryos were neither washed nor treated. All were tested for the presence of BTV using cell culture techniques. The virus was detected after three passages in BHK-21 cells only in one wash bath in the first experiment and two unwashed embryos exposed to BTV-4 at a titre of 1 x 103.5. No embryos or uterine flush fluids obtained from viraemic donors used in the second experiment were positive for BTV after the standard washing procedure had been followed. The washing procedure of the IETS protocol can thus clear sheep embryos infected with BTV either *in vitro* or *in vivo*

Venter E., Gerdes T, Wright I, Terblanche J. An investigation into the possibility of bluetongue virus transmission by transfer of infected ovine embryos *Onderstepoort Journal of Veterinary Research* 78(1): Art. #17, 7 pages. doi:10.4102/ojvr.v78i1.17

The objective of this study was to investigate methods of decontaminating early goat embryos that had been infected in vitro with bluetongue virus (BTV). Embryos were isolated from in vivo-fertilized BTV-free goats. Zona pellucida (ZP)-intact 8 to 16 cell embryos were cocultured for 36 h in an insert over a Vero cell monolayer infected with BTV serotype 8. The embryos were then treated with one of five different washing procedures. The treatment standard (TS) comprised phosphate-buffered saline (PBS) + 0.4% BSA (five times over for 10 s), Hank's +0.25% trypsin (twice for 45 s), and then PBS + 0.4% BSA again (five times for 10 s). The four other washing procedures all included the same first and last washing steps with PBS but without BSA (five times for 10 s) and with PBS + 0.4% BSA (five times for 10 s), respectively. The intermediate step varied for each washing procedure. Treatment 1 (T1): 0.25% trypsin (twice for 45 s). Treatment 2 (T2): 0.25% trypsin (twice for 60 s). Treatment 3 (T3): 0.5% trypsin (twice for 45 s). Treatment 4 (T4): 1% hyaluronidase (once for 5 min). After washing, the embryos were transferred and cocultured with BTV indicator Vero cell monolayers for 6 h, to detect any cytopathic effects (CPE). The effectiveness of the different washing techniques in removing the virus was evaluated

by RT-qPCR analysis. The TS, T1, T3, and T4 trypsin or hyaluronidase treatments did not eliminate BTV; Treatment 2 eliminated the virus from in vitro infected goat embryos. **Al Ahmad MZ, Bruyas JF, Pellerin JL, Larrat M, Chatagnon G, Roux C, Sailleau C, Zientara S, Fieni F. Evaluation of bluetongue virus (BTV) decontamination techniques for caprine embryos produced in vivo. Theriogenology. 2012 Oct 1;78(6):1286-93. doi: 10.1016/j.theriogenology.2012.05.024.**

Foot and mouth disease virus

The research was designed to investigate the risk of transmission of FMDV through embryos obtained from FMD seropositive donor sheep and FMD convalescent donor goats (experimentally inoculated) and transferred to free recipient animals.
Two experiments were performed in sheep. In experiment #1, 79 ewes and 4 rams were used, and in experiment #2, 33 ewes and 4 rams were used. In both experiments, animals wee selected from an endemic area from farms where an outbreak of FMD was reported. The ewes in both experiments were then subject to a 14 day heat synchronization. The donors were subjected to superovulatory treatment and insemination. The embryos were collected by surgical technique. A group of goats consisting of 48 does and 3 bucks were confined in the maximum isolation units and infected with FMD virus. A strain of 01 was inoculated by intradermalingual route. The animals developed clinical signs and became positive to viral infection associated antigen (VIAA) after normal course of the disease, and the virus was isolated from oesophageal-pharyngeal fluids (OPP). Heat synchronization, superovulatory treatment and insemination were performed, and the embryos were collected by surgical technique. Sheep and goat embryos were handled following the recommendation of the International Embryo Transfer Society (IETS). The presence of FMDV was evaluated in 185 ovine embryos and in 293 caprine embryos. In addition, flushing and washing fluids from all the donor animals were assessed. Results showed that no infectious virus was present in any material tested. The rest of the embryos (60 sheep and 100 goat) were frozen in liquid nitrogen for further transfer to FMDV seronegative recipients in an experimental field in Peninsular de Valdes, an area of Argentina free from FMDV since 1991. A total of 24 sheep embryos were transferred to 23 recipients 96 goat embryos were implanted in 38 goat recipients. There was no clinical sign of foot and mouth disease during the experimental period in any of the experimental animals. All the serological tests performed to determine antibodies against VIAA in the recipient ewes and does and in the newborns were negative. **(Caamano N, et al. Proceedings, 106th Annual Meeting of the United States Animal Health Association, St Louis, Missouri, USA, October 2002, pp 263-270.)**

Scrapie agent

Day 5 to 7, ZP-I embryos were transferred 1) from scrapie-infected ewes, bred naturally by scrapie-infected rams at varying time (14 days, 8 months and 9.5 months) after being infected, to uninfected recipients (to test for embryo transmission), 2) from uninfected

donors to scrapie-infected recipients (to test for vertical transmission via the uterus), 3) from scrapie-infected donors to scrapie-infected recipients (positive controls) and 4) from uninfected donors to uninfected recipients (negative controls). Two breeds of sheep, Suffolk and Cheviot, and two strains of scrapie agent; Mission, Texas with the Suffolks and SSBP/1 with the Cheviots, were used. To date, only two lambs from the positive control group developed scrapie. They showed clinical signs at approximately 33 months of age (**Foote et al, Proc 90th Annu Meet US Anim Hlth Assoc 1986;pp413-416**).

Reciprocal embryo transfers were carried out between **scrapie-inoculated** and scrapie-free sheep to obtain data on vertical (in utero) transmission of the disease and on the efficacy of ET for obtaining scrapie-free progeny. Approximately 50% of 179 scrapie-inoculated donors and recipients (mainly Suffolks and Cheviots) developed the disease after incubations averaging one year in Cheviots and two years in Suffolks. Embryo collections from the scrapie-inoculated donor ewes began at under one month post-inoculation in some and continued for over three years in others. In those that succumbed to the disease the times of scrapie onset after embryo collection varied from nil to 32 months. Embryos were not frozen before transfer and mostly they were washed three, not ten times. All viable embryos were transferred, irrespective of whether the ZP was intact or not.
None of 198 recipient ewes (106 Suffolk, 47 Cheviots, 45 Targhees), that had embryos transferred into them from the scrapie-inoculated ewes and were observed for over four years after ET developed scrapie. Following the ETs 129 recipients lambed and 56 of the ET lambs survived for at least 5 years without clinical signs of scrapie.
The reciprocal transfers (i.e. embryos from scrapie-free ewes transferred into scrapie-inoculated ewes) did not lead to *in utero* transmission of the scrapie agent to the offspring. There were 19 lambs in this category that were derived by caesarian section at term from inoculated recipients; ie recipients that did eventually develop scrapie. Immediately following their caesarian births these lambs were removed to a clean environment. None of them developed scrapie during observation periods lasting five or more years.
Another group, referred to as positive controls, consisting of 21 lambs conceived, gestated and reared naturally by their own scrapie-inoculated mothers, were kept under observation for two to five years. Two of these 21 lambs, both from the same Cheviot ewe, developed scrapie at 2. years and 3. years respectively but 16 of the lambs lived for five years or more without signs of scrapie. Confirmation of the presence of scrapie, or of its absence, was by histopathology, mouse inoculation and/or proteinase-K resistant protein (PrP) analysis. RFLP analyses were also done on stored tissues to ascertain the Sip ('scrapie short incubation period') genotypes of the lambs and their natural and surrogate parents. These showed that 25 to 44% of offspring in the groups of Cheviots and 61 to 83% of offspring in the groups of Suffolks carried the Sip allele, thus demonstrating a high level of susceptibility to the disease. Overall the study indicated that the method of ET employed prevents the transmission of scrapie (**Foote et al, Am J Vet Res 1993;54:1863-1868**).

In a 'preliminary' study designed to test a 'worst case scenario', embryos from scrapie-inoculated ewes were not washed between collection and transfer. Six Cheviot donor ewes; two homozygous and four heterozygous for the scrapie susceptibility (Sip = short incubation period) gene, were used together with one heterozygous Cheviot ram. The donors were inoculated subcutaneously with a virulent scrapie strain six months prior to embryo

collection and scrapie signs appeared in all of them between six weeks and six months after collection. Thirty-seven embryos were transferred without prior washing or freezing into 16 recipient ewes, 15 of which were genotyped as homozygous resistant and one heterozygous. Nine of these recipients, including the heterozygous one, survived for at least three years after transfer without signs of scrapie and the six others were culled for reasons unrelated to scrapie. After collection of semen (for AI) the ram was inoculated with scrapie and he developed the disease 11 months later.
From the 16 recipient ewes a total of 26 lambs were born but six died within a year of birth for reasons unrelated to scrapie. Three of the remainder were of a homozygous resistant genotype, 11 were heterozygous and six were homozygous susceptible. All six lambs of the latter genotype developed scrapie signs at between two and two and a half years of age and were confirmed scrapie positive by brain histopathology or other tests at slaughter. The remaining 14 lambs have remained healthy. The assumption that ET will always prevent transmission of scrapie is challenged by this study but the need for further work using IETS recommended washing procedures is acknowledged (**Foster et al, Vet Rec 1992;132:341-343**).

Thirty Awassi ewes and 4 Awassi rams were used to produce 163 embryos for export from Israel to New Zealand in 1991. A range of precautions were used to prevent introduction of new pathogens into New Zealand via the embryos but the main concern was scrapie. IETS recommendations were followed for hygenic collection and processing of embryos. Two trypsin washes were included. Soon after the embryo collection the donor ewes and rams were slaughtered and various tissues were taken for virological and other tests to exclude the possibility of scrapie, rabies, FMD, bluetongue, maedi/visna, SPA, nasal adenocarcinoma and brucellosis. Spleen, retropharyngeal lymph node, mesenteric lymph node and medulla oblongata were taken for scrapie bioassay. Embryos were taken to Soames Is. quarantine station, New Zealand where 157 were transferred into recipient ewes and 39 lambs were born as a result. Forty days after transfer the recipients were tested serologically for *Chlamydia*, Q-fever, *Brucella melitensis*, bluetongue and FMD with negative results. At the same time the scrapie bioassays were initiated by inoculation (intracerebral, subcutaneous and intraperitoneal) of goat kids with the tissues mentioned above. These goats and the generations of Israeli Awassi sheep offspring are being kept under observation for 3. years after which if there is no evidence of scrapie the sheep will be allowed entry into New Zealand. This appears to be the first time that scrapie bioassay has been used on a large scale in connection with ET (**Rapoport and Allison, Proc Sheep Vet Soc 1991;15:71-80**).

"This investigation studied the maternal transmission of scrapie in sheep by using embryo transfer to examine the viability of highly susceptible offspring derived from scrapie-affected and uninfected donors. The study also examined the effect of washing the embryos. Scrapie occcurred in both washed and unwashed embryo-derived *Sips*AsA progeny from both groups of donor ewes." (Table 1)
TABLE 1. Ages at death (days) of scrapie cases in the different groups of embryo transfer progeny. Numbers of donor sheep are given in brackets.

TABLE 1. Ages at death (days) of scrapie cases in the different groups of embryo transfer progeny. Numbers of donor sheep are given in brackets.

Progeny Sip genotype of embryo	Donor ewes: Uninjected sApA (3) Number born	Uninjected sApA (3) Age of death (d)	Injected sApA clinical (3) Number born	Injected sApA clinical (3) Age of death (d)	Injected pApA nonclinical (3) Number born	Injected pApA nonclinical (3) Age of death
sAsA washed	4	860,1000,2 survived	3	803,884,1267	---	---
sAsA unwashed	4	778, 886, 888, 888	2	769, 1 survived	---	---
sApA washed	3	3 survived	1	1 survived	2	2 survived
sApA unwashed	4	4 survived	2*	1 survived	3*	2 survived

*Intercurrent death
(NOTE: This table taken from paper cited below)

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The authors conclusions: "As a result, the earlier observation that scrapie might pass via the unwashed embryo to develop as disease in adult sheep has to be reassessed. Several other implications of the work are considered, including the possibility that natural scrapie is not purely a genetic disease." (**Foster et al, Vet Rec 1996;138:559-562**).

This study investigated whether the transmission of naturally occurring scrapie in sheep can be prevented using embryo transfer. Embryos were collected from 38 donor ewes in a Suffolk sheep flock with a high incidence of naturally occurring scrapie, treated with a sanitary procedure (embryo washing) recommended by the International Embryo Transfer Society and then transferred to 58 scrapie-free recipient ewes. Ninety-four offspring were produced. None of the offspring or the recipient ewes developed scrapie. Furthermore, offspring derived from embryos collected from donor ewes bred to the immunohistochemically positive ram did not develop scrapie. We conclude that scrapie was not transmitted to offspring via the embryo nor was the infective agent transmitted to recipient ewes during embryo transfer procedures. (**Wang S et al. Theriogenology 2001;56:315-327).**

The genetic sequence of the ovine prion protein (PrP) gene between codons 102 and 175 with emphasis on ovine PrP gene codons 136 and 171 was determined, and the polymorphic distribution of the ovine PrP gene in the scrapie-exposed Suffolk embryo donors and offspring from these donors that were transferred to scrapie-free recipient ewes was investigated in this study. The most common genotype was AA(136)QQ(171) (70% and 63% in the donor and offspring flocks, respectively), which is considered a high risk genotype in US Suffolk sheep. Although embryos were collected from scrapie-positive donors and many embryos had the high risk genotype, no scrapie occurred in the resulting offspring. Based upon the results of this study, we conclude that vertical transmission of scrapie can be circumvented using embryo transfer procedures even when the offspring have the high risk genotype. **(Wang S et al. Theriogenology 2002;57:1865-1875)**

The Cheviot flock at the Institute for Animal Health's Neuropathogenesis Unit (NPU) has endemic scrapie, which affects primarily VRQ/VRQ sheep and at high frequency. A new flock with a full range of PrP genotypes, including the highly susceptible VRQ/VRQ, has been produced on a separate site, from animals in the NPU breeding flock, and it remains scrapie-free after eight years. In contrast, in a parallel flock at the NPU farm, scrapie has reappeared after five years, although the animals were kept in separate accommodation from the

scrapie-affected sheep. During this time the NPU breeding flock continued to have scrapie cases. Although it is known that highly susceptible sheep can remain free of infection in a clean environment, this is the first report of the infection being removed successfully from the bloodlines of scrapie-affected sheep. The results confirm that scrapie is not a genetic disease dependent only on the PrP gene sequence, but requires both genetic susceptibility and an infectious agent. **(Note: While the abstract does not mention it, embryo transfer was used to establish the scrapie free flock. Further, in the discussion the following statement is made: "...it is considered highly unlikely that the disease can be transmitted via the embryo".) (Foster et al, Veterinary Record 2006;159:42-45).**

The objective of the study was to examine whether or not the preimplantation embryo can act as a carrier of classic scrapie infection. The study was carried out on quarantined premises with sheep of highly susceptible scrapie genotypes. Uninfected embryos, collected from New Zealand-derived Suffolk ewes, were surgically transferred into recipient ewes that were also of New Zealand origin. Seventeen negative control lambs were born on the study premises from these embryo transfers. Thirty-nine experimental lambs were from embryos collected from naturally infected donor ewes. The experimental lambs were also born on the study premises after their surgical transfer into recipient ewes of New Zealand origin. These embryos had been collected from donor ewes in a scrapie-infected flock where the ewes were clinically sick with scrapie or developed clinical scrapie after embryo collection. All lambs were confirmed as scrapie susceptible of the ARQ/ARQ genotype. Twenty-eight experimental animals survived to the end point of the study at 5 yr of age with a mean survival of 1579 d. In the negative control group, 12 of 17 sheep survived to 5 yr of age with a mean survival of 1508 d. Postmortem examinations were carried out on all animals derived by embryo transfer, and in none was histologic or immunohistochemical evidence of scrapie found. In contrast, in the originating flock the majority of scrapie cases occurred in ARQ/ARQ genotyped animals where a 56% mortality from scrapie had been recorded in animals of this genotype. Thus, the study provides no evidence for transmission of scrapie and reinforces published evidence that vertical transmission of scrapie may be circumvented by embryo transfer procedures.

Low JC, Chambers J, McKelvey WA, McKendrick IJ, Jeffrey M. Failure to transmit scrapie infection by transferring preimplantation embryos from naturally infected donor sheep. Theriogenology. 2009;72(6):809-16.

Natural scrapie transmission from infected ewes to their lambs is thought to occur by the oral route around the time of birth. However the hypothesis that scrapie transmission can also occur before birth (in utero) is not currently favoured by most researchers. As scrapie is an opportunistic infection with multiple infection routes likely to be functional in sheep, definitive evidence for or against transmission from ewe to her developing fetus has been difficult to achieve. In addition the very early literature on maternal transmission of scrapie in sheep was compromised by lack of knowledge of the role of the PRNP (prion protein) gene in control of susceptibility to scrapie. In this study we experimentally infected pregnant ewes of known PRNP genotype with a distinctive scrapie strain (SSBP/1) and looked for evidence of transmission of SSBP/1 to the offspring. The sheep were from the NPU Cheviot flock, which has endemic natural scrapie from which SSBP/1 can be differentiated on the basis of histology, genetics of disease incidence and strain typing bioassay in mice. We used embryo

transfer techniques to allow sheep fetuses of scrapie-susceptible PRNP genotypes to develop in a range of scrapie-resistant and susceptible recipient mothers and challenged the recipients with SSBP/1. Scrapie clinical disease, caused by both natural scrapie and SSBP/1, occurred in the progeny but evidence (including mouse strain typing) of SSBP/1 infection was found only in lambs born to fully susceptible recipient mothers. Progeny were not protected from transmission of natural scrapie or SSBP/1 by washing of embryos to International Embryo Transfer Society standards or by caesarean derivation and complete separation from their birth mothers. Our results strongly suggest that pre-natal (in utero) transmission of scrapie may have occurred in these sheep.
Foster JD, Goldmann W, Hunter N. Evidence in sheep for pre-natal transmission of scrapie to lambs from infected mothers. PLoS One. 2013 Nov 18;8(11):e79433.

Naturally scrapie infected VRQ/VRQ (n = 47) and ARR/ARR control (n = 11) Romanov sheep were super-ovulated and mated with matching genotype rams. Embryos were collected in dams 6 to 7 days after the oestrus and embryos were selected according to guidelines from the International Embryo Technology Society. A total of 267 transferable and 149 non-transferable embryos were collected in the VRQ/VRQ dams and 55 transferable and 23 non-transferable embryos were collected in ARR/ARR controls. The presence of prion seeding activity in non-transferable and transferable embryos from each dam was tested by Protein Misfolding Cyclic Amplification. After four amplification rounds, none of the reactions seeded with embryos displayed detectable levels of abnormal PrP. In contrast, Protein Misfolding Cyclic Amplification reactions seeded with a 10^{-8} diluted 10% brainstem homogenate from a VRQ/VRQ infected dam were found to be PrP^{res} positive. Among the 267 transferable VRQ/VRQ embryos, 204 embryos collected from 19 different VRQ/VRQ infected dams were inoculated to ovine PrP transgenic mice (tg338 mice) by intracerebral route. Nineteen embryos from two ARR/ARR dams were inoculated as controls. No clinical signs indicative of Transmissible Spongiform Encephalopathy and no PrP^{Sc} accumulation were observed in any of the tg338 mice inoculated with embryos. Within the limit of the experiment (intrinsic sensitivity of the bioassay and Protein Misfolding Cyclic Amplification) these results indicate that the residual risk of the presence of detectable infectivity or positive seeding activity in transferable embryos from other VRQ/VRQ sheep that would be infected by the Langlade scrapie agent is lower than 1.79% and 1.37%, respectively (upper bound of the exact binomial 95% confidence interval). **Olivier Andreoletti - Unité Mixte de Recherche INRA / ENVT 1225 Interactions Hôtes – Agents Pathogènes (UMR INRA ENVT 1225 – IHAP), 2017. Assessment of classical scrapie infectivity in sheep embryos. EFSA supporting publication 2017:EN-1300. 21 pp. doi:10.2903/sp.efsa.2017.EN-1300**

Campylobacter fetus

A total of 460 ZP-I, days 3-5 mice embryos were collected and used as follows: 184 were exposed to *C.fetus* ss *fetus* and 46 were controls (non contaminated); 182 were exposed to *C.fetus* ss *venerealis* and 48 were controls. In addition, 164 ZP-I, day 6 sheep embryos were collected and exposed to *C.fetus* ss *venerealis* and these were compared with 51 control embryos. The mouse embryos were exposed for 48 hours at 37°C to a range of 0.01-100 ID_{50} and the sheep embryos to a range of 0.1-100 ID_{50}. After 48 hours, the non-degenerated embryos were washed, homogenized and then cultured simultaneously to the washing fluids

to search for *Campylobacter fetus.* After 48 hours, a dose-effect on the survival rate of the embryos (p <0.001) and a duration-effect (p <0.01) of the contamination were shown both for mice and sheep embryos for the two sub-species of *Campylobacter fetus*. In neither mice nor sheep were *Campylobacter fetus* isolated from the last washing mediums or from the homogenised embryos (**Guerin et al, Bull Acad Vet France 1988;61:63-78**).

Chlamydophila abortus (formerly: Chlamydia psittaci)

"Ten ewe lambs were infected with inoculum, containing 52,000,000 inclusion forming units of chlymidia isolates T6 and G188, approximately two weeks before lambing. These animals were then maintained in isolation throughout the study. Five infected animals with high titres of compliment fixing antibodies (CFT) were used as donors for multiple ovulation embryo transfer (MOET) during the following breeding season. Four of the five donors yielded 43 normal blastocysts which were transformed to 16 recipients from a seronegative Enzootic Abortion of Ewes (EAE) free flock. The 10 infected animals were then mated to an EAE free ram. All animals were observed for signs of EAE during late pregnancy. Blood samples were collected 6 weeks before and within 2 weeks of parturition for CFT. Vaginal swabs were taken within two weeks of parturition and tested for chlymidia using an enzyme-linked immunoassay.
Nine of the ten inoculated animals lambed the following year. Six had significant CFT titres (>1:32) and positive vaginal swabs with both tests. One animal had a CFT titre >1:32 but negative vaginal swabs. Furthermore, three of the five donors had titers ranging from 1:64 to 1:128 and vaginal swabs positive for both tests. Therefore these animals were infected with EAE, although thay had apparently normal pregnancies and none aborted. Eight of the 16 recipients receiving embryos from the inoculated donors produced 14 normal healthy lambs. Furthermore, 12 of these lambs were the progeny of the three progeny positive for EAE. However, all of the recipients had undetectable CFT titres and negative vaginal swabs. These results demonstrate that it is posssible to produce EAE free lambs from infected ewes by transferring their embryos to EAE free recipients (**Williams et al, Proc 13th ICAR, Sydney, Australia 1996;p213g**).

The objective of the study was to determine if embryo transfer was a feasible method to break the cycle of infection of *Chlamydia psittaci* in sheep. Five sheep artificially exposed (per os) in late pregnancy to T76 and G188 isolates of *Chlamydia psittaci (ovis)* and seropositive to the complement fixation test were used as donors for MOET. Three ewes excreted the organism at parturition one year after exposure and one animal had a high CFT titer indicative of clinical disease. Twelve embryos from these three donors were transferred to 7 disease-free recipients and “survived” without infection of offspring or recipients. All embryos were washed ten times as suggested by IETS. Thus embryos collected from the infected animals did not appear to transmit the chlamydia causing enzootic abortion. (**Williams AFJ et al. Veterinary Journal 1998;155:79-84**)

Retroviruses

The sheep genome contains multiple copies of endogenous betaretroviruses highly related to the exogenous and oncogenic jaagsiekte sheep retrovirus (JSRV). The endogenous JSRVs (enJSRVs) are abundantly expressed in the uterine luminal and glandular epithelia as well as in the conceptus trophectoderm and are essential for conceptus elongation and trophectoderm growth and development. Of note, enJSRVs are present in sheep and goats but not cattle. At least 5 of the 27 enJSRV loci cloned to date possess an intact genomic organization and are able to produce viral particles in vitro. In this study, we found that enJSRVs form viral particles that are released into the uterine lumen of sheep. In order to test the infectious potential of enJSRV particles in the uterus, we transferred bovine blastocysts into synchronized ovine recipients and allowed them to develop for 13 days. Analysis of microdissected trophectoderm of the bovine conceptuses revealed the presence of enJSRV RNA and, in some cases, DNA. Interestingly, we found that RNAs belonging to only the most recently integrated enJSRV loci were packaged into viral particles and transmitted to the trophectoderm. Collectively, these results support the hypothesis that intact enJSRV loci expressed in the uterine endometrial epithelia are shed into the uterine lumen and could potentially transduce the conceptus trophectoderm. The essential role played by enJSRVs in sheep reproductive biology could also be played by endometrium-derived viral particles that influence development and differentiation of the trophectoderm.
Black SG, Arnaud F, Burghardt RC, Satterfield MC, Fleming JA, Long CR, Hanna C, Murphy L, Biek R, Palmarini M, Spencer TE. Viral particles of endogenous betaretroviruses are released in the sheep uterus and infect the conceptus trophectoderm in a transspecies embryo transfer model. J Virol. 2010 Sep;84(18):9078-85. Epub 2010 Jul 7.

Maedi-Visna virus (MVV)

Sheep embryos collected from persistently MVV-infected, seropositive, donors were transferred into sero-negative recipients. Twenty recipients received fresh embryos, and 16 recipients received frozen-thawed embryos. Twelve lambs were born. All of the recipients and lambs remained seronegative for 3 years **(Dawson and Wilmut, unpublished - cited by Wrathall in Proc Inter Embr Movement Symp, Montreal, Canada 1988;pp123-125).**

Efforts to eradicate MVV from a University teaching flock of 333 ewes were initiated in 1987 and, primarily by the use of serological (AGID) testing and culling positive sheep, the MVV +ve rate was reduced from 37.8% to 0.1% by 1992. Some rams that were tested MVV negative were introduced into the flock but no females were introduced. To capitalize on the genetic merit of two seropositive ewes of superior type in the flock embryos were collected from them and transferred fresh or frozen. A total of 9 lambs were produced and survivors of this group are being monitored to determine whether MVV can be transmitted via ET **(Youngs, Proc 111th Ann Mtg Iowa Vet Med Assoc, Jan 22-24, 1993).**

Preimplantation embryos were collected from 10MVV positive donor ewes seven days following oestrus and washed according to IETS standards (including trypsinisation). Nucleic acids (RNA and DNA) from these embryos, and the accompanying uterine fluids collected at surgery, were subjected to PCR amplification using MVV-specific primers and the identity of PCR products was confirmed by Southern blot hybridisation. PCR sensitivity was quantified using positively seeded control media (positive detection at 10 virus particles per embryo).

No virus was detected in either the uterine washings or associated with the embryos. These results indicate that non infective germplasm may be recovered from MVV positive ewes **(Woodall et al, Proc 3rd Int Sheep Vet Conf, Edinburgh, June, 1993;p126).**

A technique (based on sequential RNA and DNA isolation and analysis of the nucleic acids by PCR) was developed to detect RNA or DNA viruses in single embryos. As an internal standard a constituitively expressed embryo gene was also analysed. RNA extraction was more efficient than DNA extraction but positive results were obtained for the internal standard gene from 84% (RNA) and 30% (DNA) of embryos tested (**Woodall et al, J Virol Meth 1994;46:263-274**).

The aim of this study was to examine the Maedi-Visna virus (MVV) infection status of oocytes, cumulus cells, and follicular fluid taken from 140 ewes from breeding flocks. MVV proviral-DNA and MVV RNAwere detected using nested-PCR and RT-PCR MVV gene amplification, respectively in the gag gene. Nested-PCR analysis for MVV proviral-DNA was positive in peripheral blood mononuclear cells in 37.1% (52/140) of ewes and in 44.6% (125/280) of ovarian cortex samples. The examination of samples taken from ovarian follicles demonstrated that 8/280 batches of cumulus cells contained MVV proviral-DNA, whereas none of the 280 batches of oocytes taken from the same ovaries and whose cumulus cells has been removed, was found to be PCR positive. This was confirmed by RT-PCR analysis showing no MVV-viral RNA detection in all batches of oocytes without cumulus cells (0/280) and follicular fluid samples taken from the last 88 ovaries (0/88). The purity of the oocyte fraction and the efficacy of cumulus cell removal from oocytes was proved by absence of granulosa cellspecific
mRNA in all batches of oocytes lacking the cumulus cells, using RT-PCR. This is the first demonstration that ewe cumulus cells harbor MVV genome and despite being in contact with these infectedcumulus cells, the oocytes and follicular fluid remain free from infection. In addition, the enzymatic and mechanical procedures we used to remove infected-cumulus cells surrounding the oocytes, are effective to generateMVVfree-oocytes fromMVV-infected ewes. **(Cortez Romero et al, Theriogenology 2006;66:1131-1139).**

The transmission of maedi-visna virus (MVV) through embryos collected from virus infected ewes and then transferred to uninfected recipients was investigated. Early sheep embryos were isolated from four in vivo-fertilized, MVV infected donors, which were determined to be positive by ELISA and PCR. A total of 52 morula and blastocyst stage embryos were washed ten times and transferred (2 or 3 per recipient) to 22 serologically negative sheep. Three ewes became pregnant, delivering three live kids out of which one died immediately after delivery. All two kids were then kept over 72 weeks of age and neither MVV antibodies nor proviral DNA was found in the blood samples collected before colostrum suckling, as well as after 14, 32, 56 and 72 weeks of age. Additionally, no active virus particles were found when tested by syncytia formation assay. This study demonstrated that it is possible to obtain transferable stage embryos from donor ewes infected with MVV, in vivo fertilized by infected rams, and that collected embryos are unlikely to transmit MVV infection to the resulting offspring. (**Rola M, et al. Medycyna Wet, 2006; 62(8) 942-945 (paper in Polish with English summary).)**

Maedi Visna virus (MVV) causes progressive degenerative inflammatory disease in multiple organs including the lungs (pneumonia, 'maedi'), mammary gland, joints and nervous system (meningoencephalomyelitis, 'visna') in sheep. Maedi Visna Virus has been detected in macrophages of several tissues and epithelial cells in vivo: bone marrow, cells of the central nervous system, lung and bronchial tissues, milk epithelial cells recovered from milk samples and epithelial cells of mammary tissue. However, the presence of MVV in the genital tracts of naturally infected ewes has not previously been studied. The aim of this study was to use nested-PCR, targeting the gag gene, to determine whether genital tissues (ovaries, oviducts and uterus) from 83 ewes originating from various breeding herds in the South-East of France were positive for MVV-proviral DNA. Peripheral blood mononuclear cells (PBMC) tested positive for MVV-proviral DNA, using nested-PCR analysis, in 57.8% of ewes (48 ⁄ 83). The provirus was also identified in 47% (78 ⁄ 166) of the ovaries, 38.6% (64 ⁄ 166) of the oviducts and 45.8% (38 ⁄ 83) of the uteri sampled. These findings clearly demonstrate, for the first time, that tissue samples from the genital tract of ewes (ovary, oviduct and uterus) can be infected with MVV. This suggests that there is a risk of vertical and ⁄ or horizontal transmission of MVV during embryo transfer from embryos produced in vivo or in vitro.
Cortez-Romero C, Fieni F, Russo P, Pepin M, Roux C, Pellerin J. Presence of Maedi Visna Virus (MVV)-Proviral DNA in the Genital Tissues of Naturally Infected Ewes. Reprod Domest Anim. 2010 doi: 10.1111/j.1439-0531.2010.01608

Sheep pulmonary adenomatosis (SPA)

Between November 1985 and January 1990, 122 embryos, recovered from 50 donor ewes from endemically infected flocks were inserted into 82 recipient ewes. SPA was confirmed by histopathological examination in 22 of the donors. Of 71 lambs born full term, 58 have been reared including 26 derived from embryos of donors with lesions of SPA. Eleven embryos from matings between a ram with advanced SPA and four donor ewes have been inserted into seven recipient ewes. Of five lambs born full term, four have been reared. At July 1990 no evidence of SPA has been found in the recipient flock including the progeny derived by ET, whose ages range from two months to four years. In view of the long incubation period of SPA final conclusions cannot yet be drawn about the efficacy of ET in preventing transmission of the disease. The recipient ewes and the lambs derived by ET are being monitored throughout their natural life span for clinical evidence of SPA and lungs are examined post-mortem (**Parker et al, Theriogenology 1991;35:252 abstr**).

The study was carried out to determine the effectiveness of ET, conducted according to IETS Manual guidelines, for deriving SPA-free progeny from donor ewes taken from SPA-infected flocks. Between November 1985 and January 1991, 206 embryos recovered from 76 donor ewes from endemically infected flocks were inserted into 131 recipients in an isolated flock with a long history of freedom from SPA. Ninety-eight of the embryos were from 33 donor ewes in which SPA was confirmed by radiological and histopathological examination. They were transferred to 64 recipient ewes. Thirty-eight of these ewes produced 51 lambs (from 26 donor ewes) of which 43 lambs from 32 recipient ewes (and from 24 donor ewes) were reared. One hundred and eight embryos were derived from 43 donor ewes in which no lesions of SPA were found on radiological and histopathological examination. They were transferred to 67 recipient ewes. Forty-nine of these ewes produced 74 lambs (from 33

donor ewes) of which 62 lambs from 43 recipient ewes (and from 30 donor ewes were reared.
Lambs resulting from ET's and the recipient ewes were culled when at least five years old or five years from the time of embryo insertion respectively unless dying or euthanased earlier due to incidental disease. A total of 38 ET lambs from SPA positive donors and 55 from donors in which no lesions of SPA were detected, survived for at least five years. A total of 27 recipients of embryos from confirmed positive donors and 36 recipeints of embryos from donors in which no lesions were detected, survived for at least five years from ET. Radiological and histopathological examination of the lungs of all sheep was carried out post mortem and no evidence of SPA was found in any recipients and their ET offspring. (**Parker et al, Theriogenology 1997;47:379 abstr**)

Two hundred and fifteen embryos from 76 ewes from flocks with endemic SPA and bred to uninfected rams were washed using IETS procedures and transferred to 131 uninfected recipients. Recipient and progeny were housed in a closed, isolated SPA-free flock. Thirty-eight of 51 progeny from SPA-positive ewes and 55 of 74 progeny from ewes in which no lesions of SPA were detected survived for at least five years after birth. Also, 11 embryos from four uninfected ewes bred to an SPA-infected ram were transferred to 7 recipients and 4 of 5 progeny born to 4 recipients survived for at least 5 years. No evidence of SPA was found in progeny or recipients from either study. On the basis of pathological as well as clinical findings, it was concluded that embryo transfer could be used to prevent transmission of SPA from ewes in infected flocks whether or no the dam or sire showed clinical signs. (**Parker BNJ, et al. Vet Rec 1998;142:687-689**)

Brucellosis

The efforts have been made to analyses the risks associated with importing in vivo derived ovine embryos. Few studies have been made of the interaction between embryos and pathogens in small ruminants in comparison with those conducted on bovine embryos. As a consequence, few disease agents affecting sheep and goats have been categorized by the International Embryo Transfer Society Import/Export Committee (IETS) Research subcommittee for their capacity to be transmitted via ET. The characteristics of embryos and their interactions with pathogens cannot be generalized. Embryos of different species differ in the glycoprotein composition of the ZP. This structure in sheep and goats differs from that in cattle (Chen and Wrathall 1989; Dunbar et al 1991). It has been suggested that ovine ZP is 'stickier' than that of bovine embryos, and less likely to resist penetration and adherence of pathogens (Singh et al 1997). This may explain the higher probability of binding between the ZP and various pathogens in these species. In the absence of relevant information, infection patterns for sheep and goat embryos are based on studies of infection of bovine genetic material. The risk estimate for B ovis in sheep without risk management measures is negligible for embryos however in the case of B melitensis without risk management measures is high for embryos. To the best of our knowledge, no work has yet been done to investigate the possible carriage of Brucella melitensis by embryos despite the fact that this is an important cause of disease in small ruminants particularly in the peri-Mediterranean regions. The experiment was conducted to test for the recovery of Brucella oganisms from uterine flushings of sero positive embryo donor females. We used 14 donor ewes with history of being chronically seropositive to the plate serum agglutination acidified plate antigen card (B. abortus, B. melitensis). Donors were superovulated with single shot FSH (9 cc Folltropin)

combined with and eCG (500 I.U.) injection 24h prior to sponge withdrawal and artificially inseminated at 24 hours following the onset of estrus with Brucella free semen. Samples of recovered flushing medium were placed into a validated in vitro culture system to detect the presence of brucella bacteria. Uterine flushings from donor females were free from B mellitensis, ovis and abortus contamination. It was concluded that the superovulatory treatment is not likely to reactivate the release of Brucella into the uterine lumen during the period when embryos are normally collected. **ABSTRACT - Emsen, M. Kutluca Korkmaz, H. Demirezer. Detection of Brucellosis in seropositive superovulated sheep embryo flushing media E. A270E Embryology, Developmental Biology and Physiology of Reproduction. Proceedings of the 30th Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Foz do Iguaçu, PR, Brazil, August 25th to 27th, 2016, and 32nd Meeting of the European Embryo Transfer Association (AETE); Barcelona, Spain, September 9th and 10th, 2016.**

Brucella ovis (B.ovis)

Seventy-three zona pellucida-intact ova were collected surgically from 15 superovulated, *Brucella*-free mixed-breed ewes. Twenty-one groups containing one to five ova were incubated in medium containing *B.ovis*. Subsequently, seven and five groups were incubated for 24 and 4 hours, respectively, at 37°C in medium containing penicillin and streptomycin, while nine groups were not treated with antibiotics. All groups of ova were washed 10 times, and ova and sequential washes were cultured for the presence of *B.ovis*. *Brucella* were isolated from seven of the nine groups of non treated ova and from the 10th wash for six of these groups. While *Brucella* were detected in fewer washes after antibiotic treatment, the organism was still isolated from 11 of the 12 treated groups. It is concluded that standard washing techniques are not reliable for removing *B.ovis* from exposed, zona pellucida-intact, ovine ova (**Wolfe et al, Theriogenology 1988;30:387-393**).

One hundred and fourteen zona pellucida-intact embryos were collected on day 6 from *Brucella*-free ewes that had been mated with *Brucella*-free rams. The embryos were washed 10 times in PBS to which had been added a penicillin/streptomycin solution (100,000 iu and 5mg per litre) to ensure the elimination of all bacteria. To remove all traces of the antibiotics the embryos were submitted to a further 10 washes in a solution without antibiotics. The embryos were distributed into 5 groups: the first, a control group (n = 6), was not exposed to *B. ovis*; the second group (n = 34) was co-cultured for 4 h. with 1.0 x 10_9 CFU of *B. ovis* per ml and the third group (n = 40) was co-cultured with 1.7 x 10_{11} CFU/ml. In a further set of experiments 34 embryos were co-cultured with 2.5 x 10_9 CFU *B. ovis* per ml for 20 h. then washed 10 times in PBS with added antibiotics. Subsequently half these embryos (group 4) were treated with gentamycin (5mcg/ml) at 37°C for 30 minutes, and half were left untreated (group 5). After washing each group of embryos was grinded into a micropotter then inoculated onto Columbia blood agar base plates for *B. ovis* isolation.
No *B. ovis* was found in the first (control) group of embryos or in washes of these, but viable organisms were cultured from the embryos and from all ten washes in groups 2 to 5. Although strains of *B. ovis* had previously been shown to be susceptible in vitro to the doses of gentamycin used in this study the 30 minutes treatment failed to eliminate all the organisms. This raises the possibility that the treatment period was too short or the patterns of antibiosusceptibility of bacteria that are adherent to the zona pellucida may differ from those in other circumstances (**Guerin et al, Proc 12th ICAR, The Hague 1992;3:1307-1309**).

A study was designed to determine if the number of bacterial cells adhering to the ZP after in vitro exposure to *B.ovis* would constitute an infective dose for susceptible recipient ewes. Donor and recipient *Brucella*-negative ewes and three *Brucella*-negative rams were maintained in isolation from other sheep during the course of this study. Blood was collected from all sheep 30 and 15 days prior to the study, from donors and recipients on the day of embryo recovery and transfer, from donors and rams monthly thereafter, and from recipients twice monthly thereafter. Sera were examined for anti-*Brucella* antibodies using the complement fixation (CF) and enzyme-linked immunosorbent assay (ELISA) tests. Embryos were washed 10x in Dulbecco's phosphate-buffered saline (PBS) plus 2% fetal bovine serum (FBS) and then exposed for 3 hours to *B.ovis* (10^8 colony forming units per ml) suspended in PBS+20% FBS. Subsequently, the exposed embryos were washed 10x in PBS+2% FBS without antibiotics and transferred to synchronized recipient ewes. Additional ZP-I ova were likewise exposed, washed, and cultured immediately for isolation of *Brucella*. Recipient ewes were examined ultrasonographically for pregnancy at 35 and 50 days after transfer. Seventeen exposed embryos were transferred, in groups of one to three, to seven *Brucella*-negative recipient ewes and an additional 15 ZP-I ova were cultured after exposure for isolation of the organism (positive controls). *Brucella* were isolated from all positive control ova. No pregnancies were established in recipient ewes receiving the *B.ovis*-exposed ova. However, seroconversion occurred in four of the seven recipients despite the fact that rams, donors, and other ewes in the flock remained seronegative during the same period. Two recipients were positive and two were suspect on the ELISA test, while three were positive and one was a suspect on the CF test. Seroconversation initially occurred between 2 and 8 weeks after receipt of exposed embryos. High titers have persisted in three of the recipient ewes for 6 months. Results indicated that the number of bacterial cells adhering to the ZP-I ovine embryos after in vitro exposure to *B.ovis* and washing can constitute an infective dose for susceptible recipient ewes (**Riddell, MG, et al, Theriogenology 1989;31:248 abstr and , Riddell MG, et al, Theriogenology 1990;34:965-973**).

Brucella abortus (B. abortus)

Fifty-three zona pellucida-intact ova were collected surgically from superovulated, *Brucella*-free mixed-breed ewes. Groups containing two to seven ova were incubated in medium containing *Brucella abortus*. All groups of ova were then washed 10 times in antibiotic-free medium and ova and sequential washes were cultured for the isolation of *B.abortus*. *Brucella* were not found beyond the fifth wash for any group of ova, but were isolated from one of 12 groups of ova. Results indicate that mechanical washing in the absence of antibiotics is advantageous, but is not totally reliable for removing *B.abortus* from exposed, zona pellucida-intact ova (**Riddell MG, et al, Theriogenology 1989;31:895-901**).

Goats

Bluetongue virus

Seventy-eight day 7 embryos, collected at slaughter from nine donor animals, were washed 10x, frozen down in liquid nitrogen, and subsequently thawed preparatory to transfer. Sixty-three were transferred into 19 recipients of which 13 became pregnant and gave birth to 19 kids. The donor females were from a flock in Guadeloupe and 47% of them were seropositive for BTV. The recipients were dairy goats located in France. None of them and none of the kids developed antibodies to BTV. Virus isolation studies on the flush fluids and the last wash were negative (**Chemineau et al, Theriogenology 1986;26:279-290**).

The three objectives of this study were to investigate whether cells of early goat embryos isolated from in vivo fertilized goats interact with bluetongue virus (BTV) in vitro, whether the embryonic zona pellucida (ZP) protects early embryo cells from BTV infection, and whether the 10 wash cycles recommended by the International Embryo Transfer Society (IETS) for bovine embryos effectively decontaminates caprine embryos exposed to Bluetongue Virus (BTV) in vitro. Donor goats and bucks were individually screened and tested negative for the virus by RT-PCR detection of BTV RNA in circulating erythrocytes. ZP-free and ZP-intact 8-16 cell embryos were co-cultured for 36 h in an insert over a Vero cell monolayer infected with BTV. Embryos were washed 10 times in accordance with IETS recommendations for ruminant and porcine embryos, before being transferred to an insert on BTV indicator Vero cells for 6 h, to detect any cytopathic effects (CPE). They were then washed and cultured in B2 Ménézo for 24 h. Non-inoculated ZP-free and ZP-intact embryos were submitted to similar treatments and used as controls. The Vero cell monolayer used as feeder cells for BTV inoculated ZP-free and ZP-intact embryos showed cytopathic effects (CPE). BTV was found by RT-qPCR in the ten washes of exposed ZP-free and ZP-intact embryos. In the acellular medium, the early embryonic cells produced at least 10(2.5) TCID(50)/ml. BTV RNA was detected in ZP-free and ZP-intact embryos using RT-qPCR. All of these results clearly demonstrate that caprine early embryonic cells are susceptible to infection with BTV and that infection with this virus is productive. The washing procedure failed to remove BTV, which indicates that BTV could bind to the zona pellucida. **Al Ahmad MZ, Pellerin JL, Larrat M, Chatagnon G, Cécile R, Sailleau C, Zientara S, Fieni F. Can bluetongue virus (BTV) be transmitted via caprine embryo transfer? Theriogenology. 2011 Jul 1;76(1):126-32. Epub 2011 Mar 12.**

The objective of this study was to investigate methods of decontaminating early goat embryos that had been infected in vitro with bluetongue virus (BTV). Embryos were isolated from in vivo-fertilized BTV-free goats. Zona pellucida (ZP)-intact 8 to 16 cell embryos were cocultured for 36 h in an insert over a Vero cell monolayer infected with BTV serotype 8. The embryos were then treated with one of five different washing procedures. The treatment standard (TS) comprised phosphate-buffered saline (PBS) + 0.4% BSA (five times over for 10 s), Hank's +0.25% trypsin (twice for 45 s), and then PBS + 0.4% BSA again (five times for 10 s). The four other washing procedures all included the same first and last washing steps with PBS but without BSA (five times for 10 s) and with PBS + 0.4% BSA (five times for 10 s),

respectively. The intermediate step varied for each washing procedure. Treatment 1 (T1): 0.25% trypsin (twice for 45 s). Treatment 2 (T2): 0.25% trypsin (twice for 60 s). Treatment 3 (T3): 0.5% trypsin (twice for 45 s). Treatment 4 (T4): 1% hyaluronidase (once for 5 min). After washing, the embryos were transferred and cocultured with BTV indicator Vero cell monolayers for 6 h, to detect any cytopathic effects (CPE). The effectiveness of the different washing techniques in removing the virus was evaluated by RT-qPCR analysis. The TS, T1, T3, and T4 trypsin or hyaluronidase treatments did not eliminate BTV; Treatment 2 eliminated the virus from in vitro infected goat embryos. (**Al Ahmad MZ et al, Theriogenology 2012;78(6):1286-93. doi: 10.1016/j.theriogenology.2012.05.024**)

Caprine arthritis-encephalitis virus (CAEV)

Twelve attempts were made to isolate caprine arthritis-encephalitis virus (CAEV) from the uterine flushings of serologically positive superovulated does mated to serologically positive bucks. Sixteen embryos were washed 3x and transferred to eight serologically negative estrus-synchronized recipient does. The recipients were monitored serologically following embryo transfer. Virus isolation was attempted from colostrum and placental tissues from does that kidded following embryo transfers and the surviving kid was monitored serologically until four months of age. The CAEV was not isolated from any of the uterine flushings, colostrum or placental tissues. All recipients and the kid remained seronegative throughout the trial (**Wolfe et al, Theriogenology 1987;28:307-316**).

The objective of the study was to determine ovulatory response and quality of embryos collected from donor goats clinically affected with CAEV. Eight mature goats (Saanen and Alpine) were tested for CAEV using the AGID. Four were seropositive and had clinical evidence of arthritis in one or more joints. Four goats were seronegative and served as negative controls. All goats were treated for superovulation, bred by seropositive or seronegative bucks (presumably for positive and negative females, respectively), and embryos collected via laparotomy at Day 7. No significant difference was determined for any parameter examined (Table 1). A kid born after transfer of an embryo from a positive donor was still negative at six months after birth.

Table 1. Ovulatory response and embryo production in goats seropositive and seronegative for CAEV.

Donor	No. CL	Total Ova	Unfertilized Ova	Degenerated Ova	Transferrable Embryos
Seropositive	12.5 + 0.5	8.8 + 3.4	3.0 + 5.4	0.8 + 1.5	5.8 + 1.0
Seronegative	22.5 + 12.1	10.8 + 12.4	0.3 + 0.5	0.0 + 0.0	10.5 + 2.4

Cavalcant TV, et al, 14th Ann Meet AETE, Venise, September, 1998;p136.)

The objective of this study was to determine if CAEV-infected cells might be found in flushing media recovered during embryo collection from slaughtered donors. Embryos from 89 superovulated does were collected at slaughter, 36 to 48 hours after the beginning of estrus. Oviducts and uterine lumens were flushed with PBS containing heat treated fetal bovine serum and antibiotics. Mononuclear cells were recovered from flushing media by centrifugation and from blood by density-gradient centrifugation through a cushion of Ficoll. A double nested polymerase chain reaction (d-n-PCR) technique was used to detect CAEV proviral DNA in flushing media. Of the 89 goats, 64 (72%) were found to have no CAEV-DNA in blood, while 25 (28%) did have CAEV-DNA in the blood. All does that were negative to the blood test were also negative to the test on uterine flushing medium. Of the 25 infected

goats (blood positive), 44% (11/25) also had CAEV-DNA in the flushing medium. The authors concluded that washing of embryos should be highly recommended and that use of the PCR to screen for healthy donors is also indicated. **(Fieni F et al, Proceedings 15e Reunion AETE, Lyon, 10-11 September 1999, P 156 abstr.)**

Twenty-nine embryos from 11 superovulated does were collected at slaughter, 36 and 48 hours after the beginning of estrus. Five goats were infected with CAEV (blood PCR positive) and 6 were uninfected (blood PCR negative) control goats. The oviducts and uterine lumens were flushed with PBS, and embryos were placed into 0.5 mL of M199 (supplemented with 10 % fetal bovine serum, antibiotics, pyruvate, and bicarbonate) for incubation at 38.5$_{o}$C for 6 or 7 days (medium was replaced every 24 hours). After the 6 or 7 day incubation period embryos were used for d-n-PCR analyses. Blood samples were taken by jugular venipuncture. Mononuclear cells were recovered from flushing media by centrifugation and from blood by density-gradient centrifugation through a cushion of Ficoll. Flushing media, blood cells and embryo cells were then washed and lysed. Caprine arthritis-encephalitis virus proviral-DNA was detected by a double nested polymerase chain reaction (d-n-PCR) technique. Proviral-DNA of CAEV was detected in the flushing medium sample from one CAEV-infected goat. The two embryos recovered from this goat as well as the embryos recovered from the other infected and uninfected goats were CAEV d-n-PCR negative. Thus, after in vitro culture for an extended period of time, proviral-DNA was not detected in association with embryos recovered from blood and/or flushing media CAEV-positive goats. **(Fieni F et al, Proceedings, ICAR 2000 Satellite Symposium: Reproduction in Small Ruminants, Sandness, Norway, 2000;p68 abstr.)**

Embryos and fluids were collected from 10 Saanen goats that were seropositive for CAEV and that had been bred with bucks that were also seropositive. Fifty ZP-I embryos were washed (n=25) or not washed (n=25) according to IETS guidelines. Flush fluids, embryos and wash fluids were assayed for CAEV using virus isolation and nPCR. Thirty-seven and one half percent of the uterine flush fluids were positive for CAEV by virus isolation and 70 % were positive by nPCR. However, CAEV was not detected in any embryos (washed or unwashed) or in any wash solutions. **(Andrioli A et al. Theriogenology 2002;57:567 abstr.)**

To improve the knowledge on the risk of transmission of the caprine arthritis-encephalitis virus (CAEV) during embryo manipulations, we conducted a double-nested polymerase chain reaction (PCR) for CAEV proviral-DNA on flushing media recovered from the oviducts 48 h after the beginning of estrus and on blood from 89 donor does. Sixty-four does had negative blood and flushing media by PCR. Among the 25 CAEV infected goats (blood PCR positive), 11 were PCR flushing media positive ($P < 0.01$). Cell lysate from flushing media samples that were PCR positive were serially diluted 10 times at 1:100. Starting with the second 1:100 dilution all the cell lysate samples were PCR negative. The mean number of embryos recovered was not significantly different between goats with flushing media PCR positive and goats with flushing media PCR negative (6.0 +/- 5.4 versus 7.8 +/- 4.4, respectively; mean +/- S.D.) nor between goats with blood PCR positive and goats with blood PCR negative (7.0 +/- 5.0 versus 5.9 +/- 5.3; mean +/- S.D.). The presence of CAEV infected cells in oviductal flushing media from infected donor does was indicated for the first time during this study. The absence of flushing media PCR positive for goat blood PCR negative seemed to allow the use of the blood PCR test to confidently predict the absence of CAEV provirus in the oviductal fluid. **(Fieni F et al. Theriogenology 2002;57:931-940.)**

The aim of this study was to investigate whether cells of early goat embryos isolated from in vivo-fertilized goats interact with the caprine arthritis-encephalitis virus (CAEV) in vitro and whether the embryonic zona pellucida (ZP) protects early embryo cells from CAEV infection. ZP-free and ZP-intact 8-16 cell embryos were inoculated for 2 h with CAEV at the 10^4 tissue culture infectious dose 50 ($TCID_{50}$)/ml. Infected embryos were incubated for 72 h over feeder monolayer containing caprine oviduct epithelial cells (COECs) and CAEV indicator goat synovial membrane (GSM) cells. Noninoculated ZP-free and ZP-intact embryos were submitted to similar treatments and used as controls. Six days postinoculation, infectious virus assay of the wash fluids of inoculated early goat embryos showed typical CAEV-induced cytopathic effects (CPE) on indicator GSM monolayers, with fluids of the first two washes only. The mixed cell monolayer (COEC+GSM) used as feeder cells for CAEV inoculated ZP-free embryos showed CPE. In contrast, none of the feeder monolayers, used for culture of CAEV inoculated ZP-intact embryos or the noninoculated controls, developed any CPE. CAEV exposure apparently did not interfere with development of ZP-free embryos in vitro during the 72 h study period when compared with untreated controls (34.6 and 36% blastocysts, respectively, $P>0.05$). From these results one can conclude that the transmission of infectious molecularly cloned CAEV-pBSCA (plasmid binding site CAEV) by embryonic cells from in vivo-produced embryos at the 8-16 cell stages is possible with ZP-free embryos. The absence of interactions between ZP-intact embryos and CAEV in vitro suggests that the ZP is an efficient protective embryo barrier. **(Lamara A et al. Theriogenology 2002;58:1153-1163)**

Transmission of caprine arthritis-encephalitis virus (CAEV) is not completely understood, and the vertical route of infection from the goat to the embryo or to the fetus needs to be investigated. This route of infection involves the presence of CAEV in the genital tract tissues. Prior studies have detected CAEV-infected cells in genital secretions and in flushing media recovered during embryo collection from infected goats. To specify the origin of these cells, we conducted a double-nested polymerase chain reaction (PCR) test on embryo flushing media and on mammary gland, mammary lymph node, synovial membrane, pelvic lymph node, uterus and oviduct tissues from 25 CAEV-infected (blood PCR positive) embryo donor goats for the presence of CAEV proviral DNA. The presence of proviral DNA was found in 22 of 25 mammary gland samples, 14 of 25 uterus samples, and in 16 of 25 oviduct samples. Nineteen of 25 goats had at least one positive genital tract sample. Flushing media from 11 goats were PCR positive. All goats with positive-flushing media were oviduct positive. Of this group of does, except for 1 of the 11, infection of flushing media correlated with infection of almost all the other tissues examined. The frequency of positive tissues for flushing media-positive goats (61/66; 92%) was significantly higher than that for flushing media-negative goats (50/84; 60%) ($P<0.01$).This study demonstrated the presence of CAEV-infected cells in the goat genital tract. The presence of CAEV-infected cells in the uterus and oviducts suggests potential for vertical transmission of CAEV from doe to embryo or fetus. **(Fieni FF et al. Theriogenology 2003;59:1515-1523.)**

The aim of this study was to demonstrate that embryo transfer can be used to produce CAEV-free kids from CAEV-infected biological mothers. Twenty-eight goats that had tested positive for CAEV using PCR on vaginal secretions were used as embryo donors. Embryo with intact ZP were selected and washed ten times; they were then frozen and used for transfer into CAEV-free recipient goats. Nineteen of the forty-nine recipient goats gave birth,

producing a total of 23 kids. Three blood samples were taken from each recipient goat, ten days before, during, and ten days after parturition; these were tested for CAEV antibodies using ELISA and for CAEV proviral DNA using PCR. The mothers were then euthanized. Tissue samples were taken from the lungs, udder, and retromammary and prescapular lymph nodes. The kids were separated from their mothers at birth. Seven of them died. At 4 months of age, 16 kids were subjected to drug-induced immunosuppression. Blood samples were taken every month from birth to 4 months of age; sample were then taken on day 15, day 21, and day 28 after the start of the immunosuppressive treatment. The kids were then euthanized and tissue samples taken from the carpal synovial membrane, lung tissue, prescapular lymph nodes, inguinal and retro-mammary lymph nodes, and uterus. All samples from the 19 recipient goats and 23 kids were found to be negative for CAEV antibodies and/or CAEV proviral DNA. Under acute conditions for infection this study clearly demonstrates that embryo transfer can be safely used to produce CAEV-free neonates from infected CAEV donors. (**Ali Al Ahmad, MZ., et al. Theriogenology, 2008; 69(4):408-15.)**

The aim of this study was to determine the infectious status of semen and genital tract tissues from male goat naturally infected with the caprine lentivirus. Firstly, polymerase chain reaction (PCR) was used to detect the presence of CAEV proviral-DNA in the circulating mononuclear cells, semen (spermatozoa and non-spermatic cells), and genital tract tissues (testis, epididymis, vas deferens, and vesicular gland) of nine bucks. RT-PCR was used to detect the presence of CAEV viral RNA in seminal plasma. Secondly, in situ hybridization was performed on PCR-positive samples from the head, body, and tail of the epididymis. CAEV proviral-DNA was identified by PCR in the blood cells of 7/9 bucks and in non-spermatic cells of the seminal plasma of 3/9 bucks. No CAEV proviral-DNA was identified in the spermatozoa fraction. The presence of CAEV proviral-DNA in non-spermatic cells and the presence of CAEV in the seminal plasma was significantly higher ($p<0.01$) in bucks with PCR-positive blood. Two of the three bucks with positive seminal plasma cells presented with at least one PCR-positive genital tract tissue. Proviral-DNA was found in the head (3/9), body (3/9), and tail (2/9) of the epididymis. In situ hybridization confirmed the presence of viral mRNA in at least one of each of these tissues, in the periphery of the epididymal epithelium. This study clearly demonstrates the presence of viral mRNA and proviral-DNA in naturally infected male goat semen and in various tissues of the male genital tract. (**Ali Al Ahmad, MZ, et al. Theriogenology, 2008;69(4):473-80.)**

Zona-pellucida-free embryos at 8-16 cell stage were co-cultured for 6 days in an insert over a mixed cell monolayer infected with CAEV-pBSCA. Embryos were washed and transferred to an insert on CAEV indicator goat synovial membrane cells for 6 h, then they were washed and cultivated in B2 Ménézo for 24 h, finally, embryo cells were dissociated and cultivated ina feeder monolayer for 8 days. After 5 weeks, multinucleated giant cells typical of CAEV infection were observed in indicator GSM cell monolayers. In the acellular medium, the early embryonic cells produced at least $10^{3,25}$ $TCID_{50}$/ml over 24 h. The monolayer of cultivated embryonic cells developed cytopathic lesions within 8 days, and CAEV RNA, CAEV proviral DNA and protein p28 of the capsid were detected. All these results clearly demonstrate that caprine early embryonic cells are susceptible to infection with CAEV and that infection with this virus is productive. (**Ali Al Ahmad MZ, et al. Virology, 2006;353:307-315.)**

The aim of this study was to determine whether oocytes taken from ovarian follicles in 123 naturally infected goats were carrying the proviral CAEV genome. Examination of DNA

isolated from 190 batches of oocytes with intect cumulus cells and 190 batches of oocytes whose cumulus cells had been removed, taken from follicles of the same ovaries, demonstrated that 42/190 batches of oocytes with intact cumulus cells had the proviral CAEV genome, whereas none of the 190 batches of oocytes without cumulus cells were positive for the provirus. To confirm that the proviral genome was present in the cumulus cells and not in the oocyte cells, 586 oocytes from 56 different ovaries, were separated from their cumulus cells. The DNA was then extracted from each fraction and examined. The purity of the oocyte fraction was verified by searching for granulosa cell-specific mRNA, using RT-PCR; this was negative in all the batches of oocytes in which the cumulus cells were positive, whereas 22/56 of the batches with cumulus cells were found to be positive. This study clearly demonstrates that despite being surrounded by infected cumulus cells, the oocytes are not infected and that the enzymatic and mechanical technique for removing the cells surrounding the oocyte, as used in this study, is effective, thus enabling CEAV-free oocytes to be obtained from infected goats. (**Ali Al Ahmad MZ., et al. Theriogenology, 2006;64:1656-1666.)**

The aim of this study was to determine, using immunofluorescence and in situ hybridization, whether CAEV is capable of infecting goat uterine epithelial cells in vivo. Five CAEV seropositive goats confirmed as infected using double nested polymerase chain reaction (dnPCR) on leucocytes and on vaginal secretions were used as CAEV positive goats. Five CAEV-free goats were used as controls. Samples from the uterine horn were prepared for dnPCR, in situ hybridization, and immunofluorescence. The results from dnPCR confirmed the presence of CAEV proviral DNA in the uterine horn samples of infected goats whereas no CAEV proviral DNA was detected in samples taken from the uninfected control goats. The in situ hybridization probe was complementary to part of the CAEV gag gene and confirmed the presence of CAEV nucleic acids in uterine samples. The positively staining cells were seen concentrated in the mucosa of the lamina propria of uterine sections. Finally, laser confocal analysis of double p28/cytokeratin immunolabelled transverse sections of CAEV infected goat uterus, demonstrated that the virus was localized in glandular and epithelial cells. This study clearly demonstrates that goat uterine epithelial cells are susceptible to CAEV infection in vivo. This finding could help to further our understanding of the epidemiology of CAEV, and in particular the possibility of vertical transmission. **Al Ahmad MZ, Dubreil L, Chatagnon G, Khayli Z, Theret M, Martignat L, Chebloune Y, Fieni F. Goat uterine epithelial cells are susceptible to infection with Caprine Arthritis Encephalitis Virus (CAEV) in vivo. Vet Res. 2012 Jan 25;43(1):5. doi: 10.1186/1297-9716-43-5.**

The aim of this study was to determine, in vivo, whether in vitro infected cryopreserved caprine sperm is capable of transmitting caprine arthritis-encephalitis virus (CAEV) vertically to early embryo development stages via artificial insemination with in vitro infected semen. Sperm was collected from CAEV-free bucks by electroejaculation. Half of each ejaculate was inoculated with CAEV-pBSCA at a viral concentration of 10(4) TCID(50)/mL. The second half of each ejaculate was used as a negative control. The semen was then frozen. On Day 13 of superovulation treatment, 14 CAEV-free does were inseminated directly into the uterus under endoscopic control with thawed infected semen. Six CAEV-free does, used as a negative control, were inseminated intrauterine with thawed CAEV-free sperm, and eight CAEV-free does were mated with naturally infected bucks. Polymerase chain reaction (PCR)

was used to detect CAEV proviral-DNA in the embryos at the D7 stage, in the embryo washing media, and in the uterine secretions of recipient does. At Day 7, all the harvested embryos were PCR-negative for CAEV proviral-DNA; however, CAEV proviral-DNA was detected in 8/14 uterine smears, and 9/14 flushing media taken from does inseminated with infected sperm, and in 1/8 uterine swabs taken from the does mated with infected bucks. The results of this study confirm that (i) artificial insemination with infected semen or mating with infected bucks may result in the transmission of CAEV to the does genital tack seven days after insemination, and (ii) irrespective of the medical status of the semen or the recipient doe, it is possible to obtain CAEV-free early embryos usable for embryo transfer. **Al Ahmad MZ et al. Theriogenology. 2012 May;77(8):1673-8. doi: 10.1016/j.theriogenology.2011.12.012.**

Foot and mouth disease virus

The research was designed to investigate the risk of transmission of FMDV through embryos obtained from FMD seropositive donor sheep and FMD convalescent donor goats (experimentally inoculated) and transferred to free recipient animals.
Two experiments were performed in sheep. In experiment #1, 79 ewes and 4 rams were used, and in experiment #2, 33 ewes and 4 rams were used. In both experiments, animals were selected from an endemic area from farms where an outbreak of FMD was reported. The ewes in both experiments were then subject to a 14 day heat synchronization. The donors were subjected to superovulatory treatment and insemination. The embryos were collected by surgical technique. A group of goats consisting of 48 does and 3 bucks were confined in the maximum isolation units and infected with FMD virus. A strain of 01 was inoculated by intradermalingual route. The animals developed clinical signs and became positive to viral infection associated antigen (VIAA) after normal course of the disease, and the virus was isolated from oesophageal-pharyngeal fluids (OPP). Heat synchronization, superovulatory treatment and insemination were performed, and the embryos were collected by surgical technique. Sheep and goat embryos were handled following the recommendation of the International Embryo Transfer Society (IETS). The presence of FMDV was evaluated in 185 ovine embryos and in 293 caprine embryos. In addition, flushing and washing fluids from all the donor animals were assessed. Results showed that no infectious virus was present in any material tested. The rest of the embryos (60 sheep and 100 goat) were frozen in liquid nitrogen for further transfer to FMDV seronegative recipients in an experimental field in Peninsular de Valdes, an area of Argentina free from FMDV since 1991. A total of 24 sheep embryos were transferred to 23 recipients 96 goat embryos were implanted in 38 goat recipients. There was no clinical sign of foot and mouth disease during the experimental period in any of the experimental animals. All the serological tests performed to determine antibodies against VIAA in the recipient ewes and does and in the newborns were negative. (**Caamano N, et al. Proceedings, 106th Annual Meeting of the United States Animal Health Association, St Louis, Missouri, USA, October 2002, pp 263-270.)**

Coxiella burnetii

The aim of the present study was the detection and quantification of Coxiella burnetii DNA in the flushing media (oviducts and uterine horns) and genital tract tissues of non pregnant goats from 20 goats chosen at random from 86 goats originating from 56 different breeding herds in south-west France. The serological prevalence rate of C. burnetii in the study

population was 70.3%. The DNA of C. burnetii was identified using conventional PCR in the flushing media from the oviducts and uterus in 8/20 goats (40%) and in genital tract tissues (oviduct, uterus and ovary) in 5/20 goats (25%). This study clearly shows for the first time that the media used to flush the oviducts or uterine horns, collected using the standard embryo harvesting technique in goats, are susceptible to infection with C. burnetii. The 16 conventional PCR-positive samples were also analyzed using real-time PCR. The bacterial load of the oviduct and uterine flushing media varied from 2.9×10(4) to 7.5×10(6) bacteria per flushing medium, while the bacterial load of the tissue samples varied from 1.0×10(2) to 1.5×10(5) bacteria per mg of tissue. The infection of genital tract flushing media and tissues is a risk factor for the transmission of C. burnetii from donor to recipient during embryo transfer or to the embryo and fetus when gestation is pursued to term.

Alsaleh A, Pellerin JL, Rodolakis A, Larrat M, Cochonneau D, Bruyas JF, Fieni F. Detection of Coxiella burnetii, the agent of Q fever, in oviducts and uterine flushing media and in genital tract tissues of the non pregnant goat. Comp Immunol Microbiol Infect Dis. 2011 Jul;34(4):355-60. doi: 10.1016/j.cimid.2011.05.002. Epub 2011 Jun 16.

The detection of significant bacterial loads of *Coxiella burnetii* in flushing media and tissue samples from the genital tracts of nonpregnant goats represents a risk factor for in utero infection and transmission during embryo transfer. The aim of this study was to investigate (1) whether cells of early goat embryos isolated from in vivo-fertilized goats interact with *C. burnetii* in vitro, (2) whether the embryonic zona pellucida (ZP) protects early embryo cells from infection, and (3) the efficacy of the International Embryo Transfer Society (IETS) washing protocol for bovine embryos. The study was performed in triple replicate: 12 donor goats, certified negative by ELISA and polymerase chain reaction, were synchronized, superovulated, and subsequently inseminated by Q fever-negative males. Sixty-eight embryos were collected 4 days later by laparotomy. Two-thirds of the resulting ZP-intact and ZP-free 8- to 16-cell embryos (9-9, 11-11, and 4-4 in replicates 1, 2, and 3, respectively) were placed in 1 mL minimum essential medium containing 10(9)C. burnetii CBC1 (IASP, INRA Tours). After overnight incubation at 37 °C and 5% CO2, the embryos were washed according to the IETS procedure. In parallel, the remaining third ZP-intact and ZP-free uninfected embryos (3-3, 5-5, and 2-2 in replicates 1, 2, and 3, respectively) were subjected to the same procedures, but without C. burnetii, thus serving as controls. The 10 washing fluids for all batches of each replicate were collected and centrifuged for 1 hour at 13,000 × g. The washed embryos and pellets were tested by polymerase chain reaction. *Coxiella burnetii* DNA was found in all batches of ZP-intact and ZP-free infected embryos after 10 successive washes. It was also detected in the first five washing fluids for ZP-intact embryos and in the first eight washing fluids for ZP-free embryos. None of the control batches (embryos and washing fluids) were found to contain bacterial DNA. These results clearly indicate that caprine early embryonic cells are susceptible to infection by *C. burnetii*. The bacterium shows a strong tendency to adhere to the ZP after in vitro infection, and the washing procedure recommended by the IETS for bovine embryos failed to remove it. The persistence of these bacteria makes the embryo a potential means of transmission to recipient goats. Further studies are needed to investigate whether the enzymatic treatment of caprine embryos infected by *C. burnetii* would eliminate the bacteria from the ZP.

Alsaleh A, Fieni F, Rodolakis A, Bruyas JF, Roux C, Larrat M, Chatagnon G, Pellerin JL. Can Coxiellaburnetii be transmitted by embryo transfer in goats? Theriogenology. 2013 Oct 1;80(6):571-5.

Mycoplasma mycoides mycoides (LC type)

Nineteen adult goats (Saanen and Alpine breeds) were superovulated and inseminated with frozen semen from Mycoplasma-free bucks. A total of 193 embryos were collected surgically at 7d; 78 were frozen (Group 1) and 115 were used fresh (Group 2). Group 1 embryos (after thawing) and Group 2 embryos were each further distributed into 4 sub-groups and exposed for 12h. to suspensions of *M. mycoides* (LC) at the following concentrations:
Group 1a = 1.7 x 10_7 Group 2a = 2.3 x 10_7
Group 1b = 1.7 x 10_5 Group 2b = 1.7 x 10_5
Group 1c = 1.7 x 10_3 Group 2c = 1.7 x 10_3
Group 1d = 0 (control) Group 2d = 0 (control)
After the exposure period all embryos were washed ten times, according to the IETS Manual recommendations, using a PBS solution with 20% FCS.
Samples of the fluid from each wash, and embryos which had been ground after the 10th wash, were cultured to detect presence of *M. mycoides*. The organism was not detected in either of the controls but was found in the first three washes in all other sub-groups in Groups 1 and 2. It was also present in the last three washes for the 10_7 and 10_5 sub-groups in Group 1 (frozen), and the 10_7 sub-group in Group 2 (fresh). Infection was detected on embryos from all the sub-groups except for sub-group 2c and the two control sub-groups. These results indicate that pathogen adherence occurs irrespective of whether the embryos are exposed fresh or after freezing and thawing. They also demonstrate the value of testing the washing medium to detect presence of the pathogen on caprine embryos (**Guerin et al, published in part by Thibier, Proc 6e Reunion AETE, Lyon, September, 1990;pp67-81**).

Bovine spongiform encephalopathy agent (BSE)

In this “in vivo-in vivo” study, embryos were transferred from donors that had been experimentally infected with BSE to BSE-free recipients. Ten donors of goat embryos all developed TSE-like disease following experimental challenge with BSE, that was confirmed by neurohistology and PrP_{Sc} blotting. Incubation periods varied (547 to 1284 days after challenge). Twenty-two female goats were used as recipients of transferred embryos. Seventeen recipients were culled 5.5 years later with no signs of disease. Five recipients died of “intercurrent” disease but had neither clinical or histopathological sign of TSE-like disease. Recipient goats gave birth to 37 kids from 57 transferred embryos. Fifteen of these progeny died or were culled due to illness unrelated to TSE-like disease at ages less than 560 days (nine goats) or between 941 to 2130-days-old (6 goats). The remaining progeny (22 goats) were culled between 2030 and 2130 days of age with no clinical signs of TSE-like disease. Brain from all progeny were negative for TSE-histopathology and for PrP_{Sc} by western blotting. Thus, there was no evidence of TSE in any offspring or recipients. **(Foster J, et al, J Gen Virol 1999;80:517-524).**

Scrapie

Experiments, similar to those done in sheep (see above), are in progress with artificially induced scrapie in goats (**Foote et al, Proc 90th Annu Meet USAHA 1986;pp413-416**).

Pigs

Porcine circovirus

The aim of the present study was to determine if porcine circovirus type 2 (PCV2) is able to infect embryonic cells of in vivo-produced porcine embryos with and without zona pellucida (ZP). Zona pellucida-intact and ZP-free morulae (6 day post-insemination, 6 dpins) and early blastocysts (7 dpins) and hatched blastocysts (8 dpins) were inoculated with 10^5 $TCID_{50}$ PCV2 (strain 1121, 5th passage PK15). At 48 h post-incubation, the percentage of infected embryos and the percentage of viral antigen positive cells per embryo were determined by indirect immunofluorescence. Significantly different percentages of infected embryos were detected: 15 % for ZP-free morulae, 50 % for ZP-free early blastocysts and 100 % for hatched blastocysts. The percentage of cells that expressed viral antigens was similar for the three stages of development. PCV2 exposure did not affect the in vitro development of the embryos during the 46 h study period. All ZP-intact embryos remained negative for viral antigens. In an additional experiment, the diameter of the channels in the porcine ZP was determined. After incubation of early blastocysts with fluorescent microspheres of there different sizes, beads with a diameter of 20 nm and 26 nm crossed the zona, whereas the 200 nm beads did not. In conclusion, it can be stated that PCV2 is able to replicate in the in vivo-produced ZP-free morulae and blastocysts and that the susceptibility increases during development. The ZP forms a barrier to PCV2 infection, but based on the size of the channels in the ZP the possibility that PCV2 particles cross the ZP can not be excluded. **(Mateusen B et al. Reproduction in Domestic Animals 2003;38:351 abstr.)**

The aim of the present study was to determine if porcine circovirus type 2 (PCV2) is able to infect embryonic cells of in vivo produced porcine embryos with and without zona pellucida (ZP). ZP-intact and ZP-free morulae (6-day post-insemination) and early blastocysts (7-day post-insemination), and hatched blastocysts (8-day post-insemination) were exposed to 10^5 $TCID_{50}$ PCV2 per ml (strain 1121, fifth passage PK15). At 48 h post-incubation, the percentage of infected embryos and the percentage of viral antigen-positive cells per embryo were determined by indirect immunofluorescence (IF). Significantly different percentages of infected embryos were detected: 15% for ZP-free morulae, 50% for ZP-free early blastocysts and 100% for hatched blastocysts. The percentage of cells that expressed viral antigens was similar for the three stages of development. PCV2 exposure did not affect the in vitro development of the embryos during the 48 h study period. All ZP-intact embryos remained negative for viral antigens. In an additional experiment the diameter of the channels in the porcine ZP was determined. After incubation of early blastocysts with fluorescent microspheres of three different sizes, beads with a diameter of 20 nm and beads with a diameter of 26 nm crossed the zona whereas beads with a diameter of 200 nm did not. In conclusion, it can be stated that PCV2 is able to replicate in in vivo produced ZP-free morulae and blastocysts and that the susceptibility increases during development. The ZP forms a

barrier to PCV2 infection, but based on the size of the channels in the ZP the possibility that PCV2 particles cross the ZP cannot be excluded. (**Mateusen BF, et al. Theriogenology 2004;61:91-101).**

The aim of the present study was to assess the effects of porcine circovirus type 2 (PCV2) on porcine embryos and their receptor sows during the first 21 days of pregnancy. Hatched blastocysts exposed to 10(5.0) $TCID_{50}$ PCV2 per ml (strain 1121, fifth passage PK15) and negative control embryos were transferred to PCV2-immune receptor sows at the 7th day of the cycle. Two weeks after transfer (D21), the receptor sows were euthanized and embryos were recovered. They were assessed macroscopically for viability and examined for viral antigen-positive cells by immunoperoxidase staining. The embryonic survival rate of the PCV2-exposed embryos (6.4%, 7 viable embryos out of 110 transferred) was significantly lower than the survival rate of the negative control embryos (65.4%, 34 viable embryos out of 52 transferred). All of the non-viable PCV2-exposed embryos (n=9) displayed immunohistochemical positive signals for PCV2-antigen in degenerated tissues. In the PCV2-exposed embryos that were categorized as viable at D21, small clusters (n=4) or no PCV2-positive cells (n=3) were detected. The pregnancy results of the receptor sows that received PCV2-exposed embryos (1/5) were considerably different from the negative control receptors (2/2), with 3 out of 5 sows displaying a regular return to oestrus. In conclusion, it can be stated that PCV2 can replicate in embryos and might lead to embryonic death. In a small proportion of embryos, PCV2 exposure does not have a detrimental effect on embryo development before D21. (**Mateusen B, et al. Theriogenology. 2007;68(6):896-901.)**

A nested polymerase chain reaction (nPCR) protocol was applied to porcine semen to demonstrate the porcine circovirus type 2 (PCV2) shedding patterns and duration in naturally infected boars. Sperm morphology analysis was performed on a subset of samples to determine if the presence of PCV2 DNA in semen was associated with reduced semen quality. Semen was collected serially from 43 boars representing 6 breeds, aged 33.9 to 149.3 weeks. Of the 903 semen samples collected, 30 samples (3.3%) were positive for PCV2 DNA by nPCR from 13 boars. Boars shedding PCV2 DNA in semen ranged between 35.9 and 71.0 weeks of age, and shedding occurred during a period of up to 27.3 weeks. A semen nPCR test was 2.6 times more likely to be positive when collected from pigs that were < or =52 weeks of age, and 3.0 times more likely to be positive when collected from pigs that were < or =26 weeks from time of entry into the stud main unit (generalized estimating equations: P = 0.02; 95% confidence interval [CI] of the odds ratio 1.2 to 5.5, and P = 0.01; 95% CI of the odds ratio 1.3 to 6.9, respectively). These results demonstrate a sporadic and long-term shedding pattern of PCV2 DNA in semen from naturally infected boars. PCV2 DNA in semen does not appear to have detrimental effects on sperm morphology; however, boar age and, possibly, breed may contribute to the persistence of PCV2-shedding in semen. **(McIntosh KA, et al. J Vet Diagn Invest, 2006;18(4):380-4).**

Two experiments were conducted to determine the association of porcine circovirus type 2 (PCV2) with embryos and the risk of viral transmission by embryo transfer. In the first experiment, 240 embryos from uninfected donors were exposed to PCV2a 10(4)TCID50/mL in vitro before transfer to seronegative recipients; in the second experiment, 384 embryos recovered from infected donors, 10 days after donor inoculation with PCV2, were

transferred to seronegative recipients. In total, 1120 embryos and/or ova were collected from 37 viral-free donors (experiment 1) and 1019 from 59 PCV2-infected donors (experiment 2) ($P < 0.01$). The washing and/or disinfection procedure recommended by the International Embryo Transfer Society was applied to embryos in both experiments. Transfer of embryos experimentally exposed in vitro to high titers of virus caused seroconversion of recipients (58%; N = 7/12) and their piglets (81%; N = 13/16). Postmortem, PCV2 DNA was detected in various organs of embryo transfer recipients and their embryo transfer-derived piglets. In contrast, the transfer of embryos recovered from infectious PCV2 donors did not result in the seroconversion of embryo recipients (N = 24) or their embryo transfer-derived piglets (N = 76). Neither PCV2 DNA nor infectious virus was detected in the tissues of either recipients or embryo transfer-derived piglets collected postmortem in the second experiment. The results obtained in this study indicate that the transmission of PCV2 from infected donors by embryo transfer is unlikely if the sanitary recommendations of the International Embryo Transfer Society are followed. In practical terms, this means that embryo transfer can be successfully used for the intentional elimination of PCV2 and to create virus-free offspring for the safe exchange of swine genetic materials.
Bielanski A, Algire J, Lalonde A, Garceac A, Pollard JW, Plante C. Nontransmission of porcine circovirus 2 (PCV2) by embryo transfer. Theriogenology. 2013 Jul 15;80(2):77-83

Parvovirus (PPV)

Thirty-eight, day 2, ZP-I embryos were exposed to 10^4 $CCID_{50}$/ml PPV (NADL-8 strain) for 5 days in culture. Examination by the direct fluorescent antibody technique at the end of the culture period showed that viral antigen had adhered to the outer surface of the ZP, but the virus did not appear to have infected the embryonic cells. The proportion of virus-exposed embryos which developed to the blastocyst stage (53%) was significantly lower than the proportion of controls (80%) (**Wrathall and Mengeling, Br Vet J 1979;135:249-254**).

Seventy-six, day 2, ZP-I embryos were exposed to 10^4 $CCID_{50}$/ml PPV (NADL-8 strain) for 21 hrs in culture, washed twice, and then transferred to 4 seronegative recipients. All of the recipients seroconverted within 8 days and, at slaughter on day 8 post transfer, 50% of the transferred embryos were dead. What was taken to be PPV-specific fluorescent material was observed in a few trophoblast cells of 6/11 live blastocysts (**Wrathall and Mengeling, Br Vet J 1979;135:255-261**).

Twenty-five, day 2 to 3, ZP-I embryos were exposed to PPV (NADL strain) for 2, 24 or 48 hrs and then processed, without being washed, for electron microscopy. PPV was observed by EM 1) associated with amorphous masses of cellular debris at the outer surface of the ZP, 2) occasionally in pores beneath the outer surface of the ZP or 3) associated with sperm at, or near, the outer surface of the ZP (**Bolin et al, Am J Vet Res 1983;44:1036-1039**).

Thirty-nine, 4- to 8-cell stage, ZP-I embryos, in two groups, were exposed to 10^3 PFU/ml PPV (either virulent strain NADL-8 (n=20) or avirulent strain KBSH (n=19)) for 48 hrs at 37.5°C. Embryos were washed, evaluated for progression of development, sonicated (with the ZP) and assayed using 32P-radiolabeled, strand specific, RNA probes. Viral DNA was found in/on embryos exposed to NADL-8 and KBSH, but the replicative form of DNA was only found in

embryos exposed to the KBSH strain. Embryo development was not significantly affected by exposure to virus over 48 hours compared to controls. Some embryos were also exposed to these strains of PPV by microinjection through the ZP. (**Bane et al, Theriogenology 1990;33:553-561**).

ZP-I embryos (n = 46) were incubated for 2-3 h in the presence of 10^5 pfu/ml PPV (strain NADL-8) and a control group (n = 9) was similarly incubated without exposure to the virus. They were washed 10x and in some groups trypsin or pronase treatment was included in the 6th and 7th wash. Embryos were cultured for periods varying from 0 to 52h. prior to or after washing and the embryos and/or ZP were then disrupted with proteinase K and Tween-20. A polymerase chain reaction (PCR) assay, sensitive to a level of 5 pfu of virus, was utilized to ascertain whether PPV had adhered to the ZP after virus exposure and washing, and also whether it was present within the hatched embryos. The virus was detected in 17/18 unhatched embryos, 10/12 hatched embryos and 7/7 ZP from the groups that were washed 10x. It was also present in 5/5 unhatched embryos treated with trypsin and 4/4 treated with pronase. While transzonal infection could not be ruled out, embryos could have become contaminated from the ZP during hatching or from the culture media after hatching (**Gradil, Harding and Singh, Proc 12th ICAR, The Hague 1992;3:1304-1306**).

Three experiments involving a total of 253 embryos were carried out using a PCR technique to detect PPV-specific DNA. In experiment 1 ZP-I embryos were collected from seronegative donors and incubated for up to 18h in medium with or without 10^5 PFU of PPV (NADL-8 strain). Following exposure to virus the embryos were either washed immediately (10 washes in which 0.25% trypsin or 0.25% pronase was included at the 6th and 7th wash in some groups), or they were cultured for up to 52h. and then washed. After treatment hatched and unhatched embryos or ZPs (from hatched embryos) were tested by the PCR for presence of the virus. Viral DNA was detected in fragments generated from 17 of 18 unhatched, exposed embryos, 10 of 12 hatched, exposed embryos and 7 of 7 exposed ZPs, but not from 9 unexposed embryos or from samples of the 10th wash. The PCR assay was sensitive to a value of 5 PFU of PPV.

In experiment 2, 8 gilts were inoculated with $10^{6.5}$ PFU virus and 4 day old ZP-I embryos were collected 7d. p.i. by flushing them from the uterus with 30ml medium per horn. The embryos were then washed 10 x. Viral DNA was not detected in the fragments from 139 embryos from the infected donors or from 17 embryos from controls. However, the virus was present in 1 of 6 flush fluids, 5 of 16 ovarian follicle fluids and 1 of 8 oviductal swabs from the infected donors.

In experiment 3, 51 embryos from 4 PPV inoculated donors were collected, washed 10 x and transferred into uninfected, seronegative recipients. The recipients were killed when the embryos were 15 or 32 days old and these embryos (6 and 9 respectively) were retrieved and tested for PPV DNA. All were found to be negative and no evidence of infection was found in the allantoic or amniotic fluids or in the uterus. The results indicate that although PPV becomes closely associated with embryos that are exposed to the virus in vitro, they rarely become infected in vivo, even during acute maternal infection. Thus, provided recommended sanitary procedures are adopted, the risk of transmitting an infective dose by ET from infected donors is minimal (**Gradil, Harding and Lewis. Am J Vet Res 1994;55:344-347**).

The effects of PPV, PRV and PRRSV on embryonic cells of morulae and blastocysts are not well documented. Therefore, the objectives of the present study were to (i) assess the effects of PPV, PRV and PRRSV exposure on further embryo development, (ii) determine whether PPV, PRV and PRRSV are able to replicate in embryonic cells and (iii) to link these findings with receptor expression of embryonic cells. For this latter, the expression of nectin-1 and sialoadhesin, mediators for virus entry into cells for PRV (4) and PRRSV (5), respectively, was determined. Unfortunately, to date the cellular receptor for porcine parvovirus is unknown. Subzonal microinjection of ZP-intact morulae and blastocysts and incubation of hatched blastocysts with PRV hampered in vitro development in comparison to the controls. Exposure of embryos to PPV or PRRSV did not influence further embryonic development. Antigen positive cells were detected in embryos exposed to PPV and PRV, but not in embryos exposed to PRRSV. The ratio of antigen positive cells was significantly higher for embryos exposed to PRV compared to embryos exposed to PPV. The PRV receptor nectin-1 was expressed as a continuous layer in the outer membrane of cells at the hatched blastocyst stage. Sialoadhesin, the PRRSV receptor, was not expressed during the hatched blastocyst stages. Based on these results, we can conclude that preimplantation embryos up to the hatched blastocyst stage are susceptible to PPV and PRV infection but refractory to PRRSV infection and this latter finding was consistent with the absence of the PRRSV receptor sialoadhesin. **(Mateusen et al, Proceedings, International Pestivirus Symposium (IPVS), Copenhagen, 2006;Vol I:113.)**

Porcine reproductive and respiratory syndrome virus (PRRSV)

Fertilized porcine ova, collected from 14 estrus-synchronized gilts 32 h after presumed ovulation were microinjected or cultured for 72 h in Beltsville Embryo Culture Medium-3 with or without PRRSV. To detect virus in the samples, virus isolation in swine alveolar macrophage, reverse transcriptase PCR and fluorescent antibody techniques were employed. Microinjection or incubation of embryos with PRRSV did not significantly inhibit development of the porcine embryos in vitro when compared with that of controls (P=0.75 and P=0.14, respectively). Although either 10 to 20 $TCID_{50}$ were microinjected or large concentrations of virus were used for embryo exposure by incubation, PRRSV was not detected in association with the embryos. They concluded based on their experiment that 4 to 16-cell stage porcine embryos are not susceptible to productive infection with PRRSV in vitro. (**Prieto et al, Theriogenology 1996;46:687-693.**)

In situ hybridization (ISH) and immunohistochemistry (IHC) was used to study distribution of PRRSV-infected cells in ovaries at different times after infection. In addition, virus isolation (VI) was attempted on follicular fluid. A dose of 10^6 $TCID_{50}$ of PRRSV (16244B) was given intra nasally to each of 6 PRRSV-free gilts. Three uninfected gilts were subjected to sham inoculations and served as negative controls. At 7, 14 and 21 days, two exposed and one unexposed gilt were killed and tissues collected and examined as described. Virus was isolated “from ovaries of challenged gilts”. PRRSV-positive cells in ovaries appeared to be predominately macrophages. There was also evidence of PRRSV-infected stromal cells in the ovarian cortex. Also, experiments suggested infection of granulosa cells in atretic follicles

and corpus albicans. **(Sur J-H, et al, Proc 41st Ann Meet AAVLD, October 3-9, Minneapolis, MN 1999;abstr24)**

In this study embryos recovered from control gilts and gilts experimentally infected with PRRSV were analyzed in vitro or transferred to naive recipient gilts to determine whether PRRSV could be eliminated from breeding stock by embryo washing (IETS protocol) and transfer. Sixteen control gilts (no PRRSV exposure) and 24 artificially exposed (intranasal with 3 x 10_6 PFU; strain PA-8 at beginning of the study and 1 x 10_6 PFU/dose of semen, with three total doses used for insemination) were slaughtered approximately 4 days after heat and tissues and embryos collected. Embryos (and some unfertilized oocytes) from control and exposed gilts were either: 1) frozen immediately for RT-PCR analysis for PRRSV (n=430). Washed and then frozen for RT-PCR analysis (N=196), (transferred to synchronized PRRSV-negative recipient gilts (17 to 20 embryos per recipient). Tissue samples from donors and flush fluids collected at slaughter were also analyzed by RT-PCR to determine PRRSV status of each control and PRRSV-infected donor gilt. Following embryo transfer, blood samples were collected monthly from recipients and examined for PRRSV status using ELISA analysis. At farrowing, piglets were sacrificed and tissues collected and examined with RT-PCR. "Results indicated that PRRSV infection is not transmitted to embryos through the dam." Despite high concentrations of PRRSV in the infected donors' tissues (reproductive and non reproductive), all embryos (washed and unwashed) were found to be free of PRRSV. Recipients were free of PRRSV as were all piglets (n=29 from infected donors). "Results overall indicate that embryo transfer may be a highly effective clinical tool for the elimination of the PRRSV from infected breeding stock." **(Randall AE, et al, Theriogenology 1999;51:274 abstr.)**

The objective of the study was to evaluate efficacy of surgical recovery of porcine embryos from PRRSV infected gilts to generate a source of PRRSV-free germ plasm. Seven prepuberal gilts (180 days of age) that were seronegative for antibody to anti-PRRSV antibody were used. Estrus was induced using P.G. 600 (Day=0). Also, on Day 0, gilts were bled and samples submitted for PRRSV testing via reverse transcription polymerase chain reaction assay (RT-PCR). On Day 4, all gilts were artificially inseminated once. Five mL of each insemination dose was frozen in liquid nitrogen for PRRSV testing via RT-PCR. Also, on Day 4, all gilts were vaccinated intramuscularly with a single dose of modified live PRRSV vaccine (PrimePac, Schering Plough). On Day 8, embryos were recovered surgically, and uterine fluids and embryos from each gilt were tested by RT-PCR for PRRSV. Uterine flushings from each horn were kept separated for testing. Embryos from each horn were divided into two treatment groups that would be tested for PRRSV via RT-PCR after "Group I" embryos were washed with trypsin and "Group II" embryos were washed without trypsin. The RT-PCR for serum on Day 0 was negative for all seven gilts. Day 8 serum was collected from 6 of the 7 gilts and 4/6 were positive for PRRSV in the serum. No embryos from either group tested positive for PRRSV. Authors said that results suggest that pig embryo recovery and transfer might be a viable option for transporting germ plasm with reduced health risks. **(Didion BA et al, J Anim Sci Suppl 1, 1999;77:127 (abstract 41).)**

Note: This abstract provides information on multiple pathogens including PRRSV). There is a big need for genetical (international) linkage of porcine breeding herds. For nucleus herds with different health status this can be done by caesareans (costly, unfriendly), frozen sperm

(half of the genes), surgical and non surgical embryo transfer (nsET). According to the research information of the International Embryo Transfer Society (IETS), mostly based on bovine embryos, ET is the safest way to transfer genes. To investigate the level of health security of porcine embryos an *in vitro* experiment with *Mycoplasma hyopneumoniae, Streptococcus susi,* and *Pasteurella multocida* DNP+ and PRRS (American and European strain) was carried out. For each pathogen investigated 40 to 50 vital day 4 embryos were collected, washed five times, inoculated with 10^5-10^6 PFU/$TCID_{50}$ and incubated 18 hours in D-PBS with 10 % lamb serum, without antibiotics at 37^{o}C. After incubation, th eembryos were washed 12 times in groups of 10 in D-PBS with antibiotics and 0.25 % trypsin (IETS procedures). After washing the embryos were tested by culture or PCR in batches of 5. All the embryos tested were free from these pathogens after washing. It was concluded that porcine embryos exposed to these pathogens can be transferred without risk of transmission when linking nucleus herds by nsET-procedures. **(Smits JM et al. Book of Abstracts of the 52nd Annual Meeting of the European Association for Animal Production. Budapest, Hungary August 26-29, 2001, PhP6 no. 5; page 212)**

Thirteen gilts from a PRRS virus negative farm were used in the study. Gilts were divided into inoculated (n=11) and uninoculated (n=2; negative control) groups. Inoculated gilts were exposed 2-5 days before estrus, intra nasally with 3 mL of a field PRRSV isolate at a dose of $1x10^{4.5}$ $TCID_{50}$/mL. Then gilts were artificially inseminated twice based on observation of natural estrus. Gilts were euthanized 5 to 6 days after onset of estrus (about 120 hours after ovulation) for embryo collection and examination. Embryos were collected by retrograde flushing of the uterine horns, morphologically evaluated with a stereomicroscope and transferred to a holding medium. Embryos were then divided into three groups: a) unwashed, b) embryos washed with holding medium according to IETS standards, and c) embryos washed in holding medium as in part b but with 2 additional washes of a 0.25 % trypsin solution. In addition, samples of flush fluids and the 1st, 5th and 10th washes were analyzed for presence of virus. All samples were frozen (-20^{o}C) until processing. The presence of virus was determined by PCR. Also, blood samples were taken before inoculation, at 3 days post inoculation and at necropsy. Tissues collected for virus isolation included: lung, inguinal LN, ovary, oviduct and uterine horn. Only 8/11 inoculated gilts came in heat and therefore only 8 gilts were used for embryo collection. (Three of the challenged gilts became viremic at three days after inoculation, did not come into heat). PRRSV was detected in at least one of the samples taken nine of the challenged gilts. At necropsy, 5 of the 8 challenged gilts were viremic and virus was detected in the LNs and lungs of 4 of them. The ovaries of 4 gilts and uterine horns of 2 were PCR positive at necropsy. Control gilts remained negative throughout the study. A total of 118 ova/embryos (mean 14.8) were collected from the inoculated gilts and 27 embryos were collected from the 2 controls. PCR technique adapted to embryo analysis proved able to detect as low as $1x10^{1.6}TCID_{50}$/mL viral particles in experimentally exposed embryos (results not shown). Embryos collected at 5 days post insemination proved negative by PCR. All embryo groups (unwashed, washed, washed plus trypsin) yielded negative PRRSV PCRs. The flushing fluids used to collect the embryos were also negative when analyzed by PCR The 1st, 5th and 10th washes were negative as well. **(Torremorell M et al. The 16th International Pig Veterinary Society Congress, Melbourne, Australia, 17-20 September 2000, page 585) (Note: this report was also presented in abstract form in Theriogenology 2001;55:377abstr)**

Fifty known PRRSV-negative gilts were housed in an isolated facility without other animals. They were treated with altrenogest, cloprostenol, FSH and LH. Ten days before insemination they were inoculated intra nasally with 2 mL of 10^8 $TCID_{50}$ (PRRSV-EU, P6 batch 22-03-99; nr45). Four days after inoculation 45 of 50 sera were positive by virus isolation (IPMA method). Eight gilts (possibly 8 other gilts although it was unclear) were isolated in another location and used as negative controls (presumably treated in the same way). All gilts were inseminated twice and slaughtered for embryo collection after 5 days. Part of the embryos were not washed and directly subjected to virus isolation. The remainder of the embryos were washed (they stated according to IETS standards) : 5x in D-PBS plus 1% heat inactivated lamb serum plus antibiotics, 2x in o.25% trypsin in Hanks' balanced salt solution, and 5x in D-PBS plus 10% heat inactivated lamb serum plus antibiotics. After washing the embryos were assayed using virus isolation. All 23 embryos from viremic donors and tested before washing were negative. Also, all 199 embryos from viremic donors and tested after trypsin treatment were negative. All embryos from virus negative donors were negative as well. **(Smits JM et al. Theriogenology 2002;57:574)**

The objectives of the present study were to (1) determine whether PRV and PRRSV are able to replicate in embryonic cells of porcine morulae and blastocysts and (2) assess the effects of PRV and PRRSV exposure on further embryonic development. Two- and 4-cell stage embryos were harvested from 24 sows at 2 days after insemination. Embryos were cultured until they reached the morula or blastocyst stage. At those stages 2.5 % pronase was used to remove the zona pellucida from half of the embryos. Twelve hours later, 2/3rds of the ZP-free, 2/3rds of the ZP-I embryos and 2/3rds of the hatched blastocysts were inoculated with 10^5 $TCID_{50}$ PRV (strain 89v87, second passage in swine testicle cells) or PRRSV (LV, 13^{th} passage in swine alveolar macrophages) at 39^oC for 1 hour. Control (unexposed) embryos were maintained under identical conditions. After viral exposure, all embryos were washed and examined every 12 hours for assessment of embryonic development. Further development for morulae was defined as reaching the blastocyst stage and for blastocysts was defined as reaching the expanded or hatched blastocyst stage. Differences in rates of development were analyzed using Chi-square analysis or Fisher's exact test. At 48 hours after viral exposure, embryos were fixed and stained for PRV and PRRSV using indirect immunofluorescence. Using indirect immunofluorescence, PRV- or PRRSV-infected cells were not detected in morulae or blastocysts. Incubation of ZP-I and ZP-free morulae and blastocysts with PRV or PRRSV did not inhibit in vitro development in comparison to the controls ($P<0.05$). The data show that like early cleavage embryos, morulae and blastocysts are refractory to PRV and PRRSV infection. Whether hatched blastocysts are refractory to viral infection is under investigation. It is suspected that more advanced embryonic stages become susceptible. (**Mateusen B, et al. 18^{th} IPVS, Hamburg, Germany, 2004, p118.)**

In vivo-produced ZP-I and ZP-free morulae (6 days post insemination), early blastocysts (7 days post insemination), and hatched blastocysts (8 days post insemination) derived from 22 superovulated sows were exposed to 10^5 $TCID_{50}$ PRV (strain 89v87, second passage in swine testicle cells) or PRRSV (LV, 13^{th} passage in swine alveolar macrophages) at 39^oC for 1 hour. Control embryos were incubated under the same circumstances without viruses. Each group contained approximately 20 embryos. Embryonic development was assessed every 12 hours and differences analyzed using Chi-square analysis or Fischer's exact test. Also, at 48 hours

post incubation, embryos were collected and examined for viral antigen by indirect immunofluorescence. Further embryo development of ZP-intact and ZP-free morulae and blastocysts was not affected by exposure to either of the viruses compared to controls ($P<0.05$). Moreover, using indirect immunofluorescence, no virus positive cells were detected in the embryos. Exposure of hatched blastocysts to PRV inhibited further embryonic development as 100% (n=5) degenerated after viral exposure. This was significantly different from the controls and the PRRSV-incubated hatched blastocysts that did not experience any negative influence on embryo development. Based on these results, it can be concluded that embryonic cells are not susceptible to PRRSV infection up to the hatched blastocyst stage. Embryonic cells of morulae and blastocysts are refractory to PRV, but the virus had a detrimental effect on further development of hatched blastocysts. More experiments are necessary to confirm these results and to investigate whether, or at which pre-implantation stage, embryos are susceptible to PRRSV. (**Mateusen B, et al. Reproduction, Fertility and Development 2004;16:219(abstr.))**

In the present study, the in vitro interaction of embryos with pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) was investigated by viral antigen detection and by evaluating the expression of virus receptors, namely poliovirus receptor-related 1 (PVRL1; formerly known as nectin 1) for PRV and sialoadhesin for PRRSV. Embryonic cells of zona pellucida intact embryos incubated with PRV remained negative for viral antigens. Also, no antigen-positive cells could be detected after PRV incubation of protease treated embryos since the protease disrupted the expression of PRVL1. However, starting from the 5-cell stage onwards, viral antigen-positive cells were detected after sub-zonal micro injection of PRV. At this stage, the first foci of PVRL1, also a known cell adhesion molecule, were expressed. At the expanded blastocyst stage, a lining pattern of PVRL1 in the apico-lateral border of trophectoderm cells was present, whereas the expression in the inner cell mass was low. Furthermore, PVRL1 specific monoclonal antibody CK41 significantly blocked PRV infection of trophectoderm cells of hatched blastocysts, while the infection of the inner cell mass was only partly inhibited. Viral antigen-positive cells were never detected after PRRSV exposure of preimplantation embryos up to the hatched blastocyst stage. Also, expression of sialoadhesin in these embryonic stages was not detected. We conclude that the use of protease to investigate the virus embryo interaction can lead to misinterpretation of results. Results also show that blastomeres of 5-cell embryos up to the hatched blastocysts can get infected with PRV, but there is no risk of a PRRSV infection. **(Mateusen et al, Biology of Reproduction 2007;76:415-423)**

The effects of PPV, PRV and PRRSV on embryonic cells of morulae and blastocysts are not well documented. Therefore, the objectives of the present study were to (i) assess the effects of PPV, PRV and PRRSV exposure on further embryo development, (ii) determine whether PPV, PRV and PRRSV are able to replicate in embryonic cells and (iii) to link these findings with receptor expression of embryonic cells. For this latter, the expression of nectin-1 and sialoadhesin, mediators for virus entry into cells for PRV (4) and PRRSV (5), respectively, was determined. Unfortunately, to date the cellular receptor for porcine parvovirus is unknown. Subzonal microinjection of ZP-intact morulae and blastocysts and incubation of hatched blastocysts with PRV hampered in vitro development in comparison to the controls. Exposure of embryos to PPV or PRRSV did not influence further embryonic

development. Antigen positive cells were detected in embryos exposed to PPV and PRV, but not in embryos exposed to PRRSV. The ratio of antigen positive cells was significantly higher for embryos exposed to PRV compared to embryos exposed to PPV. The PRV receptor nectin-1 was expressed as a continuous layer in the outer membrane of cells at the hatched blastocyst stage. Sialoadhesin, the PRRSV receptor, was not expressed during the hatched blastocyst stages. Based on these results, we can conclude that preimplantation embryos up to the hatched blastocyst stage are susceptible to PPV and PRV infection but refractory to PRRSV infection and this latter finding was consistent with the absence of the PRRSV receptor sialoadhesin. **(Mateusen et al, Proceedings, International Pestivirus Symposium (IPVS), Copenhagen, 2006;Vol I:113.)**

Pseudorabies virus (PrV)/Aujeszky's Disease

Seventy-three ZP-I and 17 ZP-free, day 1 to 3 embryos, in 18 embryo groups, exposed to 104 and to 108 TCID50/ml of PrV (field strain, Sullivan) and then washed five times, were found to be negative for infectivity in cell culture. Eighty-two ZP-I and 29 ZP-free, day 1 to 2 embryos under the same exposure and washing conditions were found to be negative by direct F.A. after 16 to 48 hrs post exposure culture in fresh medium (Bolin and Bolin, 1984). The same workers, however, later found that PrV could be transmitted by embryo transfer: 79, day 1 to 3 ZP-I embryos exposed to 108 TCID50/ml in vitro, washed and then transferred to seronegative recipients caused 5/5 recipients to develop antibodies to PrV (Bolin et al, 1985). In addition, in another experiment, seroconversion occurred in 2/5 recipients that had received 70 ZP-I embryos recovered from donors infected both intranasally (2ml 10^5 $TCID_{50}$/ml PrV) and intrauterinely (10^5 $TCID_{50}$/ml PrV in 25 ml) (Bolin et al, 1985). On the other hand, 45 ZP-I embryos exposed to 10^4 $TCID_{50}$/ml in vitro and 45 ZP-I embryos derived from donors infected only intranasally (2 ml 10^5 $TCID_{50}$/ml PrV) did not cause seroconversion when transferred to 4 and 3 recipients, respectively (**Bolin et al, Am J Vet Res 1982;43:278-280**).

One hundred and twenty-seven ZP-I embryos were exposed to 10^4-10^8 $TCID_{50}$/ml PrV (Weybridge strain) for 24 hours, washed, cultured and then assayed for PrV. In three experiments, none of the exposed embryos were positive for PrV, but in another three experiments, all of the embryos were positive for PrV (**Singh and Thomas, unpublished - cited by Singh, Theriogenology 1987;27:9-20**).

Combining the results obtained with exposure to 10^4-10^8 $TCID_{50}$/ml PrV, 24% of exposed embryos were positive for PrV (**Singh, Theriogenology 1987;27:9-20**).
Forty-six ZP-I embryos exposed to 10^6 $TCID_{50}$/ml PrV and then treated with 0.25% trypsin at pH 7.6-7.8 were negative for PrV on assay (**Singh and Thomas, unpublished - cited by Singh, Theriogenology 1987;27:9-20**).

Twenty-three, day 2 to 3, ZP-I embryos were exposed to 10^8 $CCID_{50}$/ml PrV for 1, 24 or 72 hours in culture. Then, without being washed, they were fixed and processed for EM examination. Virus particles were observed associated with the outer surface of the ZP. The number of particles observed increased with exposure time. Particles were also observed in sperm tracks (**Bolin et al, Am J Vet Res 1983;44:1036-1039**).

Twenty, day 2 to 3, ZP-I embryos were exposed to PrV by cocultivating in infected cultures for 2, 24 or 48 hrs. The majority of embryos were processed for EM immediately following

incubation without washing, but a few were washed five times prior to processing. EM showed PrV adsorbed to the outer surface of the ZP as well as in sperm tracts. Washing failed to remove adsorbed virus (**Bolin et al, Am J Vet Res 1983;44:1036-1039**).

Eight hundred and five, day 3 to 4, ZP-I embryos, collected from 38 donors seropositive with antibodies to PrV, were transferred to 34 recipients from a herd seronegative for PrV. All of the recipients remained seronegative. 22/34 recipients farrowed 208 piglets (189 live) that were also seronegative for PrV. PrV could not be isolated from the flush fluids or uterine and oviductal cells recovered with the embryos (**James et al, JAVMA 1983;183:525-528**).

Nine susceptible gilts were exposed to pseudorabies virus (PrV) by intrauterine inoculation immediately after breeding. Embryos were collected from each of three gilts on days 3, 6, and 10 following exposure to PrV. The number of embryos collected from each gilt was compared with the number of corpora lutea (CL). On days 6 and 10, there were substantially fewer embryos collected than there were CL. The embryos were examined for the presence of viral particles by electron microscopy. PrV was observed in 0/33 day 3 embryos, 1 (hatched)/13 day 6 embryos and 1/9 day 10 embryos. The fluids used to flush the embryos from the uterus during collection were tested for PrV by virus isolation and direct fluorescent antibody procedures. PrV was isolated from the uterine flush fluids of one of three gilts at each time of embryo collection (**Bolin and Bolin, Theriogenology 1984;22:101-107**).

Pseudorabies virus (PrV) was inoculated into the uterus of 15 gilts within 6 hours after natural breeding and gilts were necropsied 3, 6, 10, 14, and 28 days postbreeding (DPB); 3 control gilts were treated similarly except for inoculation with PrV and were necropsied 6, 10 and 14 DPB. Tissues were collected for virus isolation, fluorescent antibody staining and histopathology. Pseudorabies virus was isolated variously from all parts of the reproductive tract for varying periods of time and from the ovary of two gilts up to 14 DPB. Lesions in the reproductive tract consisted of multifocal to diffuse lymphohistiocytic vaginitis and endometritis, and lymphoplasmacytic aggregates in the corpora lutea. Multiple ulcers were seen in the vagina or endometrium of several gilts at 3, 6, and 10 DPB. Corpora lutea of 1 gilt at 14 DPB were necrotic and contained large numbers of inflammatory cells. Focal aggregates of lymphocytes and plasma cells were seen in vagina and endometrium of 3 gilts, and in the ovary of 1 gilt at 28 DPB (**Bolin et al, Am J Vet Res 1985;46:1039-1042**).

Six hundred and fourteen embryos were collected from 47 gilts with antibody titres for PrV of 1:16 to 1:64 that had been bred naturally or AI'd with fresh semen. Embryos were washed 10x and 563 were transferred to 47 seronegative recipients held in isolation. Recipients did not seroconvert based on sampling to 150 days after the transfers. The 172 live born offspring were seronegative at 30 days of age. Virological examination of flush fluids, wash fluids, unused ova/embryos and tissues from stillborn pigs were negative for PrV (Veselinovic, 1988). A report by the same group on a similar project with similar outcome refers to the transfer of 277 embryos from 35 naturally-infected gilts to 35 seronegative gilts (**Veselinovic, Final Report Proj No: YO-ARS-92-JB-105) (IFP-618), Beltsville MD:USDA Ntl Agric Lib, 1988 and Veselinovic et al, World Rev Anim Prod 1991:26:67-68**).

Fifty-six, day-5, ZP-I embryos were collected from donors that had been inoculated intranasally and intravaginally at the time of insemination with 3 ml of 10^7 $TCID_{50}$/ml PrV. Twenty-eight embryos were trypsin-treated (2 min;0.25% trypsin solution) and washed 10x prior to being transferred to 2 seronegative recipients (15 to one, 13 to the other). Another 20 embryos were transferred to a third recipient after washing, but without trypsin treatment. Virus was recovered from the nasal cavity, ovaries, uterus and vagina of the donors, as well as from the flush fluids. Recipients receiving the trypsin-treated embryos were seronegative 50 days post transfer. One became pregnant and gave birth to 7 piglets which were negative on test, 7 and 14 days after birth. The recipient receiving the embryos that were not trypsin-treated was seropositive at 50 days post transfer (**Haraszti et al, Magyar Allatorvosok Lapja 1989;44:325-327**).

Fifty-nine, day-5, ZP-I embryos were collected from 4 of 6 donors that were inoculated intranasally with 3 ml of 10^7 $TCID_{50}$/ml PrV and administered 10 ml of 10^6 $TCID_{50}$/ml PrV orally at the time of hormone treatment (ie, 5 days prior to insemination). Forty embryos were trypsin-treated and washed 10x prior to being transferred to 2 seronegative recipients. Another 19 were transferred to a third seronegative recipient after washing, but without trypsin treatment. Four donors received a virulent strain and 2 donors a mildly virulent strain of PrV. Virus was recovered from the nasal cavity and ovaries of the donors receiving the virulent strain, but only from the nasal cavity of the donors receiving the mildly virulent strain. Virus was also recovered from the flush fluids from 2 of the 4 donors receiving the virulent strain. No pregnancies resulted from the transfers, but the 2 recipients receiving the trypsin-treated embryos were seronegative at 50 days post transfer, while the recipient receiving the embryos that were washed but not trypsin-treated was seropositive at 50 days post transfer (**Haraszti et al, Magyar Allatorvosok Lapja 1989;44:325-327**).

One hundred and twenty-three embryos were collected from 10 virus-positive sows. After trypsin treatment and washing 10x, 93 embryos were transferred to 5 negative recipients. The recipients remained seronegative at repeated testing up to 50 days post transfer (**Haraszti et al, Magyar Allatorvosok Lapja 1989;44:325-327**).

Oocytes collected from sows vaccinated and revaccinated with an inactivated vaccine against Aujeszky's disease (AD) were examined for the presence of specific antibodies of the IgG class against AD virus in the cumulus oophorus-oocyte complex by means of immunocytochemical methods. No specific antibody of the IgG class was detected in the cumulus-oocyte complexes collected from sows before vaccination. On the other hand, the specific IgG antibody was found in all immunocytochemically examined cumulus-oocyte complexes collected from revaccinated sows. The specific antibody of the IgG class against AD virus and the porcine IgG were immunocytochemically visualized under both light and electron microscopes as a fine granular product disseminated in oocytes and follicular cells (**Jerabek et al, Acta Histochem 1989;85:101-108**).

Four seronegative Hungarian Large White sows from a farm free of Aujeszky’s disease (AD) were artifically inseminated and concurrently infected by intranasal and intrauterine administration of 10^5 $TCID_{50}$ of a virulent strain of Aujeszky’s disease virus (ADV). The embryos were recovered surgically 5 days later. The virus was reisolated from nasal and

vaginal swabs and from different parts of the genital organs (ovary, oviduct, uterus, cervix) of all infected sows. In 2 of the infected sows ADV was also found in the flushing medium used for embryo recovery. The results of virus isolation were confirmed by dot-blot hybridization and histological findings. Embryos with an intact zona pellucida (ZP) collected from the donors were treated twice with 0.25% trypsin solution for 1 minute each time before transfer into a seronegative sow. The recipient sow and her offspring remained ADV-seronegative on day 28 after farrowing, indicating that the transmission of ADV was blocked during the embryo transfer. Untreated control embryos transferred into AD-free recipients caused seroconversion based on the serum neutralization test carried out 40 days later. (**Medveczky et al, Theriogenology 1996;46:1357-1365.**)

The objectives of the present study were to (1) determine whether PRV and PRRSV are able to replicate in embryonic cells of porcine morulae and blastocysts and (2) assess the effects of PRV and PRRSV exposure on further embryonic development. Two- and 4-cell stage embryos were harvested from 24 sows at 2 days after insemination. Embryos were cultured until they reached the morula or blastocyst stage. At those stages 2.5 % pronase was used to remove the zona pellucida from half of the embryos. Twelve hours later, 2/3rds of the ZP-free, 2/3rds of the ZP-I embryos and 2/3rds of the hatched blastocysts were inoculated with 10^5 $TCID_{50}$ PRV (strain 89v87, second passage in swine testicle cells) or PRRSV (LV, 13^{th} passage in swine alveolar macrophages) at 39^oC for 1 hour. Control (unexposed) embryos were maintained under identical conditions. After viral exposure, all embryos were washed and examined every 12 hours for assessment of embryonic development. Further development for morulae was defined as reaching the blastocyst stage and for blastocysts was defined as reaching the expanded or hatched blastocyst stage. Differences in rates of development were analyzed using Chi-square analysis or Fisher's exact test. At 48 hours after viral exposure, embryos were fixed and stained for PRV and PRRSV using indirect immunofluorescence. Using indirect immunofluorescence, PRV- or PRRSV-infected cells were not detected in morulae or blastocysts. Incubation of ZP-I and ZP-free morulae and blastocysts with PRV or PRRSV did not inhibit in vitro development in comparison to the controls ($P<0.05$). The data show that like early cleavage embryos, morulae and blastocysts are refractory to PRV and PRRSV infection. Whether hatched blastocysts are refractory to viral infection is under investigation. It is suspected that more advanced embryonic stages become susceptible. (**Mateusen B, et al. 18^{th} IPVS, Hamburg, Germany, 2004, p118).**

In vivo-produced ZP-I and ZP-free morulae (6 days post insemination), early blastocysts (7 days post insemination), and hatched blastocysts (8 days post insemination) derived from 22 superovulated sows were exposed to 10^5 $TCID_{50}$ PRV (strain 89v87, second passage in swine testicle cells) or PRRSV (LV, 13^{th} passage in swine alveolar macrophages) at 39^oC for 1 hour. Control embryos were incubated under the same circumstances without viruses. Each group contained approximately 20 embryos. Embryonic development was assessed every 12 hours and differences analyzed using Chi-square analysis or Fischer's exact test. Also, at 48 hours post incubation, embryos were collected and examined for viral antigen by indirect immunofluorescence. Further embryo development of ZP-intact and ZP-free morulae and blastocysts was not affected by exposure to either of the viruses compared to controls ($P<0.05$). Moreover, using indirect immunofluorescence, no virus positive cells were detected in the embryos. Exposure of hatched blastocysts to PRV inhibited further

embryonic development as 100% (n=5) degenerated after viral exposure. This was significantly different from the controls and the PRRSV-incubated hatched blastocysts that did not experience any negative influence on embryo development. Based on these results, it can be concluded that embryonic cells are not susceptible to PRRSV infection up to the hatched blastocyst stage. Embryonic cells of morulae and blastocysts are refractory to PRV, but the virus had a detrimental effect on further development of hatched blastocysts. More experiments are necessary to confirm these results and to investigate whether, or at which pre-implantation stage, embryos are susceptible to PRRSV. (**Mateusen B, et al. Reproduction, Fertility and Development 2004;16:219(abstr.))**

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PRRSV infection and this latter finding was consistent with the absence of the PRRSV receptor sialoadhesin. **(Mateusen et al, Proceedings, International Pestivirus Symposium (IPVS), Copenhagen, 2006;Vol I:113.)**

African swine fever virus (ASFV)

Eighty, day 3 to 4, ZP-I embryos were exposed to $10^{6.6}$ hemadsorption dose 50%/ml ($HAdD_{50}$/ml) ASFV (Portugal vaccine strain) for 18 hours, washed ten times, cultured and then assayed. Ninety-five percent of the embryos retained infectious virus. Papain, versene or ficin treatment of the embryos (n= 24) had no effect on the retained virus, whereas trypsin-EDTA and pronase treatment of the embryos (n=141) were found to be effective in reducing the number of embryos carrying virus (30% instead of 95%) and lowering the amount of virus on the other embryos. To date, it has not been determined whether ASFV enters the embryonic cells, but evidence suggests that most of the virus, and perhaps all of it, is bound to the ZP (**Singh et al, Theriogenology 1984;22:693-700**).

Enteroviruses (ECPO-3; ECPO-6)

Twenty-four, day 2 to 3, ZP-I embryos were exposed to ECPO-3 virus and the same number and age of embryos to ECPO-6 virus by cocultivating them with infected cells for 2, 24 or 48 hours. Following culture the embryos were fixed and processed for EM without washing. Virus particles were observed in pores beneath the outer surface of the ZP and only rarely at the surface of the ZP (**Bolin et al, Am J Vet Res 1983;44:1036-1039**).

Foot and mouth disease virus (FMDV)

One hundred and sixteen, day 3 to 4, ZP-I embryos were exposed to 10^6 pfu/ml FMDV (type 0_1 strain) for 4, 12 or 48 hrs, washed ten times and then assayed in tissue culture. Approximately 5% of the embryos were positive in contrast to similar studies with ZP-I bovine embryos where none of the embryos carried infectious virus after washing. Exposure to the virus had no morphologically detectable effect on development of the embryos (**Singh et al, Theriogenology 1986;26:587-593**).

Sixty-two porcine embryos and 8 porcine ova were exposed in vitro to foot-and-mouth disease virus (FMDV), then assayed individually in vitro. FMDV was recovered from 2 embryos (**Mebus, Proc 91st Ann Mtg USAHA 1987;p10**).

Two hundred and sixty-seven porcine embryos were collected from 14 FMDV viremic donors and assayed by intradermal lingual (IDL) inoculation into steer tongues. No FMDV was detected. Recovery of FMDV from the in vitro exposed embryos is believed to be due to exposure of the embryos to high titre ($10^{7.6}$) FMDV (**Mebus, Proc 91st Ann Mtg USAHA 1987;p10**).

Swine vesicular disease (SVD)

Eighty-three, day 3 to 4, ZP-I embryos were exposed to 10_6 pfu/ml of SVDV, washed ten times, cultured and then assayed. Infectious virus was isolated from all of the embryos. Replication of the virus did not take place in the embryos and it appeared that most, if not all of the virus, was attached to the ZP. When 100 embryos were exposed to 10_7 pfu/ml SVDV and then treated with trypsin-EDTA or pronase after washing, there was a reduction both in the number (64%) of positive embryos and in the amount of virus on the embryos. However, no treatment was found to be effective in rendering all of the embryos "clean" (**Singh and Thomas, Theriogenology 1987;27:443-449**).

Fourteen pigs were infected with SVD. Two hundred and five ZP-I embryos collected from them were transferred to nine seronegative recipients. All of the recipients and all of the piglets produced remained SVD-seronegative. Thus, although SVD sticks to embryos under in vitro conditions (Singh and Thomas, Theriogenology 1987;27:443-449), these experiments indicate that the transmission of SVD through embryo transfer is unlikely since significant amounts of virus are not secreted into the reproductive tract during infection **(Singh et al, Theriogenology 1987;27:451-457**).

Hog cholera virus (HCV); classical swine fever virus

One hundred and seventy-one pellucida-intact porcine embryos were exposed to hog cholera virus (HCV) for 2 or 18 hrs. After washing the embryos 10 times in phosphate buffered saline (PBS), HCV was isolated from 165 of them. The amount of virus on each embryo was small, and when 24 embryos were cultured after viral exposure to allow replication to occur, all of the embryos were rendered noninfectious (**Dulac and Singh, Theriogenology 1988;29:1334-1341**).

When 232 embryos were exposed to HCV, washed and treated with either 0.05% trypsin-0.02% EDTA or 0.05% trypsin, only 29 embryos retained the virus. The effectiveness of trypsin/EDTA and trypsin in reducing the number of embryos carrying virus was similar. Both treatments rendered all embryos noninectious when they were exposed to less than 10_6 focus forming unit (FFU)/ml of HCV but failed to remove the virus from 21 to 27% of the embryos when they were exposed to levels of virus exceeding 10_6 FFU/ml (**Dulac and Singh, Theriogenology 1988;29:1334-1341**).

When 216 embryos/unfertilized eggs were exposed to HCV, washed, treated with trypsin or trypsin-EDTA and assayed in groups of 14-20, virus was isolated from 3 samples that had been exposed to more than 10_6 FFU/ml of HCV. The amount of virus detected on these group samples was similar to that detected on individual embryos (**Dulac and Singh, Theriogenology 1988;29:1334-1341**).

Three hundred and eighty-five, day 3-4, ZP-I embryos were collected from HCV-infected donors and transferred to 17 recipients. None of the recipients or their offspring seroconverted. Nineteen embryos and 74 eggs collected from the HCV-infected donors and assayed in vitro were negative. Six of 33 uterine flushes from the infected donors were

positive for HCV on assay (**Singh and Dulac, unpublished - cited in Singh, Theriogenology 1987;27:9-20**).

Six sows were infected intranasally with a Korean isolate of classical swine fever virus (CSFV). The distribution of virus in ovarian tissues was then assessed for 21 days by in-situ hybridization and immunohistochemistry. The virus was detected in ovaries between 7 and 21 days post inoculation (dpi) by both methods, but the labeling was particularly intense and widespread at 7 dpi. Viral nucleic acid and antigen were located almost exclusively within the cytoplasm of cells that were determined to be macrophages. Such cells were numerous in atretic follicles. Viral nucleic acid and antigen were not observed in primordial, primary or secondary follicles from infected sows at 7, 14 or 21 dpi. Results suggest that the virus replicates in circulating peripheral monocytes and gains access to ovarian tissue from the bloodstream, and that this contributes to the distribution of virus in macrophages throughout the atretic follicles. **(Choi C, Chae C. Journal of Comparative Pathology 2003;128:60-66.)**

The objective of this study was to investigate the susceptibility of in vivo- and in vitro-produced (IVP) porcine embryos to classical swine fever virus (CSFV). IVP zona pellucida (ZP)-intact porcine embryos (n = 721) were co-cultured with CSFV for 120 h. After washing according to the International Embryo Transfer Society guidelines (without trypsin) and transferring embryos to CSFV-susceptible porcine kidney cells (PK15 cell line), no virus was isolated. However, when 88 IVP ZP-intact porcine embryos were co-cultured with CSFV for only 48 h before being transferred to PK15 cells, virus was isolated in three of six replicates. Similarly, 603 in vivo-produced porcine embryos were co-cultured with CSFV for 120 h. Subsequently, CSFV was isolated in eight of 50 groups (16%) and the ability of these to form a blastocyst was significantly reduced when compared with the control group (68.2 +/- 19.9% vs 81.9 +/- 9.7%; p < or = 0.001). In contrast, the development of CSFV-exposed IVP porcine embryos was not affected when compared with control embryos (19.1 +/- 10.8% vs 18.9 +/- 10.6%; p > or = 0.05). After removal of the ZP of IVP embryos and subsequent co-culture with CSFV, the virus was isolated from all groups of embryos. These data suggest that virus replication had occurred in the embryonic cells. In conclusion, data indicate that in vivo- and in vitro-produced ZP-intact porcine embryos differ in their susceptibility to CSFV. Hatched or micro-manipulated embryos may increase the risk of transmission of CSFV by embryo transfer, which has to be confirmed by in vivo tests under isolation conditions. (**Schuurmann E, et al. Reprod Domest Anim. 2005;40:415-421).**

Vesicular stomatitis virus (VSV)

Sixty-six porcine ZP-I embryos were exposed to 10^5-10^6 pfu/ml of VSV and then washed 10x. Eighty percent of the embryos were subsequently found to be carrying the virus when assayed. Exposure to the virus had no detectable effect on development and there was no evidence of viral replication. Eighty-two ZP-I embryos exposed to 10^6 pfu/ml of VSV and then treated with 0.25% trypsin at pH 7.6-7.8 for 60-90 sec were negative for VSV on assay (**Singh and Thomas, unpublished - cited in Singh, Theriogenology 1987;27:9-20**).

Porcine Rubulavirus (PoRV; causative agent of blue eye disease)

Porcine rubulavirus (PoRV), also known as blue eye disease (BED) of swine, causes respiratory and reproductive problems in pigs at several developmental stages. To study the effect of PoRV infection on semen production, five boars were infected with 1 x 10(6) TCID(50)/ml of PoRV strain PAC-3 and evaluated for 59 days post inoculation (DPI). Infected boars developed reproductive tract pathology that included swelling of the testes and epididymides. Analysis of the semen showed that the infection had little effect on semen production in four animals, but semen from one boar showed severe alterations in sperm concentration, motility, and morphology. When motility was analyzed in BTS-diluted semen after 24, 48, or 72 h, alterations were detected in all boars. Furthermore, viral antigen was detected in semen, the seminal plasma fraction, or sperm fraction from all boars. These results showed that PoRV is excreted via semen and, therefore, artificial insemination is a potential route of dissemination. **(Solís M, et al., Res Vet Sci, 2007;83(3):403-9.)**

Mycoplasma hyopneumonia, Streptococcus suis, Pasteurella multocida

Note: This is the same abstract included under PRRSV. It contains information on multiple pathogens. There is a big need for genetical (international) linkage of porcine breeding herds. For nucleus herds with different health status this can be done by caesareans (costly, unfriendly), frozen sperm (half of the genes), surgical and non surgical embryo transfer (nsET). According to the research information of the International Embryo Transfer Society (IETS), mostly based on bovine embryos, ET is the safest way to transfer genes. To investigate the level of health security of porcine embryos an *in vitro* experiment with *Mycoplasma hyopneumoniae, Streptococcus suis,* and *Pasteurella multocida* DNP+ and PRRS (American and European strain) was carried out. For each pathogen investigated 40 to 50 vital day 4 embryos were collected, washed five times, inoculated with 10^5-10^6 PFU/$TCID_{50}$ and incubated 18 hours in D-PBS with 10 % lamb serum, without antibiotics at 37^{o}C. After incubation, the embryos were washed 12 times in groups of 10 in D-PBS with antibiotics and 0.25 % trypsin (IETS procedures). After washing the embryos were tested by culture or PCR in batches of 5. All the embryos tested were free from these pathogens after washing. It was concluded that porcine embryos exposed to these pathogens can be transferred without risk of transmission when linking nucleus herds by nsET-procedures. **(Smits JM et al. Book of Abstracts of the 52nd Annual Meeting of the European Association for Animal Production. Budapest, Hungary August 26-29, 2001, PhP6 no. 5; page 212).**

Chlamydophila/Chlamydia

Chlamydial infections of the genital organs cause reproductive failure in female pigs, and the uterus is recognized a target tissue for an infection. In contrast, information on the effect of chlamydiae on the porcine oviduct is patchily and inconclusive, although the bacteria are known to cause severe tubal defects in humans and laboratory animals. The aim of this study was to examine the segments ampulla (A), isthmus (I) and utero-tubal junction of the left (n = 20) or both (n = 22) oviducts, and uteri (U) from 42 culled repeat breeder pigs for chlamydiae using ompA-PCR, partial ompA gene sequencing, immunohistochemistry (IHC) and microscopy of tissue specimens for histopathology. As revealed by PCR, among a total of 26

chlamydia-positive females, 19 were tested positive in one or more segments of one or both oviducts, 14 were found positive in the uterus, and concomitant infections of both organs were observed in 7 of them. Sequencing of 33 PCR products revealed the following chlamydial species: Chlamydophila (Cp.) psittaci (n = 18), Cp. abortus (n = 2), Chlamydia (C.) suis (n = 10), and C. trachomatis (n = 3). Immunopositive staining was observed within the surface epithelium (in A, I, U), stromal tissue (in I, U) and muscular layer (in A, I, U). A total of 24 females had inflamed oviductal segments (in A and/or I) and 36 inflamed uteri. However, there was no relationship between histopathology and results of PCR or IHC. In conclusion, chlamydiae were found to infect oviducts and uteri of pigs. Further studies are required to clarify whether chlamydial infection causes specific histopathology and alters tubal function. **(Kauffold et al, Theriogenology 2006;66:1816-1823.)**

Horses

Equine Herpes Virus 1 (EHV1)

The objective of this study was to determine whether the 10 wash cycles proposed by IETS for bovine embryos efficiently decontaminated equine embryos exposed to equine herpes virus 1 (EHV1) in vitro. Donor mares and stallions were individually screened and shown to be negative for the virus by PCR detection of EHV-1 DNA in blood leukocytes, semen, and uterine lavages in which embryos were recovered. Twenty embryos were recovered and randomly assigned to one or two groups: 10 embryos were exposed for 24 h to infectious EHV-1 at 10_6 $TCID_{50}$/ml and 10 embryos were used as negative controls. Exposed embryos were washed in accordance with IETS recommendations for ruminant and porcine embryos, before being incubated for 24 h with semi confluent rabbit kidney (RK13) cells to detect any cytopathogenic effects (CPE) and finally tested for the presence of EHV-1 viral DNA by PCR. The embryo washing media were also assayed for the virus on RK13 cells and by PCR. Control embryos were neither exposed to the virus nor washed. EHV-1 was not found in the control embryos, or in the last five washes of the exposed embryos. However, the virus was detected in 7/10 of the embryos exposed to EHV-1 for 24 h, as well as in the first five washes of the embryos. The gradual disappearance of EHV-1 from the 10 successive wash solutions from the exposed embryos and the detection of viral DNA in 7/10 washed embryos by PCR, demonstrated that the washing procedure was unable to remove EHV-1 and suggested that EHV-1 could be attached to the acellular layer surrounding embryos (zona pellucida or capsule) or had penetrated the embryo. (**Hebia I., et al. Theriogenology, 2007;67:1485-1491.)**

The objective of the study was to determine whether the standard embryo washing protocol used following collection and prior to transfer, effectively decontaminates equine embryos exposed to EHV-1 in vitro. Twenty embryos were collected from 40 pony mares inseminated from 3 stallions. Mares and stallions were vaccinated. 2 groups (Group I and II) of 10 ZP intact embryos were constituted. Group I embryos (Group 1) were individually contaminated by contact (24 h) with EHV-1 (Kentucky D strain;10_6 $TCID_{50}$/ml) in separate wells. Group II embryos were used as negative controls. After viral exposure, they were individually washed ten times (IETS guidelines). Immediately after the 10 wash cycles (only for Group I), embryos (Group I and II) were incubated individually for 24 h on rabbit kidney cells cultures (RK13) to detect any pathogenic effects. After freezing DNA was extracted from embryos (Group I and II), samples of wash media, semen, uterine flushes and leucocytes from the mares and

stallions. PCR used in this study was a type-specific PCR for EHV-1 and EHV-4. EHV-1 DNA was detected by PCR in 7/10 embryos exposed toEHV-1 and in the first three washes from all embryos in Group I, in the fourth wash of five embryos (5/10) and in the fifth was of one embryo (1/10) ; no DNA was detected in the next washes (6 to 10). A cytopathic effect was observed in the RK13 culture incubated with the five first washes of those embryos. No virus was detected in RK13 cells cocultured with any embryo of either group. This study clearly demonstrates that the standard embryo washing protocol as recommended by the IETS did not completely eliminate EHV-1 after in vitro contamination of equine embryos. The elimination of EHV-1 with the 10 successive washes and the absence of detection of viral DNA in the final washes of all embryos from Group I demonstrates that the virus detected in the seven embryos was either adherent to the surface of the embryos, or had penetrated inside. (**Hebia I., et al. J Anim Reprod Sci, 2006;94:387-390.)**

Detection, by PCR, of EHV-1 DNA in an equine embryo collected from a healthy donor mare was reported by Carvalho et al. (Arq Bras Med Vet Zootec 2000; 52-54). In a previous study, we have demonstrated that, after an in vitro contamination of equine embryos, EHV1 cannot be removed from all (7/10) equine blastocysts (D7) by the washing procedure recommended by sanitary European regulations and IETS guidelines (Hebia et al, Theriogenology 2007; 67:1485-1491). As equine D7 blastocysts are surrounded by a capsule and a ZP or only by a capsule, the interactions between EHV1 and the capsule or the ZP could be different.

The objective of this study was to evaluate, in equine early-blastocysts (D6.5) surrounded by ZP, if the standard embryo washing procedure, or treatment with trypsin recommended by IETS guidelines for bovine embryos contaminated by BHV1, effectively decontaminate equine early-blastocysts exposed to EHV-1 in vitro.

Fifteen embryos were collected non-surgically on Day 6.5 after ovulation from pony mares previously inseminated with fresh semen from 3 stallions. The mares and stallions were regularly vaccinated against EHV1. The embryos were in a first step contaminated with the virus (strain Kentucky D) and in a second step randomly split into two groups: 5 embryos were individually washed in accordance with International Embryo Transfer Society (IETS) guidelines (Group 1), and 10 embryos (group 2) were individually, before the washing procedure, passed into a PBS without albumin bath and placed into a trypsin (0.25%) bath for 90 sec. The embryos (Groups 1 and 2), samples of wash media, semen, uterine flushes, and leukocytes from the mares and stallions, were then frozen and stored until DNA extraction using NucleoSpin®RNA Virus. The PCR used in this experiment was a type-specific PCR (Kirisawa et al, Vet Microbiol 1993; 36:57-67). The sequences of two primers were selected from the nucleotide sequence of EHV-1 and EHV-4 glycoprotein B genes.

EHV-1 DNA was not detected by PCR in any of the leukocyte samples from the mares and stallions. No EHV-1 DNA was found in the semen, uterine flushing media.

EHV-1 DNA was detected by PCR in 5/5 embryos (Group1) exposed to EHV-1 for 24 hours and only washed but not detected in the 10 embryos exposed to EHV1 and treated with trypsin. EHV-1 DNA was detected by PCR in the first 3 washes from all Group 1 embryos, in the 4^{th} and 5^{th} washes of 3 embryos (3/5), and in PBS bath, trypsin bath and two first washes from all Group 2 embryos, in the 3^{rd} wash of 7 embryos (7/10) and in the 4^{th} and 5^{th} washes of 2 embryos (2/10); however no viral DNA was detected in the 6^{th} to 10^{th} washes from any embryos of 2 groups.

This study clearly demonstrates that the standard embryo-washing protocol as recommended by the European rules did not completely eliminate EHV1 after in vitro contamination of D6.5 equine embryos surrounded by ZP. The elimination of EHV-1 with the 10 successive washes and the absence of detection of viral DNA in the final washes of all embryos from group 1, and the efficacy of treatment with trypsin to remove EHV1 from all group 2 embryos demonstrate that the virus was adherent on the equine ZP.

Further studies are needed to evaluate the interaction between EHV1 and equine blastocysts surrounded only by the capsule and if the enzymatic treatment could effectively decontaminate them, as for equine early blastocysts (D6.5) in the present study and as for bovine embryos infected by BHV-1.

(Hebia I, et al. Proceedings of 23rd Annual AETE meeting, Alghero, Sardinia. 2007: 174 abstr.)

Equid herpesvirus 3

Equine coital exanthema (ECE) is an acute, venereal discase caused by equid herpesvirus 3 (EHV-3), characterized by the formation of papules, vesicles, pustules, and ulcers on the vaginal and vestibular mucosa as well as on the skin of the penis, prepuce, and perineal region of mares and stallions. The present work describes an outbreak of ECE characterized by typical lesions around the anus and on the perineal skin, discomfort, and anorectal lymphadenopathy, which occurred in a large number of donor and recipient mares at an embryo transfer center in Argentina. We found that the horses' movement pattern, the environmental conditions, and the management procedures could have favored the introduction and/or reactivation from latency and spread of EHV-3. Although this is the first report of an outbreak of ECE in an embryo transfer facility, it reinforces the need to introduce additional hygienic and preventive measures to avoid EHV-3 spread and future ECE outbreaks.

Barrandeguy M, Perkins J, Mac Donough J, Vissani A, Olguin C, Thiry E Occurrence of Equine Coital Exanthema in Mares from an Embryo Transfer Center Journal of Equine Veterinary Science 2010 (30), P 145-149

Taylorella equigenitalis (causative agent of contagious equine metritis [CEM])

Contagious Equine Metritis (CEM) is an equine venereal disease caused by the bacterium *Taylorella equigenitalis*. CEM reduces fertility by causing acute vaginitis and endometritis in mares bred by infected stallions. Stallions are asymptomatic carriers and mares can pass the bacteria back to stallions. This disease caused massive financial losses in the thoroughbred breeding industry of the United Kingdom and United States in 1977 and 1978. CEM is considered a foreign animal disease in the United States. Fresh or chilled semen from three stallions, unknowingly CEM positive, was used for AI as part of an embryo transfer (ET) program. Positive diagnosis for *T. equigenitalis* was by culture. Cultures were carried out on chocolate agar under 5–10% CO_2. Test mare breeding per the U.S. Code of Federal Regulations (2006 9 CFR) was performed, and stallions did infect these mares. Real-time PCR was used to distinguish between *T. equigenitalis* and *T. asinigenitalis*, with a 97% homology for *T. equigenitalis*. Kirby-Bauer antibiotic sensitivity testing was performed. Of note, the bacterium was sensitive to gentamicin. Semen was collected using a Minitube artificial vagina (Minitube of America, Inc., Verona, WI, USA), analyzed using CASA (SpermVision™, Minitube), and prepared according to published guidelines. Semen was extended using Minitube EquiPRO® CellGuard™ extender with amikacin and penicillin. Six mares were used for breeding. Donor and recipient mares were examined using transrectal palpation and ultrasound to confirm estrus, follicular size, and ovulation synchrony. Mares were examined for signs of vaginitis, cervicitis, or endometritis. Embryo recovery was performed using EquiPRO recovery media on Day 7. Recovered embryos were washed twice in EquiPRO holding medium at dilution rate of 5 µL recovery media to 3 mL holding media. Media contained gentamicin and kanamycin. No mares were treated with systemic antibiotics. Recovered embryos were transferred to recipients for the purpose of producing live foals. Seventeen embryos were recovered in 19 attempts, yielding an 89% embryo recovery rate. Fourteen of the embryos were transferred to recipient mares. Three embryos were vitrified. Ten of 14 (78%) transfers yielded pregnancies by Day 14 of gestation. Seven live foals were born. After CEM diagnosis in the stallions, the donor and recipient mares were tested. Testing was performed per 2006 9 CFR. The clitoral fossa and

sinuses and, in addition, the cervix were cultured. Culture methods were as previously described. No mare sample returned a positive culture. To our knowledge, this is the first report in the United States demonstrating transfer of genetics of CEM-infected stallions without transfer of disease or reduction of fertility since eradication. The use of AI with extended semen is likely the greatest contributor to this success. It is felt that ART (Assisted Reproductive Technology(s)) may be a beneficial tool for propagating genetics of animals confronted with pathogen presence; however, further controlled studies are necessary. (**Hayna JH, et al. Reproduction, Fertility and Development, 2008;20:157-158 abstr.)**

Equine arteritis virus

The objective was to evaluate the potential risks associated with embryo transfer from mares bred with equine arteritis virus (EAV) infective semen. Twenty-six mares were embryo donors, whereas 18 unvaccinated and EAV antibody seronegative mares were embryo recipients. Of the 26 donor mares, 15 were unvaccinated and seronegative for antibodies to EAV and 11 were vaccinated for the first time with a commercially available modified live virus vaccine against EVA before breeding and subsequent embryo transfer. All donor mares were bred with EAV-infective semen from a stallion persistently infected with the virus. Twenty-four embryos were recovered 7 d post-ovulation; all were subjected in sequential order to five washes in embryo flush medium, two trypsin treatments, and five additional washes in embryo flush medium (prior to transfer). Twelve and seven embryos (Grades 1 or 2) were transferred from the non-vaccinated and vaccinated donors, respectively, and pregnancy was established in 3 of 12 and 2 of 7. Perhaps trypsin reduced embryo viability and pregnancy rate. The uterine flush fluid of 11 mares (9 of 15 and 2 of 11 from non-vaccinated and vaccinated donor groups, respectively) was positive for EAV by VI (confirmed by real-time RT-PCR); the wash fluid from the embryos of nine of these mares was negative following 10 washes and two trypsin treatments. However, the embryo wash fluid from two mares was still positive for EAV after all 10 washes and the two trypsin treatments, and one embryo was positive for EAV. Two of 18 recipient mares had seroconverted to EAV 28 d after embryo transfer. Virus was not detected in any fetal tissues or fluids harvested after pregnancies were terminated (60 d). In conclusion, we inferred that the washing protocol of 10 washes and two trypsin treatments did not eliminate EAV from all embryos; due to limitations in experimental design, this requires confirmation. Furthermore, there may be a risk of EAV transmission associated with in vivo embryo transfer from a donor mare inseminated with EAV infective semen.

Broaddus CC, Balasuriya UB, Timoney PJ, White JL, Makloski C, Torrisi K, Payton M, Holyoak GR. Infection of embryos following insemination of donor mares with equine arteritis virus infective semen. Theriogenology. 2011 Jul 1;76(1):47-60.

Human

The aim of this preliminary experimental study was to test the stability of cryopreservation straws to human immunodeficiency virus-1 (HIV-1). Three kinds of straws were tested: four polyvinyl chloride (PVC), four polyethylene terephthalate glycol (PETG) and 20 high-security ionomeric resin (IR). The PVC and PETG straws were sealed ultrasonically, and the IR straw by thermosoldering. Each sealed straw was cut in half to produce two demi-straws and then filled with 100 microl of HIV-1-containing supernatant (reverse transcriptase activity: 15 000 c.p.m./50 microl). The unsealed cotton end of PVC and PETG straws and the two halves of the IR straws (cotton and plastic plug ends) were tested. Each demi-straw was two- thirds submerged in RPMI medium at 37 degrees C, and RPMI samples were withdrawn on days 3, 7 and 11. Viral RNA was extracted from the medium and then amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) followed by nested PCR using primers specific to HIV-1 protease. On day 7, no HIV-1 RNA was detected in any of the different samples of medium that had surrounded the unsealed PVC and PETG straws with cotton ends, but three IR specimens were positive. On day 11, PVC and PETG remained negative but HIV-1 RNA was detected in RPMI samples for two more IR demi-straws (n = 5). In conclusion, under these experimental conditions (at 37 degrees C), the unsealed cotton end PVC, PETG and thermosoldered cotton end IR demi-straws appeared to be safe for HIV-1, while IR straws, sealed or unsealed with a plastic plug and with unsealed cotton ends, leaked. **(Benifla JL et al. Hum Reprod 2000:15:2186-2189)**

Hamster

Antibiotics are commonly added to embryo culture media, but effects on embryo development have not been examined thoroughly. Hamster ova were used to investigate whether penicillin, streptomycin or gentamicin affect embryo development in vitro. Ova were collected 10 h post activation by spermatozoa in vivo and cultured in five treatments: 1) Control: chemically-defined medium HECM-9 with no antibiotics; 2) HECM-9 with 100 IU/mL penicillin; 3) HECM-9 with 50 microg/mL streptomycin; 4) HECM-9 with 10 microg/mL gentamicin and 5) HECM-9 with both 100 IU/mL penicillin and 50 microg/mL streptomycin. Individually, penicillin, streptomycin and gentamicin did not affect embryo development to the 8-cell stage at 58 h post oocyte activation, or morula/blastocyst stages, or blastocysts alone at 82 h post activation. However, when penicillin and streptomycin were both present in the culture medium the percentages of 8-cell embryos at 58 h and blastocysts at 82 h were significantly lower than the control. No antibiotic treatment improved hamster embryo development in vitro. We caution against the use of penicillin and streptomycin together for hamster embryo culture, and show that it is not necessary to include any antibiotics in embryo culture media for up to 72 h if proper sterile technique is used with an oil overlay%. **(Zhou H et al. Theriogenology 2000;54:99-106)**

Mice

Mouse - The technique of embryo transfer has been evaluated for the purpose of changing the mouse stocks to a germfree (GF) status. Our results show reproducible and qualityassured conversion of animals to those which are negative for the presence of microorganisms. Rapid and easy access to GF mice is advantageous for studies of selected microflora and their crosstalks with the host, when applying, e.g. genomic, proteomic and metabolic methodology. The study involved embryo transfer in an isolator environment, thereby allowing implantation of cleansed embryos into GF recipients under wellcontrolled conditions. The recipient females gave birth normally and took care of the offspring as if they were their own pups, thus enhancing the survival rate. Access to full technical resources required to maintain GF isolators are, however, a prerequisite. In this study, we used stainless steel isolators designed by Gustafsson (1959), on which a stereomicroscope was mounted to facilitate embryo transfer inside the isolator. The use of embryo transfer and isolator techniques will facilitate the availability of various mouse mutant models under different gnotobiotic conditions, GF, monoxenic or polyxenic animals, to enable comparison with conventional animals for physiological and pathophysiological studies. **(Inzunza J et al, Lab Animal 2005 Oct;39:421-427.)**

The present study investigated the presence and location of fluorescent microspheres having the size of mouse hepatitis virus (MHV) and mouse minute virus (MMV) in the zona pellucida (ZP) of *in vivo*-produced murine embryos, transmission of these viruses by embryos during embryo transfer, and the time of seroconversion of recipients and pups. To this end, fertilized oocytes and morulae were exposed to different concentrations of MMVp for 16h while 2-cell embryos and blastocysts were co-incubated for 1h. In addition, morulae were exposed to MHV-A59 for 16h. One group of embryos was washed and the remaining embryos remained unwashed prior to embryo transfer. To detect antibodies to MHV and MMV in recipients and progeny, serological analyses were performed via ELISA on d14, 21, 28, 42, and 63 and on d42, 63, 84, 112, 133, and 154 post embryo transfer, respectively. Co-incubation with a minimum of 10^5/ml fluorescent microspheres showed that particles with a diameter of 20 but not 100 nm crossed the ZP of murine blastocysts. Washing generally led to a 10 to 100-fold reduction of MMVp. Washed MMV but not MHV exposed embryos led to the production of antibodies, independent of embryonic stage and time of virus exposure. Recipients receiving embryos exposed to a minimum of 10^7 $TCID_{50}$/ml MHV-A59 and 10^2 $TCID_{50}$/ml MMVp seroconverted by d42 post embryo transfer. The results indicate that MMV but not MHV can be transmitted to recipients even after washing embryos 10 times prior to embryo transfer. **(Mahabir E et al, Biology of Reproduction 2007;76:189-97)**

Lymphocytic choriomeningitis virus (LCMV)

Persistent LCMV infection in wild-derived MAI/Pas mice housed under conventional conditions remained undetected for a decade, despite periodic health monitoring using dirty-bedding sentinels. When MAI/Pas mice were rederived by embryo transfer, recipient mothers produced antiLCMV antibodies, which first revealed the presence of the virus in the colony. Before this information was obtained, MAI/Pas mice had been shipped to another facility, undergone cesarean rederivation there, and been introduced into the recipient

barrier. The foster mothers of rederived pups were LCMV-negative according to enzyme-linked immunosorbent assay, but sera of both cesarean-rederived MAI/Pas mice and their foster mothers were positive for LCMV infection by immunofluorescent assay (IFA). LCMV was isolated from the MAI/Pas mice, and its genomic RNA was sequenced. Examination of animal technicians in contact with LCMV-infected mice and of other mouse samples by IFA or a reverse transcriptase-polymerase chain reaction test (or both) revealed that neither the workers nor other animals had been infected with LCMV. Experimental data showed that LCMV transmission from persistently infected mice to naïve ones occurred only after direct contact of animals housed in the same cage. This experience demonstrates the importance of careful viral monitoring in the transfer of laboratory rodents between institutions, the limitation of dirty-bedding sentinels for detection of LCMV infection, and the inadequacy of cesarean rederivation for elimination of enzootic LCMV infection. **(Ike F, et al., Comp Med, 2007;57(3):272-81.)**

Mouse Parvovirus (MPV)

We used primary and nested polymerase chain reaction (PCR) assays to determine the presence of mouse parvovirus (MPV) in mouse sperm, oocytes, preimplantation embryos, and ovarian tissues collected from MPV-infected mice. The primary PCR assay detected MPV in 56% of the sperm samples. MPV was not eliminated by passing sperm samples through a Percoll gradient. After Percoll treatment, MPV was still present in 50% of the samples according to primary PCR assay. Oocyte samples that did not undergo extensive washing procedures had detectable MPV in 7% of the samples based on the primary PCR assay, but nested PCR assay detected higher (28%) infection rate. However, MPV was not detected in oocytes that underwent extensive washing procedures, as assessed by either primary or nested PCR assay. Although primary PCR did not detect MPV in embryos, a nested PCR assay determined that 50% of the embryos were positive for the virus. In addition, ovarian tissues were collected from 3 different mouse colonies with enzootic MPV infection. Ovarian tissue collected from 129CT, 101/R1, and Sencar mice had high incidence (38%, 63%, and 65%, respectively) of MPV infection on the basis of nested PCR amplification. These results demonstrate that mouse gametes, embryos, and ovarian tissues may be contaminated with MPV and therefore caution is necessary when infected germplasm is used for assisted reproductive technologies such as embryo transfer, establishing embryonic stem cell lines, in vitro fertilization, ovary transplantation, and intracytoplasmic sperm injection. **(Agca Y, et al. Comp Med, 2007;57(1):51-6.)**

Murine parvoviruses, including minute virus of mice (MVM), represent major infectious disease problems encountered in contemporary laboratory animal research facilities with embryo transfer (ET), one of the most widely used techniques for rederivation. Using an in vivo approach, the objectives of this study were to assess the risk of MVM transmission during rederivation and to provide data that allow recommendation of preventive measures. Therefore, we determined whether immunosuppressive variant MVMi viral DNA is detectable in reproductive organs, gametes (oocytes and spermatozoa), and embryos collected from experimentally infected mice and whether washing as recommended before ET eliminates MVMi sufficiently from gametes and embryos. Fractions of reproductive organs tested positive from Day 5 to Day 30 postinoculation, demonstrating a risk for a minimum period of 4 wk; the highest incidence of positive organs was found between Day 9

and Day 13 postinoculation. Real-time PCR detected viral DNA to a lesser extent in male than in female reproductive organs. MVMi DNA was detected in oocytes and sperm cells derived after in vivo infection but not in two-cell embryos. In vitro contamination studies revealed that the virus firmly adheres to the zona pellucida after 10 wash steps, indicating that even extensive washing might not eliminate MVMi completely from embryos. According to this systematic in vivo approach, recommended measures to prevent transmission of MVM during rederivation include sufficient washing of embryos, accompanying testing using adequate (PCR) methods, and using embryos rather than in vitro fertilization techniques; furthermore, the exchange of gametes should be considered a risk factor.

Janus LM, Smoczek A, Hedrich HJ, Bleich A. Risk assessment of minute virus of mice transmission during rederivation: detection in reproductive organs, gametes, and embryos of mice after in vivo infection. Biol Reprod. 2009;81(5):1010-5.

****DISEASE TRANSMISSION THROUGH IN VITRO PRODUCED EMBRYOS****

Cattle

Akabane virus

Bovine oocytes surrounded by cumulus were exposed to BHV-1, bluetongue virus (BTV) or akabane virus (AV), matured in culture for 24 h and then fertilized in vitro. BHV-1 was isolated from medium 3 d after IVF, cytopathic effect in the cumulus cells was obvious, and embryos failed to develop beyond the 8-cell stage. BTV replication was obvious in cumulus while embryos developed to blastocysts. No effect of AV replication could be observed in cumulus during in vitro development. They concluded that the viruses had not infected the ZP-I oocytes or embryos. (**Tsuboi T and Imada T. Vet Micro 1997;51:135-142**)

Bluetongue virus

Bovine oocytes surrounded by cumulus were exposed to BHV-1, bluetongue virus (BTV) or akabane virus (AV), matured in culture for 24 h and then fertilized in vitro. BHV-1 was isolated from medium 3 d after IVF, cytopathic effect in the cumulus cells was obvious, and embryos failed to develop beyond the 8-cell stage. BTV replication was obvious in cumulus while embryos developed to blastocysts. No effect of AV replication could be observed in cumulus during in vitro development. They concluded that the viruses had not infected the ZP-I oocytes or embryos. (**Tsuboi T and Imada T. Vet Micro 1997;51:135-142**);

The objective of this study was to expose bovine IVF embryos to bluetongue virus (BTV), which has been shown not to adhere to in-vivo-derived embryos, and then determine if IETS washing procedures would be effective for removing the virus. In 5 weekly trials, oocytes from an abattoir were matured, fertilized and cultured to day 7. The Day 7 embryos (zona pellucida-intact morulae and blastocysts) without visible adherent material (at 50x magnification) were divided equally into 2 groups. One half was exposed to BTV serotype 17 ($8x10^5$ to $2x10^8$ plaque forming units/ml) for 2 hours. The other half was held in the same medium without virus for 2 hours. Time of exposure was short to simulate many previous studies on in-vivo-derived embryo-pathogen interactions. A total of 77 BTV-exposed embryos (9 groups, containing 5 to 10 embryos each) and 77 unexposed embryos in identically sized groups were then washed in Ham's F10 medium with 10% FBS according to IETS guidelines except 12 instead of 10 washes were used. Subsequently, washes 10, 11 and 12 were overlaid with bovine embryonic cells (BE12-6), incubated and observed for cytopathic effect for 5 days. Also, sonicate fluids from each washed group were inoculated onto monolayers of BE12-6 cells that were overlaid with agarose, incubated and observed for appearance of plaques for 5 days.
After washing exposed groups, virus was found in washes 10 and 11 for 6 of 9 groups and in wash 12 for 8 of 9 groups. No cytopathic effect was observed in plaque assays of unexposed groups. However, 5 to 10 plaques were observed in assays of all 9 groups of expose embryos after washing. Plaques were expanded by subsequent culture in BE12-6 cells and virus determined to be BTV by neutralization with specific antisera. Thus, a washing protocol that

was slightly more vigorous than the IETS protocol for in-vivo-derived embryos was ineffective for removal of BTV from IVF embryos after artificial exposure. **(Langston NL, et al, Theriogenology 1999;51:273 abstr)**

Bovine herpes virus 1 (BoHV-1)

The aim of this study was to evaluate the efficiency of trypsin treatment on the inactivation of bovine herpesvirus type 1 (BoHV-1) on in vitro produced by fertilization and artificially infected bovine embryos. Bovine embryos on day 7 were exposed with 10 microl of BoHV-1, Los Angeles strain 10(7.5) TCID. These embryos and control embryos were divided in two groups: submitted to the sequential washes or to the trypsin treatment according to the International Embryo Transfer Society (IETS) guidelines. The embryos and the last washing drop of each group were used as inoculum to infect Madin Darby bovine kidney (MDBK) cells and submitted to nested PCR reaction using the primer that encodes the gene conserved region of virus glycoprotein gB. The data have shown that the control embryos and their last washing drop were negative. The exposed embryos that were treated with trypsin have shown positive results on the n-PCR and MDBK culture, and their last washing drop were negative. Our data have demonstrated that the trypsin treatment was not able to eliminate the BHV-1 of the embryos, suggesting an interaction between virus and embryo.
D'Angelo M, Visintin JA, Richtzenhain LJ, Gonçalves RF. Evaluation of trypsin treatment on the inactivation of bovine herpesvirus type 1 on in vitro produced pre-implantation embryos. Reprod Domest Anim. 2009;44(3):536-9.

In this study, the polymerase chain reaction (PCR) was used to evaluate the presence of viral DNA in ovarian tissue, in the *cumulus*-oocyte complex (COC), follicular liquid, and blood of animals naturally infected with bovine herpesvirus-1 (BoHV-1). The serum profile of the sampled animals was also evaluated. Samples of serum, blood, ovarian tissue, follicular liquid, and COC were collected from 147 slaughterhouse animals that were not vaccinated against BoHV-1. Contaminated or insufficient samples were disregarded. Serological tests allowed the identification of serum-positive animals with neutralizing antibodies against BoHV-1. Analysis of samples by PCR revealed the presence of viral DNA in 0.9% (1/115) of the COC samples, in 4.3% (5/117) of the ovarian tissue samples, and in 2.8% (3/108) of the blood samples. Viral DNA was not detected in any of the follicular liquid samples. In serological samples, a positivity of 83.6% (117/140) was observed for BoHV-1. All PCR-positive animals, regardless of the samples analyzed, showed positivity in the serum neutralization test for the detection of BoHV-1-specific antibodies. According to these results, a high prevalence of antibodies against BoHV-1 was detected in naturally infected animals from different herds, and the molecular tests revealed the presence of viral DNA in bovine ovarian tissue, providing evidence that this might be a site of BoHV-1 infection in naturally infected animals.
Pereira E C M ; Da Costa E P ; Junior A S ; De Araujo Mendes V R ; Dos Santos G M ; Da Costa S L ; Santos M R . Natural infection in ovarian structures by bovine herpesvirus 1: molecular and serological detection Semina: Ciências Agrárias, Londrina 2015 36 (2):863-870

The objective of this work was to investigate the presence of bovine herpesvirus type 1 (BoHV-1) in follicular fluid and in cumulus-oocyte complexes (COC) recovered from naturally

infected cows but with no clinical signs of the disease. Cows that were seropositive (n=38) or seronegative (n=8, control) to infectious bovine rhinotracheitis were selected after a serum neutralization test in microplates. The presence of the virus was investigated by PCR in COC and in follicular fluid. Viral DNA was not found in any of the samples. The obtained results suggest that serologically positive cows with no clinical signs of the disease offer negligible risk of transmitting BoHV-1 by COC or follicular fluid.

Penido Oliveira A, Bryan Heinemann M, Cortez A, Pires do Nascimento PM, Romulo Cerqueira Leite R, Moreira Viana JH. Bovine herpesvirus type 1 in cumulus-oocyte complexes. Pesq. agropec. bras., Brasília, v.51, n.5, p.676-679, maio 2016.

Bovine herpesvirus 1 (BHV1) is an important bovine pathogen, responsible for respiratory diseases and reproductive problems. This study investigated the penetration capacity of BHV1 into oocytes after co-incubation for either 1 h or 24 h. Immunofluorescence assays in cumulus-oocyte complexes (COCs) and denuded oocytes (without the presence of cumulus cells) were performed and evaluated using confocal laser scanning microscopy. Blood samples and ovaries from BHV1 seronegative cows were used. The oocytes recovered were divided into two groups. Group I comprised COCs (n = 312) and denuded oocytes (n = 296), which were experimentally infected with BHV1 and incubated for 1 h at 38.5˚C and 5% CO2. Group II comprised COCs (n = 425) and denuded oocytes (n = 405), which were co-incubated with BHV1 under the same conditions for 24 h. The negative control of these two groups was respectively subjected to the same protocol, except for exposure to BHV1. To our knowledge, this study provides the first evidence of BHV1 detection within COCs and denuded oocytes exhibiting intact zona pellucida when co-incubated with the virus for 24 h. Immunolocalization also confirmed the presence of BHV1 in the cytoplasm of the cumulus cells of all COCs exposed to the virus after both incubation periods. In conclusion, detection of BHV1 inside oocytes has a great meaning for the field of animal reproduction. The detection of BHV1 in different layers of cumulus cells also demonstrates that these cells are sources of viral infection.

Queiroz-Castro VLD, da Costa EP, Alves SVP, Machado-Neves M, Guimarães JD, Gomes LL, et al. (2019) Bovine herpesvirus 1 can cross the intactzona pellucida of bovine oocytes after artificial infection. PLoS ONE 14(7): e0218963.
https://doi.org/10.1371/journal.pone.0218963

Highlights

- •Bovine herpesvirus 1 (BoHV-1) impaired in vitro oocyte development.
- •In vitro-infected and naturally infected oocytes presented lower maturation rates.
- •Oocyte developmental capacity was dependent of the cow's BoHV-1 antibody titer.
- •BoHV-1 was detected in cumulus cells, zona pellucida and inside the oocyte.
- •BoHV-1 may compromise reproductive performance due to impairment on maturation.

Abstract

Bovine herpesvirus 1 (BoHV-1) disseminates easily, is difficult to control, and is widely spread in cattle herds worldwide. BoHV-1 causes a broad range of losses to the cattle industry, mainly concerning reproduction. Previous studies involving experimental infection of BoHV-1 in an in vitro embryo production system have reported impairment of embryonic development by BoHV-1. In this study, we evaluated the interference of BoHV-1 in the in vitro maturation system of cumulus-oocyte complexes (COCs) and denuded oocytes (DOs)

cultured with a cumulus cell suspension. Blood samples and ovaries were collected from slaughterhouse cows unvaccinated against BoHV-1. Using virus neutralization assays, the seropositive animals were classified according to their antibody titers. The oocytes were recovered by follicular aspiration and divided into two groups, COCs and DOs, which were evaluated for their nuclear maturation capacity using immunofluorescence assays by laser scanning confocal microscopy. Two experiments were carried out: (I) in vitro maturation of COCs and DOs after artificial infection of seronegative animals and (II) in vitro maturation of COCs and DOs of seropositive animals. In experiment I, a difference ($P < 0.01$) was observed between the maturation rates of the control group COCs (78.2%) and the infected COCs (43.6%). In experiment II, there was a difference ($P < 0.01$) in the maturation rate between animals with antibody titers ≥16 (56.9%) and the control group (79.4%). Immunofluorescence assays identified BoHV-1 in the COCs and DOs. Therefore, it was concluded that BoHV-1 affects the in vitro maturation process in both in vitro and natural infections.

Alves, S.V., da Costa, E.P., Queiroz-Castro, V.L., Machado-Neves, M., Guimarães, J.D., Gomes, L.L., Junior, M.A.A. and Júnior, A.S., 2019. Bovine herpesvirus 1 can impact the bovine oocyte development during in vitro maturation. *Research in veterinary science*, *123*, pp.135-140. https://doi.org/10.1016/j.rvsc.2018.12.020

Highlights

- •Cumulus-oocyte complexes (COCs) from asymptomatic cows presented the BoHV-1.
- •The virus may be present in cumulus cell of COCs.
- •BoHV-1 was detected in the cytoplasm of cumulus cells.
- •Infected COCs could elucidate the reproductive failures shown in seropositive cows.

Abstract

Bovine herpesvirus 1 (BoHV-1) is the causative agent of infectious bovine rhinotracheitis (IBR) and is also associated with reproductive failure. This study investigated the presence of BoHV-1 in cumulus-oocyte complexes (COCs) of naturally-infected cows without clinical signs of IBR. The presence of BoHV-1 in COCs was evaluated by immunofluorescence using confocal laser scanning microscopy. Blood samples and ovaries from 82 cows that had not been vaccinated against BoHV-1 were collected for serological analysis. COCs were divided into two pools: COCs derivate from seropositive cows and from seronegative cows. Then, the samples were processed for confocal microscopy analysis. The results indicated that 61% (50/82) of cows were seropositive for BoHV-1. A total of 719 COCs were obtained from the cows and processed. None of 276 COCs from the 32 seronegative cows presented BoHV-1. However, BoHV-1 was present in the cytoplasm of cumulus cells from 158 out of 443 COCs aspirated from the seropositive cows. The detection of BoHV-1 in the COCs of seropositive cows suggests that the COCs of naturally-infected, asymptomatic cows may be infected with BoHV-1.

Queiroz-Castro, V.L., da Costa, E.P., Alves, S.V.P., Júnior, A.S., Machado-Neves, M. and Guimarães, J.D., 2018. Detection of bovine herpesvírus 1 in cumulus-oocyte complexes of cows. Research in veterinary science, 120, pp.54-56. https://doi.org/10.1016/j.rvsc.2018.08.010

Bovine herpesvirus-4

The objective of the present study was to assess whether bovine herpesvirus 4 (BHV-4) is able to infect in vitro-produced bovine embryos. A green recombinant BHV-4 (BHV-4EGFPΔTK), obtained by insertion of an EGFP gene into the TK locus of BHV-4, was used. The presence of this marker protein made it possible easily to detect infected cells under physiological conditions, without harmful manipulation of the cells or the addition of exogenous substrates, so that the spread of the virus could be followed in real time. Zona pellucida intact (ZP-I) and zona pellucida open (ZP-O) blastocysts were exposed to 10_6 $TCID_{50}$ viral particles and infection was monitored by fluorescent microscopy for 48 h. Expression of EGFP and degeneration of embryonic cells was observed in three of the 18 ZP-O embryos, but in none (n=21) of the ZP-I embryos. It was concluded from this preliminary study that BHV-4 has only low ability to infect in vitro produced bovine embryos, depending on the absence of the ZP, the amount of virus present and the stage of embryonic development. However, embryonic stem cells could be transduced by BHV-4EGFPΔTK just after differentiation, as shown by expression of EGFP. **(Donofrio G et al. Veterinary Research Communications 2003;27:415-424)**

Bovine herpesvirus-5

Bovine herpesvirus-5 (BoHV-5), which is the second most important infectious brain disease affecting livestock in Latin America, has been detected in bull semen and aborted fetus; however, no reports are found regarding its presence in bovine embryos. Because it has 90% genomic similarity to BoHV-1, it is possible that BHV-5 can infect the genital system after viral reactivation, leading to reproductive disorders. This study was designed to investigate the effects of infection of bovine blastocysts (B) by BoHV-5. Hormones and fetal calf serum were tested by PCR and considered free of virus. Selected oocytes, obtained from ovaries at a local slaughterhouse, were washed in PBS with 10% fetal calf serum (Nutricell®, Campinas, Brazil). The oocytes were transferred to 100-µL drops of maturation medium consisting of TCM 199 (Gibco®, Grand Island, NY, USA), 0.5 µg mL-1 of FSH (Pluset®, Calier, Spain), and 50 µg mL-1 of LH (Lutropin®-V, Bioniche Inc., Belleville, Ontario, Canada) for 24 h at 39°C and 5% CO2 in air. Afterward, frozen semen (500 µL) was thawed and placed on a Percoll gradient (45 and 90%) and centrifuged at 700 × g for 30 min. The resultant pellet was centrifuged (200 × g for 5 min) in TALP medium and the sperm pellet was then diluted in TALP plus phenylalanine (PHE) and heparin. After an 18-h fertilization period, presumptive zygotes were transferred to culture in CR2 medium up to Day 7 post-fertilization. The procedures were carried out under the same conditions previously reported for IVM. A total of 150 intact B were assigned into 2 experimental groups: I (free of virus) and II (with virus; 102 TCID50/mL for a 1-h period). Then, B of both groups were washed and re-cultured for 72 h in drops of CR2 without virus. The percentage of embryos reaching the hatched blastocyst (HB) stage was observed and analyzed by the chi-square test. At this time, HB were fixed to investigate the presence of virus, degree of apoptosis, and oxidative stress. The virus detection was performed by using an in situ hybridization assay with a specific probe to the glycoprotein C gene of BoHV-5 labeled to biotin. The apoptosis was determined by the annexin V, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), and propidium iodide (PI) markers, using immunofluorescence technique. The oxidative stress was realized by using monoclonal anti-AOP1 (antioxidant-like protein 1; Sigma®, St. Louis, MO, USA) through immunoassay. More HB ($P > 0.05$) were found in group II (75.0%) than for group I (55.0%). In both groups, positive signs for the presence of the apoptosis and oxidative stress markers were observed. The mechanism of apoptosis was initiated independently of virus

presence, as evidenced by positive signs also observed in group I. However, oxidative stress was intense in group II, suggesting an evident viral effect on the host cell without compromising embryonic development. These findings might indicate that BoHV-5 uses some mechanisms that keep the cell viable to allow its replication, as seen by the greater hatching rate of infected embryos (75%) compared with the control (55%). **Silva-Frade C, Martins Jr. A, Borsanelli AC, Frade MC, and Cardoso TC. In vitro development of blastocysts continues after artificial infection with bovine herpesvirus type 5. Reproduction, Fertility and Development. 2010; 22(1): 254 (abstr.).**

BACKGROUND: Bovine Herpesvirus type-5 (BoHV-5) is a neurovirulent α-Herpesvirus which is potentially pathogenic for cows and suspected to be associated with reproductive disorders. Interestingly, natural transmission of BoHV-5 by contaminated semen was recently described in Australia. Additionally, BoHV-5 was also isolated from the semen of a healthy bull in the same country and incriminated in a natural outbreak of reproductive disease after artificial insemination. In contrast with BoHV-1, experimental exposure of in vitro produced bovine embryos to BoHV-5 does not affect embryo viability and seems to inhibit some pathways of apoptosis. However, the mechanisms responsible for these phenomena are poorly understood. In this study, we examined mitochondrial activity, antioxidant protection, stress response and developmental rates of in vitro produced bovine embryos that were exposed and unexposed to BoHV-5.
METHODS: For this purpose, bovine embryos produced in vitro were assayed for cell markers after experimental infection of oocytes (n = 30; five repetitions), in vitro fertilization and development. The indirect immunofluorescence was employed to measure the expression of superoxide dismutase 1 (SOD1), anti-oxidant like protein 1 (AOP-1), heat shock protein 70.1 (Hsp 70.1) and also viral antigens in embryos derived from BoHV-5 exposed and unexposed oocytes. The determination of gene transcripts of mitochondrial activity (SOD1), antioxidant protection (AOP-1) and stress response (Hsp70.1) were evaluated using the reverse transcriptase polymerase chain reaction (RT-PCR). MitoTracker Green FM, JC-1 and Hoechst 33342-staining were used to evaluate mitochondrial distribution, segregation patterns and embryos morphology. The intensity of labeling was graded semi-quantitatively and embryos considered intensively marked were used for statistical analysis.
RESULTS: The quality of the produced embryos was not affected by exposure to BoHV-5. Of the 357 collected oocytes, 313 (+/- 6.5; 87.7%) were cleaved and 195 (+/- 3.2; 54.6%) blastocysts were produced without virus exposure. After exposure, 388 oocytes were cleaved into 328 (+/- 8.9, 84.5%), and these embryos produced 193 (+/- 3.2, 49.7%) blastocysts. Viral DNA corresponding to the US9 gene was only detected in embryos at day 7 after in vitro culture, and confirmed by indirect immunofluorescence assay (IFA). These results revealed significant differences ($p < 0.05$) between exposed and unexposed oocytes fertilized, as MitoTracker Green FM staining Fluorescence intensity of Jc-1 staining was significantly higher ($p < 0.005$) among exposed embryos (143 +/- 8.2). There was no significant difference between the ratios of Hoechst 33342-stained nuclei and total cells in good-quality blastocysts (in both the exposed and unexposed groups). Using IFA and reverse transcriptase polymerase chain reaction (RT-PCR) for the set of target transcripts (SOD1, AOP-1 and Hsp 70.1), there were differences in the mRNA and respective proteins between the control and exposed embryos. Only the exposed embryos produced anti-oxidant protein-like 1 (AOP-1). However, neither the control nor the exposed embryos produced the heat shock protein Hsp 70.1. Interestingly, both the control and the exposed embryos produced superoxide dismutase (SOD1), revealing intense mitochondrial activity.

CONCLUSION: This is the first demonstration of SOD1 and AOP-1 production in bovine embryos exposed to BoHV-5. Intense mitochondrial activity was also observed during infection, and this occurred without interfering with the quality or number of produced embryos. These findings further our understanding on the ability of α-Herpesviruses to prevent apoptosis by modulating mitochondrial pathways. **Brenner MP, Silva-Frade C, Ferrarezi MC, Garcia AF, Flores EF, Cardoso TC Evaluation of developmental changes in bovine in vitro produced embryos following exposure to bovine Herpesvirus type 5. Reprod Biol Endocrinol. 2012 Jul 23;10:53.**

Bovine immunodeficiency virus (BIV)

Cell-free supernatant from BIV-infected fetal lamb kidney cells at a final titer of 10^5 $TCID_{50}$/mL was added to maturation medium (24 hours) containing oocytes or to semen prior to swim-up separation. Corresponding non-exposed oocytes inseminated with virus-free sperm served as a negative control. A standard IVF system was used. Briefly, COCs were matured in TCM199 and inseminated with 10^6/mL sperm resulting from swim-up. After 18 hours in IVF, cumulus-free presumptive zygotes were co-cultured on monolayers of granulosa cells in TCM199 with 10 % fetal bovine serum for 9 days prior to microscopic examination.

Viral exposure		Total Oocytes	% Cleaved	% Blastocyst	% Hatched
BIV	+IVM	67	66	23	8
	+semen	87	65	24	8
Control		274	65	23	7

Values for cleavage and development in the same column are not significantly different from the uninfected control group (chi-square test, P>0.05)
It was concluded that presence of BIV had no apparent affect on embryonic development. Transferrable stage embryos were not tested for presence of infectious virus. **(Bielanski A, Theriogenology 2000;53:318 abstr)**

Bovine IVF embryos (produced using standard techniques) in a mixture of 20% ethylene glycol, 20% ME_2SO, and 0.6% sucrose were vitrified in either unsealed standard 1/4 mL straws, modified open pulled straws or in plastic cryovials and then plunged into liquid nitrogen (LN) that was contaminated with bovine immunodeficiency (BIV) by immersing an open vial containing 1 mL of cell culture suspension of BIV (10^5 $TCID_{50}$/mL; R29 isolate) into the same storage tank. After storage for 3 to 5 weeks, embryos were thawed, sequentially washed and batches of 3, ZP-I embryos were assayed for BIV by nested PCR. All control embryos that were stored in sealed vials were negative. Furthermore, none (0/22) of the batches of embryos stored in unsealed containers were positive for BIV. **(Bielanski A et al, Cryobiology 2000;40:110-116)**

The association of bovine immunodeficiency virus (BIV) with embryos derived by in vitro fertilization from oocytes of experimentally infected heifers or oocytes/embryos exposed to the virus in vitro was investigated. Using a nested-PCR assay, proviral DNA of BIV was not detected in follicular fluid or in embryos derived from BIV-infected donors. In vitro exposure of oocytes to BIV during maturation or insemination with BIV-infected semen resulted in zona pellucida-intact embryos testing negative for BIV provirus. However, exposure of zona

pellucida-free day-7 embryos to the virus resulted in a positive BIV assay for 28% of the batches of embryos, suggesting that the zona pellucida has a role in protecting against BIV infection. The presence of BIV in the IVF system had no apparent effect on the development of bovine embryos to the blastocyst stage. **(Bielanski A et al. Vet Res Commun 2001;25:663-673)**

Bovine leukemia virus

Bovine leukemia virus (BLV) propagated in fetal lamb kidney cells was transmitted by cell contact to bovine and ovine embryo cells from different organs Transmission of the BLV genome was achieved by coculture of mitomycin C-killed "virus-producer" fetal lamb kidney cells with the bovine and ovine embryo cells or by cell fusion. The procedure was only "exceptionally" successful for viral transmission when "nonproducer" cells were used. Donor cells contained 3 integrated BLV proviruses while recipient cells contained only one provirus. In cells containing transmitted BLV, the virus genome was expressed to its protein products. Some cells produced virus particles and reverse transcriptase activity in the medium. (**Altanter C et al. Folia Biologica 1987;33:400-409**)

The sanitary status and development of IVF embryos were reported after artificial exposure to BLV during maturation, fertilization and culture of the embryos. For the study, a standard method for IVF embryo production was used. Briefly, COCs were collected by aspiration of follicles on slaughterhouse-origin ovaries and subsequently matured for 24 hours in TCM199 with Earle's salts and 20% estrous cow serum. Then mature oocytes were inseminated with semen from a BLV-free bull (in modified Tyrode's medium for 18 hours). Subsequently, cumulus free presumptive zygotes were placed in wells with monolayers of granulosa cells in TCM199 with 10% fetal bovine serum and incubated for 8 days. In 3 different experiments, cell-free supernatant of a BLV-infected fetal lamb kidney (FLK) cell line (titer of 10^5 $TCID_{50}$/ml) was added to maturation medium (number of COCs = 165), to semen used for insemination (number of matured oocytes = 170), or to embryo culture medium (seven days after fertilization) containing cleaved ZP-intact (n = 115) or ZP-free embryos (n = 20). At the end of the procedures, embryonic development was assessed and embryos were washed 10 times using procedures recommended by the IETS for in vivo derived embryos. Then PCR was used to test groups of five for association with "proviral DNA".
In total, 52 samples (comprising 260 IVF embryos) resulting from oocytes exposed to BLV during maturation, fertilization or culture were tested by PCR. Proviral DNA was not detected in any samples of embryos from BLV-exposed oocytes (n=14) or from samples of oocytes inseminated with exposed semen (n=15). Also, no BLV proviral DNA was detected in samples of ZP-I (n=19) and ZP-free (n=4) embryos.
There was no apparent effect on morphology of embryos from trials in which BLV was added. Results indicate that it is possible to produce transferrable embryos in the presence of BLV (maturation, fertilization, or culture). Since there were no assays for viral RNA, it is not know whether the washing procedure applied to oocytes and zygotes was sufficient to prevent the non replicating virus from being transferred through the stages of the IVF system. It was concluded that IVF embryos that were produced in the presence of BLV and then were properly washed did not appear to be associated with infectious BLV. Authors

indicated a need to transfer such embryos to confirm this conclusion. **(Bielanski A, Vet Rec 2000;146:255-256)**

Viral exposure		Total Oocytes	% Cleaved	% Blastocyst	% Hatched
BLV	+IVM	165	63	28	8
	+semen	170	64	29	9
Control		265	64	28	8

Values for cleavage and development in the same column are not significantly different from the uninfected control group (chi-square test, P>0.05).
It was concluded that presence of BLV had no apparent affect on embryonic development. Transferrable stage embryos were not tested for presence of infectious virus. **(Bielanski A, Theriogenology 2000;53:318 abstr.)**

Infectious bovine rhinotracheitis virus/bovine herpesvirus -1

Ten cows, intravenously inoculated with $10^{7.89}$ $TCID_{50}$/ml of BHV-1 were ovariectomized 8-10 days post inoculation. BHV-1 assays were carried out, using cytopathic effect in fetal bovine kidney cell cultures, to detect the virus in ovaries, follicular fluid, granulosa cells, corpus luteum and oviducts of infected cows. In addition, 100 oocytes were collected from the ovaries of 9 of these animals. Eighty of the 100 oocytes were matured and fertilized in vitro and 27 that initiated segmentation were then cultured on oviductal cells. After culture of the in vitro fertilized embryos was completed, the oviductal cells were examined for the presence of BHV-1.
BHV-1 was found in association with the oocytes from 5 of the 9 cows and 4 had BHV-1 in their ovaries, follicular fluids, granulosa cells, corpora lutea and oviductal cells. All samples from the fifth cow were positive for BHV-1 with the exception of the oviductal cells. BHV-1 was also isolated from the medium that had been used to mature the oocytes from 7 of these infected cows. In addition, virus was isolated from embryos derived from 4 of these animals, and from the oviductal cells that had been used in culturing the in vitro fertilized embryos derived from 6 of these cows. The maturation medium and direct assay of the embryos had been found to be negative for BHV-1 infectivity for one of these 6 animals. Embryos derived from the BHV-1 infected cows semed to have a lower rate of embryonic development compared with controls (34% of fertilized oocytes reached the two cell stage vs 51% for controls, p<0.05).
It was concluded that the oocytes, and the in vitro fertilized embryos derived from them, can be associated with BHV-1 when oocytes are collected from cows that are in the acute phase of IBR/IPV infection. The samples that should be monitored for the presence of this virus when in vitro fertilization procedures are carried out are the oocyte maturation medium and/or the oviductal cells used in embryo culture (**Guérin et al, Rec Med Vet 1989;165:827-833**).

Four hundred and seventy oocytes from 45 ovaries were distributed into two groups to study the effect of in vitro exposure to BHV-1 on in vitro maturation (IVM) (Group I; n=172) and in vitro fertilization (IVF) (Group II; n=278). In each group, oocytes were further distributed as a) unexposed controls; b) exposed controls; c) exposed experimental for virus detection following maturation (Group I) or maturation and fertilization (Group II), and d) exposed experimental for fixation, staining and microscopic examination following

maturation (Group I) or maturation and fertilization (Group II). Oocytes were exposed to $10^{2.7}$, $10^{3.7}$, $10^{5.7}$ and $10^{6.7}$ $TCID_{50}$/ml of BHV-1 (Los Angeles strain). Oocytes and fertilized zygotes were washed 10x prior to being ground and inoculated onto fetal bovine kidney (FBK) cells for virus detection. The first two and last two washes were also inoculated onto the FBK cells for virus detection.
Immediate contamination was observed on exposed control oocytes exposed to $10^{3.7}$ $TCID_{50}$ or more, and also in wash fluid no. 1 for all levels of exposure, and wash fluids no. 9 and 10 at the $10^{6.7}$ $TCID_{50}$ level. Virus was isolated from the IVM oocytes and IVF zygotes when infected with concentrations greater than or equal to $10^{3.7}$ $TCID_{50}$ in the former case and $10^{5.7}$ $TCID_{50}$ in the latter. Virus was isolated from wash fluids no. 1, 9 and 10 when the exposure levels were $10^{3.7}$ $TCID_{50}$ or more. The virus appeared to have no effect on IVM but did reduce the IVF rate (85% vs 62.5% respectively for control and infected zygotes; $p < 0.01$). Virus contamination also increased the level of abnormalities in sperm decondensation (4% vs 49% respectively for control and infected zygotes; $p < 0.001$).
In conclusion, the in vitro contamination of bovine oocytes by BHV-1 results not only in absorption of the virus to the gametes but also impairs their ability to undergo IVF, suggesting viral penetration and subsequent interaction with the intracellular mechanisms of gamete fusion (**Guerin et al, Rec Med Vet 1990;166:911-917**).

Cumulus-oocyte complexes were matured for 24h. in the presence or absence of 10^6 $TCID_{50}$/ml of BHV-1; they were then fertilized in vitro and co-cultured on oviductal cells to the blastocyst stage. The percentages of oocytes that cleaved in the presence or absence of the virus were 48% (n = 905) and 51% (n = 1004) respectively, and percentages that developed to the blastocyst stage were 29% and 31% respectively ($p > 0.05$). Embryos produced in the presence of the virus tested positive for BHV-1, and this was despite the fact that the number of washes and transfers involved in their production exceeded the IETS Manual recommendations. While decreased fertilization and cleavage rates were not observed in this study (as they have been in others) this might have been due to the use of different strains of BHV-1 or different fertilization procedures (**Bielanski and Dubuc, Reprod Dom Anim 1993;28:285-288**).

Oocytes from 21 heifers that 8d previously had been experimentally infected with BHV-1, and from dexamethasone-treated (stressed) BHV-1 seropositive animals, were matured, fertilized and co-cultured in vitro for 7d. prior to being tested for presence of the virus. Nineteen of the 21 infected donors yielded embryos and follicular fluids that were BHV-1 positive. Oviductal cells (17/21) and uterine fluids (14/21) were also positive. Titres of virus in positive samples ranged from $10^{1.6}$ to $10^{9.6}$ $TCID_{50}$/ml. Cleavage rate and the proportion of blastocysts that developed from oocytes of BHV-1 infected heifers were 26% (n = 361) and 6% compared with 56% (n = 112) and 26% for uninfected controls ($p < 0.05$). In contrast, embryos from the dexamethasone-treated heifers were found to be BHV-1 negative and yielded 11% blastocysts as compared with 25% in the control group. This work showed that oocytes/embryos had been exposed to high titres of virus by contact with contaminated follicular fluid and gramulosa cells. Also, despite the number of manipulations and washings applied during the procedure, they remained infected and had a potential for disease transmission. While a significantly decreased rate of embryo development occurred in presence of the virus, a proportion of normal (transferable) blastocysts was nevertheless

obtained from some of the infected heifers, ie those that did not show a post-inoculation febrile response (**Bielanski and Dubuc, Theriogenology 1994;41:1211-1217**).

(BVDV and IBRV included)"Since bovine embryo production in vitro has become possible, sanitary precautions must be taken while transferring such embryos. In a large field trial, in vitro produced Belgium Blue embryos were transferred to dairy cattle recipients housed on four different farms. Blood samples of oocyte and oviduct donor cattle were screened for the prevalence of BVD-virus and for BHV-1 antibodies. Also, follicular fluid, maturation medium, oviduct medium and medium with degenerated embryos were screened for the presence of common bovine viral infectious agents. 95.6% of the oocyte donors showed high virus antibody titers against BHV-1. None of them were viraemic for BVD-virus. Of the 96 sampled fluids, 4.1% was positive for noncytopathic BVD-virus. The probable source of infection was the serum used for in vitro embryo culture. No other viruses were found. Pregnancy and calving rates did not differ between BVD-virus infected and BVD-virus free groups. Three calves, born after transfer of an embryo out of a BVD-virus infected group were BVD-negative at six months of age." (**Van Soom A, et al., Vlaams Diergeneeskd Tijdschr 1994;63:139-145**).

Bovine COCs or IVF embryos were exposed to BHV-1 during in vitro maturation or coculture with uterine tubal cells, respectively. Trypsin at a concentration of 0.25%, was applied (for approximately 90 seconds) to disinfect either COCs or cumulus free oocytes (CFO) 18 h after insemination, or on day 7 to embryos resulting from infected oocytes. In total, virus was not detected in 71% of 93 samples containing 233 embryos exposed to BHV-1 and trypsin treatment. BHV-1 was detected in 14% and 54% of samples containing a single embryo and five embryos, respectively. In corresponding groups of embryos exposed to BHV-1, then washed but not treated with trypsin (70 samples), 85% and 96% of samples containing one embryo and pooled embryos, respectively, were positive for the virus. There was no effect of trypsin treatment on the development of IVF-embryos. It was concluded that IVF-generated embryos have a greater tendency to carry BHV-1 after experimental exposure to the virus than in vivo derived embryos and they are more difficult to disinfect by means of the standard trypsin treatment use. (**Bielanski et al, Anim Reprod Sci 1997;47:1-8**)

The purpose of the study was to determine if BHV-1 is to replicate in IVF embryos and investigate the degree to which the ZP is able to protect IVF embryos against infection with BHV-1. Both ZP-I and ZP-F matured oocytes, zygotes (1 d post insemination; 1dpi), 8-cell stage embryos (3-dpi), morulae (6 dpi) were incubated for 1 hr in 1 ml of MEM containing $10^{7.7}$ $TCID_{50}$ /ml BHV-1 (Cooper strain). Three titers ($10^{5.7}$, $10^{6.7}$, and $10^{7.7}$ $TCID_{50}$/ml) of the Cooper strain were used for incubation of hatched blastocysts (9dpi). Bovine embryonic lung cells (BEL) on microcarriers were inoculated following the same protocol as for the embryos. At 0, 12, 24, and 36 hr post inoculation (hpi), groups of embryos and BEL cells were collected for virus titration and for the determination of the percentage of viral antigen positive cells by immunofluorescence. For the 3 developmental stages in ZP-F embryos, similar maximal intracellular virus progeny titers were obtained at 24 to 48 hpi ranging from $10^{1.32}$ to $10^{1.43}$ $TCID_{50}$/ 100 embryonic cells. The intracellular virus titer in the BEL cells peaked at $10^{3.08}$ $TCID_{50}$/100 BEL cells. The percentage of cells which expressed viral antigens was 13% in ZP-F morulae and 100% in BEL cells. In ZP-I embryos, no replication of BHV-1 was detected. These results clearly show that only after removal of the ZP, BHV-1 is able to replicate within the in

vitro produced embryos, with only a subset of embryo cells being fully susceptible. (**Vanroose et al, Theriogenology 1997;47:1389-1402**)

(BHV-1 and BHV-4 included) Hatched blastocysts (n=100) were produced in vitro. Fifty hatched blastocysts were incubated for 1 hour in MEM (25 embryos/ml) containing $10^{5.5}$ $TCID_{50}$/ml BHV-4 (LVR 140 strain) and 50 hatched blastocysts were incubated in MEM (25 embryos/ml) containing $10^{5.7}$ $TCID_{50}$/ml BHV-1 (Cooper strain). After incubation the embryos were washed and groups of 5 hatched blastocysts were transferred in Menezo B2 medium for further incubation. At 0, 12, 24, 36, and 48 hours post inoculation (hpi), groups of 5 embryos were collected for virus titration. For this purpose, the embryos were placed in 0.5 ml MEM, rapidly frozen and thawed twice and vortexed thoroughly. Tenfold dilutions of the embryo suspensions were inoculated on cell cultures. The suspensions obtained from blastocysts incubated in BHV-4 were inoculated onto monolayers of secondary fetal calf testicle cells at 50% confluency, whereas dilutions of blastocysts incubated in BHV-1 were inoculated onto monolayers of bovine embryonal lung cells at 100% confluency. The inoculated cells were examined for detection of cytopathic effects over 6 days for BHV-1 and over 10 days for BHV-4. The inoculated secondary calf testicle cells were stained at 10 days using an immunoperoxidase technique. At 12 hpi, 50 hatched blastocysts (25 exposed to each virus) were collected for determination of the percentages of viral antigen positive cells by immunofluorescence.
For the hatched blastocysts incubated with BHV-1, maximal viral titers were obtained at 36 and 48 hpi, ranging from $10^{1.35}$ to $10^{1.38}$ $TCID_{50}$/5 embryos. This infection resulted in degeneration of the embryos. For the hatched blastocysts incubated with BHV-4, virus was not isolated during the course of the experiment and the embryos showed no morphologial changes. The percentage of cells that expressed viral antigen was 13% in hatched blastocysts inoculated with BHV-1 and 0% in hatched blastocysts inoculated with BHV-4.
From the results they concluded that IVF hatched blastocysts are susceptible to BHV-1 and refractory to BHV-4 infections. (**Vanroose et al, Proc 12e Reunion AETE, Lyon, 13-14 September, 1996;abstract 206**)

Bovine oocytes surrounded by cumulus were exposed to BHV-1, bluetongue virus (BTV) or akabane virus (AV), matured in culture for 24 h and then fertilized in vitro. BHV-1 was isolated from medium 3 d after IVF, cytopathic effect in the cumulus cells was obvious, and embryos failed to develop beyond the 8-cell stage. BTV replication was obvious in cumulus while embryos developed to blastocysts. No effect of AV replication could be observed in cumulus during in vitro development. They concluded that the viruses had not infected the ZP-I oocytes or embryos. (**Tsuboi T and Imada T. Vet Micro 1997;51:135-142**)

COCs or IVF bovine embryos were exposed to BHV-1 during IVM and IVC (with uterine tubal cells), respectively. Trypsin was applied (0.25% for about 90 seconds) to either the exposed COCs or cumulus-free oocytes at 18 h after in vitro insemination, or at 7 d to embryos resulting from exposed COCs. For comparison, corresponding exposed groups were not trypsin treated. In one trial, COCs were vortexed as part of the treatments. Finally, development was monitored. Embryos were assayed for virus either singly or in groups of five.
Overall, of 93 samples (containing 233 exposed embryos or embryos from exposed oocytes) representing trypsin treated embryos/oocytes, 29% (27/93) were positive for virus. The virus was found in 14% (8/58) and 54% (19/35) of samples containing single or five embryos,

respectively. Overall, of 70 samples (containing 166 exposed embryos or embryos from exposed oocytes) representing non trypsin treated embryos/oocytes, 88% were positive for virus (85% of single embryos and 96% of groups of five embryos). Overall, trypsin treatment reduced the number of positive samples ($P<0.05$). There was no effect of trypsin treatment on development. The authors concluded that IVF embryos have a greater tendency to carry BHV-1 after artificial exposure to the virus than in vivo derived embryos and trypsin treatment is less effective for removal of the virus. (**Bielanski et al, Anim Repro Sci 1997;47:1-8**)

Association of BHV-1 with bovine oocytes and IVF embryos after artificial exposure to the virus and treatment with a cocktail of monoclonal antibodies and/or guinea pig complement was examined.
In the first experiment, treatment of COCs with antibody or a combination of antibody and complement did not remove virus from most oocytes and 7 d embryos. Further, after fertilization only 54% of oocytes cleaved and there was no development to blastocysts.
In the second experiment, treatment of oocytes after fertilization with antibodies and complement or only complement resulted in 100% virus free embryos on 7 d when no uterine tubal cells were used in IVC (25/25 virus negative when treated with antibody and complement; 15/15 negative when treated with only complement). Treated zygotes that cleaved and cleaved embryos that developed to blastocysts in either presence or absence of uterine tubal cells were 56% and16%, and 54% and12%, respectively (no difference).
In the third experiment, 7 d embryos exposed to BHV-1 were all free of virus after treatment with the combination of antibody and complement (12/12 were negative). The treatment of infected embryos with only antibody was not effective in neutralizing the virus (9/9 positive). One sample of nine treated with complement alone was virus positive (1/9 positive). (**Bielanski A et al, Reprod Dom Anim 1998;33:89-92**)

It has been reported that bovine herpesvirus-1 (BHV-1) remains associated with in vitro-produced (IVP) bovine embryos after exposure to the virus and either washing or trypsin treatment. However, it is not known if the quantity of virus associated with an exposed IVP embryo is likely to infect a recipient cow after transfer. The specific objective of this study was to determine if IVP embryos that were exposed to BHV-1 would infect uterine tubal cells (UTC) in a co-culture system. In vitro-produced, Day 7 embryos were exposed to BHV-1 and then washed or trypsin treated according to the IETS guidelines. These embryos were then co-cultured individually or in groups with UTC in microdrops of tissue culture medium 199 (TCM 199) supplemented with 10% equine serum. Following co-culture for 48 h, virus isolation was attempted on the embryos and the UTC from each drop. Virus was detected in washed individual embryos, groups of washed embryos, groups of trypsin-treated embryos and the UTC co-cultured with each of these treatments. However, BHV-1 was not detected in the individual, trypsin-treated embryos or the UTC co-cultured with them. It is concluded that trypsin treatment might effectively prevent infection of recipients if individual, Day 7, exposed embryos were transferred into the uterus. (**Edens MS, et al. Theriogenology 2003;60:1495-1504.)**

This research evaluated the ability of phosphonoformic acid to inhibit bovine herpesvirus 1 (BHV1) in cumulus cells commonly used in coculture with bovine in vitroproduced embryos.

At 200 and 400 microg/ml, phosphonoformic acid inhibited 4 logs of BHV1. Subsequently, phosphonoformic acid (200 and 400 microg/ml) added to both in vitro fertilization and culture medium resulted in a decrease in the proportion of developed blastocysts, and the number of cells per blastocyst was lower in the treated embryos. Therefore, while phosphonoformic acid can effectively inhibit replication of BHV1 in coculture cells, it also inhibits development of in vitroproduced bovine embryos. **(Marley MS et al, Veterinary Therapeutics 2006;7:156-166)**

The world market for bovine embryos has increased in the past few years. However, sanitary problems such as foot and mouth disease in Brazil, vesicular stomatitis in South America, and bovine spongiform encephalopathy (BSE) in North America and Europe have increased concerns regarding the risk of introducing exotic diseases and/or more virulent serotypes of endemic diseases by embryo transfer. Many countries are trying to develop and/or improve new techniques for infectious disease detection, with the scientific basis to support the import and export of animal germplasm. Therefore, the epidemiology of the diseases and the interaction between pathogens and cumulus–oocyte complexes (COCs), embryos, and semen must be investigated. Despite the many studies that have been carried out to evaluate the possibility of transmission of infectious agents by the embryo, few data are available regarding COC susceptibility (Tsuboi *et al*. 1992 J. Vet. Med. Sci. 54, 1179–1181). The aim of this study was to evaluate the presence of bovine herpes virus serotype 1 (BHV-1) in COCs and follicular fluid (FF) collected from naturally infected animals in a low stress condition. Blood samples of non-lactating Gyr breed (*Bos indicus*) cows were collected and evaluated for BHV-1 antibodies by the serum neutralization microplate test, performed as described in the Manual for Standards for Diagnostic Tests and Vaccines (OIE, 1992). The cows were diagnosed as serologically positive (*n* = 38) or serologically negative (*n* = 8), and kept under grazing in *Brachiaria decumbens* pasture with mineral supplementation. The cows considered as positive showed titers greater than 1/4. COCs and follicular fluid (FF) were obtained by ovum pick-up (OPU) using sterile and disposable materials for each animal. Virus detection was performed by the PCR technique. PCR specificity was assessed using COCs and FF recovered from eight BHV-1 serologically negative animals. These samples were either artificially exposed on plates with $10^{6.5}$ TCID in 50 µL of IBR Colorado 1 reference serotype (ATCC, VR-864) or used as a negative control. The PCR analitical sensitivity was $10^{0.5}$ TCID. The presence of BHV-1 in COCs and FF was not detected in any of the animals, despite the high sensitivity of the PCR technique. In the present *in vivo* model, results show that COCs collected from serologically BHV-1 positive cows presenting no clinical signs of the illness and managed in a low stress condition could be used as donors for *in vitro* fertilization procedures with minimal sanitary risks. Also, the absence of the virus in COCs and FF cannot be used as a predictor of BHV-1 infection status in bovine herds. (**Oliveira AP, et al. Reproduction, Fertility and Development, 2008;20:158 (abstr))**

(Includes IBRV [BHV-1] and BVDV)
The aims of the present study were to investigate whether a prolonged presence (24 h or 8 days) of $10^{6.3}$ $TCID_{50}$/ ml BHV-1 or $10^{5.3}$ $TCID_{50}$/ ml BVDV in an in-vitro embryo production system affected rate of cleavage and embryonic development of ZP-intact embryos, and to point out eventual causes of adverse effects. When virus was present in each step of an IVP system, significant lower rates of cleavage and blastocyst formation of virus-exposed embryos were observed, in comparison with control embryos ($P<0.01$). When embryos were

only exposed to virus during the in-vitro fertilization (IVF), the rates of cleavage and blastocyst formation were significantly affected. The introduction of BHV-1 or BVDV during in vitro maturation (IVM) or in-vitro culture (IVC) resulted only in significantly lower rates of blastocyst ($P<0.01$). In all experiments, virus replication was not detected in the embryonic cells. On the other hand, virus replication was clearly demonstrated in oviductal cells in the co-culture system, resulting in a degeneration of these cells.
In an additional experiment, synthetic oviduct fluid (SOF) without somatic cells was used as an alternative culture system. Even when SOF-embryos were exposed to $10^{6.3}$ $TCID_{50}$/ ml BHV-1 or $10^{5.3}$ $TCID_{50}$/ ml CP and NCP BVDV, the rates of blastocyst formation of the BHV-1, CP and NCP BVDV-exposed embryos were not different from the unexposed control embryos, 23%, 24% and 24%, respectively vs 27%. Taken together, it can be concluded that the virus-induced adverse effects on embryonic development in conventional co-cultures were due to changes in the embryonic environment caused by infection of oviductal cells. **(Vanroose G et al, Mol Reprod Dev 1999;54:255-263. 1999)**

Bovine herpesvirus-1 (BHV-1) has been associated with reproductive failure such as infertility and early embryonic mortality. Previously, we have demonstrated that in vitro fertilization (IVF) in the presence of BHV-1 results in significantly lower cleavage and blastocysts rates. The experiments in this study were designed to determine whether BHV-1 had an adverse effect on the IVF process.
The effect of four varying concentrations of BHV-1 (0, 10^5, 10^6, and 10^7 $TCID_{50}$/ml) present during IVF of cumulus-free zona pellucida (ZP)-intact oocytes with different sperm cells concentrations (10^4, 10^5, and 10^6 sperm cells/ml) was investigated and reflected in the rate of fertilization. For each combination (sperm cell concentration x concentration BHV-1), 20 presumed zygotes were examined at 24 hour post fertilization. To determine if fertilization was completed, the presumed zygotes were fixed and stained with Hoechst 33342. To explore the effect of the presence of BHV-1 (10^5 $TCID_{50}$/ml) on the binding capacity of sperm cells on the ZP, the number of sperm cells bound on the ZP of each presumed zygote was determined. This experiment was repeated three times.
The results are given in Table 1. For all concentrations of sperm cells, the fertilization rate decreased with the increase of the presence of BHV-1 titres. For all concentrations of BHV-1, the percentage of fertilization increased with the increase of the concentration of sperm cells. From multiple logistic regression analysis it was shown that a linear relationship exists between log odds of fertilization and the logarithms of the sperm cell concentration and of the viral concentration. At any given concentration of BHV-1, the expected odds of fertilized oocytes increased 3.14 times ($e^{1.15}$, $P<0.001$) when the sperm cell concentration is increased by a factor of 10. At any given concentration of sperm cells, the expected odds of fertilized oocytes drops to 0.54 times ($e^{-0.62}$, $P<0.001$) its previous level whenever the viral concentration is increased by a factor of 10. There was a significant difference between the number of sperm cells bound on the ZP of oocytes incubated with sperm cells in the absence of BHV-1 and incubated in the presence of BHV-1 ($P<0.05$). The mean numbers of attached sperm cells were 182, 91 or 63 for incubation in the presence of 10^5, 10^6, or 10^7 $TCID_{50}$ BHV-1 / ml, respectively. In the control group, a mean number of 197 sperm cells were found on the surface of the ZP of a presumed zygote.
These preliminary results indicate that an infective dose of BHV-1 was associated with individual developing embryos that had been washed but not with individual developing

embryos that had been trypsin treated. Thus, trypsin treatment might effectively prevent infection of recipients when individual, Day 7, exposed embryos are transferred into the uterus. **(Edens MSD et al. Theriogenology 2002;57:570 abstr.)**

The aim of this study was to evaluate the efficiency of trypsin treatment on the inactivation of BHV-1 that is associated with in vitro derived embryos. Bovine embryos at "about 8 or 9 days" were exposed to $10^{7.5}$ DICT of BHV-1 (Los Angeles strain). These embryos as well as unexposed embryos were divided into two groups. One group was washed and one group was trypsin treated according to IETS standards. The embryos and last wash were assayed by virus isolation and PCR. All control embryos and their last washes were negative. The exposed embryos (presumably both washed and trypsin-treated?) were positive both by virus isolation and PCR, although the last washes were negative. **(D'Angelo M et al. Theriogenology 2002;57:569 abstr.)**

Bovine virus diarrhoea virus (BVDV)

Frozen semen from a persistently BVDV infected bull was used for the IVF procedure. Oocytes were matured in vitro for 26h at 39°C then distributed into 2 groups. Group I (64 oocytes) was fertilized with semen from a control bull negative for BVDV and for antibodies. Group II (81 oocytes) was fertilized with semen from the infected bull. Virus was isolated from the following media used for IVF of Group II oocytes: the upper fraction after "swim-up"; the supernatant and pellet of swim-up sperm after centrifugation; the fertilization medium. BVDV was also isolated from the washing medium of the fertilized ova and from the embryos and culture medium. Fertilization and cleavage were significantly reduced in Group II and development to blastocyst stage in Group II was 2.1% compared with 19.6% in Group I. The probability of transmitting BVDV by transfer of such embryos to recipient cattle is low because embryonic development is drastically reduced. To further limit the risk of BVDV transmission the method of choice is to test the embryo culture media for the virus. (**Guerin et al, Theriogenology 1992;37:217 abstr**).

Semen from 3 persistently infected bulls were used for production of embryos by IVF. The percentages of oocytes that cleaved and percentages of embryos that developed to blastocysts were 20%, 47% and 51%, and 13%, 17% and 20% respectively for the 3 bulls. Pooled semen from 3 uninfected bulls resulted in 53% oocytes cleaved and 22% embryos developing to blastocysts. No infectious virus was isolated from any of the embryos produced by IVF. When a non-cytopathic BVDV strain (10^5 $TCID_{50}$/ml) was added to sperm from uninfected bulls prior to IVF 49% of oocytes cleaved and 22% developed to blastocysts. Again, all the embryos tested negative for BVDV. (**Bielanski & Loewen, Anim Reprod Sci 1994;35:183-189**).

Ovaries, granulosa cells and oviduct epithelial cells were obtained from 3 persistently viraemic cattle with BVDV and from some uninfected controls. Viral antigen was demonstrated by an immunofluorescent antibody technique and viral RNA was demonstrated by in situ hybridisation. Viral antigen and viral RNA were found in ovarian tissues, including granulosa cells, and in oviduct epithelial cells from the persistently viraemic cattle but not in those from controls. Primary cultures of the two cell types from control cattle were shown to be permissive to in vitro infection with BVDV. These results

demonstrate that BVDV is an important threat to IVF and IVC procedures in cattle (**Booth et al, J Reprod Fertil, 1992;Abstract Series 9:42 abstr and Booth et al, J Reprod Fertil 1995;105:17-24**).

Frequency of virus contamination in a routine in vitro embryo production system in Denmark was studied by inoculating a) follicular fluid from the oocyte aspirations, b) oocyte collection medium, c) media from oocyte maturation and insemination, d) supernatant from cultures of oviduct cells, and e) medium containing sperm from the swim-up preparation. Samples were inoculated onto primary calf kidney monolayers and cultured for 4d., inspected for CPE, then passaged once again. The cultures were fixed and stained in indirect immunofluorescence assays for BHV-1, BVDV, BRSV, PI-3, adenovirus and reovirus. Samples from 49 consecutive (weekly) experiments were tested for virus contamination. The average number of animals used for each experiment was 24 (range 8 to 38) and the total number of animals was 1163. Samples from 6 (12%) of the experiments contained BVDV; 3 involved insemination medium alone and 3 involved media from oocyte collection, oocyte maturation and insemination. No other viruses were detected in any of the samples. Cleavage and blastocyst rates from the BVDV positive experiments were not different from the virus negative experiments. The authors point out that a 12% frequency of BVDV positive experiments did not necessarily mean that 12% of animals were infected; since all collected oocytes from each experiment were pooled, a single infected animal or oocyte might have contaminated the whole batch (**Avery et al, Vet Rec 1993;132:660**).

Oocytes (n = 247) from 10 cows were matured and fertilized in vitro and the presumptive zygotes were cultured for 7d. on oviductal epithelial cells (BOEC). Primary BOEC cultures for IVF and culture were divided into 2 groups: one group was infected with a noncytopathic biotype of BVDV (SD-1) and the other consisted of uninfected controls. After oocytes were matured approximately equal numbers from each cow were inseminated and the presumptive zygotes were cultured using the infected or control BOEC. After 7d. culture ZP-I morulae/blastocysts and degenerated ova were washed, sonicated and tested for presence of the virus by inoculating onto bovine kidney cell cultures followed by immunoperoxidase labelling. Virus was not recovered from the morulae/blastocysts but was isolated from some groups of degenerate ova. Infections of the BOEC were inapparent and the rates of cleavage and development of embryos cultured on the infected BOEC were not significantly different from those on non-infected BOEC. While it was encouraging that no virus was isolated from morulae/blastocysts (of transferable quality) after washing, the presence of infection in groups of washed degenerate ova indicates that the conventional washing procedures recommended for ensuring that in vivo produced bovine embryos are free from BVDV may not be adequate for IVF embryos (**Zurovac et al, Theriogenology 1994;41:841-853**).

Semen samples from bulls that were persistently infected with BVDV, and which contained 10^5 - 10^6 $TCID_{50}$/ml semen, were subjected to sperm separation procedures (ie washing, swim-up, Percoll gradient, glass wool filtration, glass bead filtration) that are commonly used prior to IVF. The final sperm pellets from both fresh and frozen ejaculates were tested for the virus by the immunoperoxidase technique. All the tests for BVDV were positive in the range of 10^3 - 10^4 $TCID_{50}$/ml. The work shows that BVDV is not completely removed by the simple physical methods used to prepare semen for IVF (**Bielanski, Dubuc and Hare, Reprod Dom Anim 1992;27:303-306**).

"Virus neutralization was performed in "swim-up" medium (TALP with non-specific IgG) to which was added specific bovine anti BVDV IgG purified from a naturally infected animal. Oocytes were collected from a slaughterhouse and matured in vitro for 26 h at 39C under 5% CO_2. The oocytes were then divided into 3 groups: Group 1 oocytes (n=246) were exposed to semen from persistently BVD infected bull (positive control), group 2 (n=220) were exposed to same semen but previously treated with specific IgG during the swim up process and group 3 oocytes (n=209) were exposed to semen from a control bull free of BVDV (negative control, i.e. negative for virus and antibodies). In vitro fertilization and culture were performed as described previously (Marquant-Le Guienne, et al., 1990. Reprod. Nutr. Develop. 30:259-266). The presence of the BVDV was confirmed using an ELISA technique on bovine turbinate cells.
The results demonstrate that the fertilization and cleavage rates of groups 1 and 2 were not significantly different, but both were significantly lower than those of the negative control group (group 3: $P<0.05$). By contrast, the developmental rate of the non treated semen from the positive control group (group 1) was significantly lower than those of the other two groups (treated and negative control, $P<0.05$) resulting in 4% of the overall rate of development (blastocyst/inseminated oocytes) in group 1 oocytes as opposed to 8.2% and 13.9% respectively in groups 2 and 3.
These results confirm a bull effect on IVF and moreover suggest additional effect of the BVDV on in vitro development. The neutralization of virus from this persistently BVD infected bull improved the development rate up to a level similar to that of the negative control group. This study also shows that the likelihood of succeeding in producing transferable embryos is much less with the presence of BVDV in the semen." (**Allietta M, et al., Theriogenology 1995;43:156 abstr**)

"Bovine viral diarrhea virus infection was induced in 16 heifers by inoculation of a noncytopathic strain of bovine viral diarrhea virus (BVDV). Six BVDV-free heifers served as controls. On day 8 after inoculation, cumulus-oocyte complexes were collected from ovaries of animals at the second peak of fever preceded by leukopenia. The oocytes were then matured and fertilized in vitro. There was no significant difference (48% vs. 54%) ($P>0.05$) in the percentage of cleaved oocytes between infected and non-infected animals. However, the proportion of embryos that developed to the blastocyst stage was significantly higher for the control group than for BVDV group (29% vs. 14%) ($P<0.01$). All follicular fluids and cumulus-oocyte complexes collected from infected animals tested positive for the presence of virus, but embryos produced by in vitro fertilization 7 days after in vitro co-culture tested negative. (**Bielanski A and Dubuc C., Anim Reprod Sci 1995;38:215-221**)

"We have assessed the efficacy of protocols in the IETS Manual (1990) for the washing of in vivo produced embryos (including two intermediate trypsin washes) in removing virus from in vitro produced (IVP) embryos deliberately exposed to BVDV for various time periods during the IVP process." Following standard methods for IVM and IVF, "Presumptive zygotes were transferred to primary cultures of granulosa cells for in vitro culture (IVC) for 6-7 days. In experiments 1 and 2 these cells were infected with ncp BVDV 2-3 days before use.
Prior to the recommended washing procedure, all groups (one group = 10 zona-intact blastocysts or morulae) of IVP embryos were vortexed for 2 min to remove any attached

granulosa cells and/or adherent debris likely to be a vehicle for viral transmission. After washing, each group was sonicated and inoculated onto BVDV-susceptible Cte cells for virus isolation. After 3 in vitro passages, the cells were stained and examined by indirect immunofluorescence. All serum used was BVDV antibody and virus free.
Experiment 1: IVP was performed as described except COCs were incubated with BVDV (strain Pe 515 $10^{6.25}$TCID/ml) for 2h to infect cumulus cells before transfer to IVM medium for 22h. The cumulus cells presumably were a source of virus during IVF, further to the granulosa cells. Results: BVDV was isolated from 6/6 embryo groups. The use of BVDV antibody positive serum in the latter part of washing procedure did not reduce the contamination.
Experiment 2: IVP was BVDV-free apart from the IVC system, the granulosa cells had been infected as before. Results: 4/4 groups were BVDV positive.
Experiment 3: Embryos generated by IVP in BVDV-free conditions were incubated with virus for 2-4h before washing. Result: 7/12 groups were BVDV positive. In every experiment, BVDV contamination of abattoir tissues was not detected in control embryos, oocytes, follicular fluid or microdrops but was detected in the same materials from experimentally infected groups. (**Booth PJ et al., Proc 10e Reunion AETE, Lyon, 9-10 September,1994;abstract 154**)

"Granulosa cell (GC) feeder layers are commonly employed to support IVP bovine embryos, unfortunately the source material is frequently infected with bovine viral diarrhea virus (BVDV). Cryopreservation of GC would permit an initial screening for freedom from BVDV. This study assessed the ability of cryopreserved GC to support embryo development." Their results demonstrated "that cryopreserved bovine GC cultured for up to 10 days can support embryo development and, therefore, facilitate the preparation of BVDV free IVP bovine embryos." (**Fray MD, et al, J Reprod Fertil 1995;abstr series 15:210**)104

(BVDV and IBRV included)"Since bovine embryo production in vitro has become possible, sanitary precautions must be taken while transferring such embryos. In a large field trial, in vitro produced Belgium Blue embryos were transferred to dairy cattle recipients housed on four different farms. Blood samples of oocyte and oviduct donor cattle were screened for the prevalence of BVD-virus and for BHV-1 antibodies. Also, follicular fluid, maturation medium, oviduct medium and medium with degenerated embryos were screened for the presence of common bovine viral infectious agents. 95.6% of the oocyte donors showed high virus antibody titers against BHV-1. None of them were viraemic for BVD-virus. Of the 96 sampled fluids, 4.1% was positive for noncytopathic BVD-virus. The probable source of infection was the serum used for in vitro embryo culture. No other viruses were found. Pregnancy and calving rates did not differ between BVD-virus infected and BVD-virus free groups. Three calves, born after transfer of an embryo out of a BVD-virus infected group were BVD-negative at six months of age." (**Van Soom A, et al., Vlaams Diergeneeskd Tijdschr 1994;63:139-145**).

"The aim of this study was to evaluate the effect of a noncytopathic biotype of bovine viral diarrhea virus (BVDV isoalte PT810) on the development of in vitro produced embryos. Bovine oocytes (n=1007) were collected by aspiration from 2-8 mm follicles of ovaries from local slaughterhouse. Donor ovaries were tested for intracellular BVDV-antigen with indirect

immunofluorescence by FACSCAN system. Only oocytes without BVDV antigen were used for this experiment. Cumulus oocyte complexes (COCs) with multilayered (>4 layers) of cumulus cells and dark evenly granulated cytoplasm were used for in vitro production of embryos. After selection the COCs were randomly divided for maturation into 2 culture treatments: 1) the oocytes (control, n=378) were cultured in nonexposed tissue culture medium 199 (TCM 199), 2) the oocytes (n=629) were cultured into droplets which were exposed to $1X10^4$ TCID50/ml of BVDV-PT810. Oocytes were matured in TCM 199 supplemented with 10% estrous cow serum (ECS) and 10□g/ml FSH-p at 39^{o}C in 5% CO_2 and maximum humidity. Following maturation, both experimental groups were fertilized in vitro (Day 0) using frozen-thawed semen in a concentration of $1X10^6$ sperms/ml. After fertilization the presumptive zygotes were vortexed to remove cumulus cells and cultured with granulosa cells (0.8 to $1.0X10^6$). Media from maturation (IVM), fertilization (IVF) and culture (IVC) were inoculated (50□l to primary bovine embryonic lung fibroblast cells (BFL) and after 5 days of incubation BVDV-antigen was scored with the monoclonal antibody C16 (Hoechst, Germany). The embryos were evaluated on days 2 and 7 after fertilization to determine the number of cleaved embryos, morulae and blastocysts, respectively.

Replication of BVDV in the system was demonstrated by reisolation from IVF (10/14) and IVC (9/10), but not from IVM (0/14) 8 day after BVDV-inoculation. In vitro culture results are presented in Table 1.

Table 1. Effect of BVDV (PT 810) on the developmental capacity of in vitro produced embryos (%).

	No. of oocytes	No. of cleaved	No. of Mor/bcyst
Control	378	256 (67.7%)	134 (35.4%)
PT810	629	425 (67.5%)	237 (37.6%)

NOTE: This table was taken from reference listed below.

"These results indicate that the exposure of oocytes to noncytopathic BVDV antigen in vitro has no effect on subsequent embryonic development over an in vitro culture period of 8 days." (**Palma et al., Proc 13th ICAR, Sydney, Australia 1996;2:13g**)

Bovine oocytes enclosed within follicular epithelial (FE) cells were exposed to noncytopathogenic or cytopathogenic strains of bovine viral diarrhea-mucosal disease virus (BVD-MDV). After culture at 39^{o}C in humidified air with 5% CO_2 for 24 hours, the oocytes matured and then in vitro fertilization was performed. Some of the fertilized oocytes developed into blastocysts from day 8 to day 10 (day 1: date of insemination), and the rate of development to blastocysts was the same as for the unexposed control oocytes. In the developmental medium, each strain of BVD-MDV was present at 10^3 to 10^6 $TCID_{50}$/0.25 ml from day 3 to day 10, respectively and the virus was isolated from the FE cells at high titers and from the embryos at low titers on day 10. BVD-MDV antigen was detected in the cytoplasm of the FE cells by indirect immunofluorescence. These findings indicate that BVD-MDV replicates well in FE cells. The results of this study suggest that BVD-MDV replication in

cells around embryos has no effect on bovine embryo development. (**Tsuboi T & Imada T., Vet Micro 1996;49:127-134**)

The risk of the transmission of BVDV to bovine embryos and via embryo transfer was investigated by obtaining oviductal epithelial cells and granuloma cells, which are commonly used as feeder cells, from cattle persistently infected with BVDV and examining them for the presence of BVD viral antigen (p80 nonstructural protein and gp 53 envelope glycoprotein) by indirect immunofluorescent histochemistry, and also viral RNA (encoding the p80 region) by in situ hybridization. In addition, titers of virus present in oviduct, ovary and blood were assayed by immunodetection on calf testis cell cultures. Luminal epithelial cells from the oviduct and primary cultures of granulosa cells and oviduct epithelial cells from such cattle were shown to contain both viral antigen and RNA. The susceptibility of both cell types to BVDV infection was further established by inoculating primary cultures of cells derived from cattle not infected with BVDV with a cloned isolate of noncytopathic BVDV (Pe515). RNA encoding BVDV and the antigen were detected 12 h after inoculation. Viral titres present in oviduct, ovary and blood were between $10^{2.2}$ and 10^{7}; $10^{2.2}$ and $10^{6.75}$; and $10^{3.5}$ and $10^{4.25}$ $TCID_{50}$/g, respectively. Control tissues from cattle not infected with BVDV, tested in each of the preceeding techniques, were negative. It was concluded that ovary and oviduct of persistently infected animals harbor noncytopathic BVDV and that granulosa cells and oviductal epithelial cells, which are used as coculture cells during bovine embryonic development in vitro and which, in the case of granulosa cells, constitute the cumulus investment surrounding the oocyte, are a vehicle for the potential transmission of BVDV for developing embryos. (**Booth et al, J Reprod Fertil 1995;105:17-24**)

In a series of experiments, a noncytopathic strain of BVDV (New York-1; 10^{8} $TCID_{50}$/ml) was introduced into the maturation, fertilization or embryo culture medium during the IVF procedure. The virus was isolated 1) from all 40 samples containing unfertilized washed or unwashed oocytes (n=360); 2) from 46% of 52 samples of day 7 embryos (n=307) produced by IVF, which were washed 10 times; 3) from 66% of the 44 samples of unwashed embryos (n=325); and 4) from 50% of the 20 samples of embryos (n=111) exposed to washing and trypsin treatment. In addition, 5 heifers inoculated intravenously with single unwashed day 7 embryos developed antibodies to BVDV. Three of 5 other heifers seroconverted following inoculation with single embryos that had been washed 10 times. Two of the 4 heifers inoculated intravenously with 2 trypsin-treated embryos seroconverted, while the remaining 2 heifers which received a single embryo did not develop antibodies. They concluded that under in vitro conditions, BVDV may be associated with groups of unwashed and washed oocytes and embryos, and may persist through the IVF process; and the standard washing procedure with or without trypsin treatment is not fully effective rendering IVF embryos free of BVDV. (**Bielanski et al, Theriogenology 1996;46:1467-1476**)

Follicular oocytes (n=645) collected from slaughterhouse ovaries were matured and fertilized in vitro, and presumptive zygotes were cultured for 7 days. Primary cultures of uterine tubal cells for use during IVF and IVC were divided into 2 groups. One-half of the cultures were infected with noncytopathic BVDV while the other half was not exposed to the virus. Approximately, equal groups of mature oocytes were inseminated, and the presumptive zygotes were cultured with infected or noninfected uterine tubal cells. After 7 days in IVC, zona pellucida-intact (ZP-I) morulae and blastocysts and degenerated ova were

washed and assayed for the presence of infectious virus. Infections of uterine tubal cells were not apparent and did not reduce rates of cleavage and development (P>0.05;Chi-square test for heterogeneity). After washing, BVDV was isolated at significantly higher rate from groups of virus-exposed degenerated ova (79%) than from individual virus-exposed morulae and blastocysts (37%;P=0.0002; Mantel-Haenszel summary, Chi-square). (**Stringfellow et al, Theriogenology 1997;48:171-183**)

Follicular oocytes from slaughterhouse ovaries were matured and fertilized in vitro and presumptive zygotes were cultured for 7 days. During each of 14 trials, four equal groups of ten or less ZP-I embryos/ova were assembled [two groups of morulae and blastocysts (M/B) and two groups of nonfertile or degenerate ova (NFD)]. All groups were prewashed with MEM with 10% equine serum and exposed to 10^4 to 10^6 $TCID_{50}$/ml of either noncytopathic (SD-1) or cytopathic (NADL) BVDV for 2 hours. One M/B and one NFD group were trypsin treated (IETS standard). Two remaining groups were washed 10 times in MEM with 10% equine serum and 1% PSF. Virus isolation from each group was attempted in Madin Darby Bovine Kidney cells with presence of virus confirmed by immunoperoxidase assay utilizing anti-BVDV antibodies. Data was stratified by embryo type and virus biotype and results were compared using the Mantel-Haenszel Chi Square test (Table 1).

Table 1. Percentage of groups of morulae and blastocysts (M/B) or degenerate ova (NFD) that were BVDV positive after standard washing or trypsin treatment.

	Noncytopathic BVDV		Cytopathic BVDV	
	M/B*	NFD	M/B	NFD
Washing	100% (9/9)	78% (7/9)	40% (2/5)	40% (2/5)
Trypsin treatment	44% (4/9)	67% (6/9)	20% (1/5)	20% (1/5)

*Percentage positive for virus of groups that were washed were significantly different from percentage of groups that were treated with trypsin.($P<0.05$).

They concluded that neither standard washing or trypsin treatment was effective for removal of BVDV from ZP-I, IVF bovine M/B and NFD after artifical exposure to the virus. (**Trachte et al, Theriogenology 1997;47:383 abstr and Trachte et al, Theriogenology 1998;5:717-726**)

Ovaries were collected at post mortem from three heifers that were persistently infected with BVDV. A sample from each ovary was taken for virus isolation, and the rest of the ovaries were stored at -70^{o}C. Cryosections from each ovary were examined for the presence of BVDV antigen by indirect immunofluorescence assay using two monoclonal antibodies, WB103 and WB 163, previously produced against nonstructural protein NS3 and envelop glycoprotein E2, respectively. 6.97+0.17 log_{10} $TCID_{50}$/ml of BVDV were recovered from all ovarian samples. In total, 362 0f 1,939 (18.7%) of oocytes contained BVDV antigens. There was no significant difference in proportion of BVDV-infected oocytes in primordial (18.2%), primary (19.4%) and secondary (21.0%) follicles. Developmental potential of oocytes could not be determined, but it was concluded that the bovine oocyte and cumulus are susceptible to BVDV infection. (**Fray MD et al. Vet Path 1998;35:253-259**)

Follicular fluid and cumulus cells were collected from ovaries of two heifers that were persistently infected with BVDV. A higher titer of virus was present in follicular fluid than in serum. IVM and IVF was performed on oocytes and IVC was continued for 10 d. Titers of virus in culture medium were $10^{2.25}$ to $10^{3.25}$ $TCID_{50}$/0.1ml. Virus was detected in cumulus cells at 10 d. Virus was detected in cumulus by indirect immunofluorescence, but none was detected in embryos at 10 d. They concluded that developed embryos were unlikely to be infected with BVDV, but cumulus cells from persistently infected cells did support replication of the virus. (**Tsuboi T and Imada T. J Vet Med Sci 1998;60:569-572**)

In one experiment, heifers were artificially infected with BVDV-type II (strain CD87) and subsequently, IVF embryos were produced from oocytes collected on 4, 8 and 16 d after infection. From a total of 29 heifers, infectious BVDV was detected in 55% of follicular fluid samples, in 10% of uterine tubal cells, in 10% of uterine flushes and in 41% of the IVF embryos. The highest number of embryos associated with BVDV was from animals slaughtered on 8 d after infection (58%). The amount of virus associated with single embryos generated from the oocytes of heifers 8 and 16 d after infection constituted an infective dose when given intravenously to seronegative recipients (6/15).
In a second experiment, uninfected oocytes were exposed in vitro to BVDV (10^5 $TCID_{50}$/ml) in the maturation medium and then fertilized and cultured prior to virus isolation. Virus was detected in 4/7 samples containing embryos. The presence of BVDV in the IVF system did not appear to affect embryonic development in vitro. (**Bielanski A et al. Theriogenology 1998;49:1231-1238**)

Zona pellucida-free oocytes, zygotes, 8-cell embryos, morulae, and blastocysts were incubated for 1 h at 38.5^{o}C in MEM containing $10^{6.00}$ $TCID_{50}$ NCP BVDV isolate 22146. Subsequently, groups of 5 embryos or 200 oocytes and zygotes were transferred into 50µl droplets of Menezo B2 with 10% FBS (negative for antibodies to BVDV). Calf testicle (CT) cells on beads were used as controls at 0, 12, 24, 36, 48, 60 and 72 h after inoculation groups of 5 embryos and CT-cell coated beads or 200 oocytes and zygotes were titered for virus. Replication of NCP BVDV varied with stage of development. No BVDV replication was detected in oocytes, zygotes and 8-cell embryos. Maximal intracellular virus for ZP-free morulae and blastocysts was detected at 72 h with $10^{1.6}$ and $10^{1.9}$ $TCID_{50}$/ml, respectively. In CT controls, peak intracellular virus was detected at 48 h ($10^{4.5}$ $TCID_{50}$/5 beads). Results appeared to indicate that cells of earlier stages were not susceptible to NCP BVDV infection. Morulae and blastocysts supported a low level of virus replication. All stages of development appeared to be less susceptible to NCP BVDV than was shown earlier with CP BVDV. (**Vanroose G et al. Theriogenology 1998;49:254abstr**)

In five weekly trials, morulae and blastocysts produced through IVM/IVF/IVC were washed and assigned to one of the following treatments: negative control, positive control, embryos placed in IVC immediately or embryos placed in IVC after cryopreservation. With exception of negative controls, all embryos were incubated in $10^{4.5}$ to $10^{5.5}$ $CCID_{50}$ of BVDV (strain SD-1) per ml of medium for 2 h and washed 12 X in MEM plus 10% equine serum. Attempts were made to isolate virus from positive and negative control groups after washing. Individual embryos not in the control groups were cocultured, immediately or after cryopreservation, with BVDV-negative bovine uterine tubal cells (UTC) in medium free of anti-BVDV antibody.

After 2 d in coculture, the IVC medium, UTC, and washed embryos were assayed separately for presence of infectious virus. Susceptibility of UTC was confirmed for each trial. BVDV was not isolated from any individual embryos or UTC following viral exposure, washing and 2 days of coculture. Virus was isolated from one sample of IVC medium and all positive control embryo groups but was not isolated from any negative control groups. The authors concluded that based on their results, virus did not penetrate the ZP and infect embryonic cells of developing embryos. In addition, in the artificial environment there was insufficient embryo-associated virus to infect the susceptible UTC. Thus, an infective dose of BVDV might not be associated with viral-exposed, washed, individual embryos. (**Givens MD et al. Theriogenology 1998; 49:252 abstr**)

In this study, they attempted to determine hazards of using COCs from BVDV persistently infected cattle in IVF. In the first experiment, 1 COC from a persistently infected animal was included with 10 COCs from healthy cattle. Oocytes were then subjected to IVM/IVF/IVC. BVDV was isolated from development medium, mixed cumulus cells (9 d after fertilization), and embryos in 1 of 4 replicates.
In the second experiment, 100 COCs were exposed to NCP BVDV strain 12 (10^6 $TCID_{50}$/ml). Then oocytes were cultured as in exp 1. FBS either without or with neutralizing antibody (titer of 1:8??) was used in the IVF system. The survival of infectious BVDV in medium up to 264 h was determined. BVDV titer of medium without antibody was $10^{3.53}$ to $10^{6.03}$ $TCID_{50}$/0.25 ml and that of embryos was $10^{2.53}$ $TCID_{50}$/0.25 ml. However, in presence of antibody virus titers of medium and embryos were “markedly decreased”. BVDV was inactivated by 72 h when cultured with medium alone. They concluded that inclusion of COCs from persistently infected cows was hazardous, but that using FBS with anti-BVDV antibody and removing FE cells was effective to prevent BVDV transmission. (**Tsuboi T et al. Theriogenology 1998;49:253 abstr**)

The objective was to determine if CP and NCP BVDV are able to replicate in IVF bovine embryos and whether the virus affects development. ZP-free oocytes, zygote, 8-cell embryos, morulae and hatched blastocysts were incubated 1 h in MEM with 10^6 $TCID_{50}$/ml NCP BVDV (isolate 22146) or $10^{6.25}$ $TCID_{50}$/ml CP BVDV (strain Oregon C24V). Groups were collected for virus titration at 0, 12, 24, 36, 48, 60 and 72 h after exposure. A small quantity of replicated CP BVDV was detected in 8-cell embryos at 60 h. For ZP-F morulae and blastocysts, maximal intracellular virus titers were, respectively, $10^{1.47}$ and $10^{2.33}$ $TCID_{50}$/100 cells at 48 h for CP BVDV and $10^{0.64}$ and $10^{0.84}$ $TCID_{50}$/100 cells at 72 h for NCP BVDV. CP infection inhibited development. They concluded that susceptibility of ZP-F IVF embryos increases with advancing developmental stage. No signs of virus replication or degeneration were seen when ZP-I embryos were exposed to virus and assayed in similar fashion. (**Vanroose G et al. Bio of Reprod 1998;58:857-866**)

Ovarian oocytes and follicles of 3 cows persistently infected with BVDV were examined for BVDV antigen by immunohistochemistry and RNA using an in situ hybridization technique. Over 18% of the follicles were infected with BVDV and over 6% of follicles possessed oocytes that were positive for viral antigen. It is noted that if the proportion of oocytes that are infected are eventually proven to be non-viable and thosewhich are not infected can be effectively disinfected the risk of transmitting BVDV by embryo transfer is very slight. However, until this is established, the health status of donor cows should be considered to

prevent the potential transmission of BVDV through embryo transfer. **(Brownlie J, et al, Vet Rec 1997;141:335-337)**

The effect of bovine viral diarrhea virus on in vitro embryonic development was investigated. BVDV was introduced by incubating COCs with virus (strain Pe515; titre $10^{6.2}$ $TCID_{50}$/ml) or vehicle for 2 hours, and then during in vitro culture by the use of BVDV-infected granulosa cells. Exposure to BVDV throughout in vitro production reduced (P=0.01) cleavage rates but increased (P = 0.05) the number of embryos that reached the 8-cell stage when expressed as a percentage of cleaved oocytes. Blastocyst yield was increased by the presence of virus when expressed as a proportion of oocytes (P=0.0034) or of cleaved ova (P<0.0001). The total blastocyst yield on days 7, 8 and 9 was 20, 51 and 29% respectively for the control and 29, 41, and 29% for the virus treatment, indicating that the rate of blastocyst development was not significantly faster in the virus-treated group (P=0.06). It is concluded that the presence of non-cytopathic BVDV in an in vitro production system may reduce blastocyst cleavage rates but allow cleaved ova to develop at a higher rate. **(Booth PJ, et al, Theriogenology 1998;50:769-777.)**

The effect of high concentrations of cryoprotectants on the passage of BVDV through the zona pellucida (ZP) of intact bovine embryos during the pre-freezing step of cryopreservation was investigated in a series of experiments. In vitro fertilized embryos at the blastocyst stage were exposed to 10^6 $TCID_{50}$ BVDV (non-cytopathic NY-1 strain) in 30% suspension of either ethylene glycol, glycerol, DMSO, or 2 M sucrose in physiological saline for 10 minutes at 20^oC . Subsequently, the embryos were washed free of residual unbound viral particles, and the ZP of some of the embryos were removed by micromanipulation. Groups of ZP-intact embryos, ZP-free embryonic cells and their respective ZP were then tested separately for the presence of virus. The infectious virus was detected in association with 81% (17/21) of samples containing non-micromanipulated ZP-intact embryos which were exposed to the virus and cryoprotectants and then washed 10 times and in 83% (43/53) of the samples containing only ZP from micromanipulated embryos. The virus was not found in the samples containing the corresponding embryonic cells of embryos exposed previously to the virus and cryoprotectants. It was concluded that the transfer of embryos from the isotonic PBS solution into highly hypertonic cryoprotectant solution did not cause the passage of BVDV through ZP and its entry to embryonic cells. **(Bielanski A, et al, Anim Reprod Sci 1999;55:83-90.)**

Oocytes were aspirated from abattoir origin ovaries and used for IVM, IVF and IVC for eight days. Then, 5 experiments were carried out. In experiments 1 and 3, embryos were tested for BVDV after viral exposure throughout the complete in vitro production process. In experiment 2 testing was carried out after exposure during in vitro culture only. In experiments 4 and 5, shorter periods of exposure were used (4 hours and 5 minutes, respectively). It was concluded that brief exposure of in vitro-produced embryos to non-cytopathic BVDV leads to levels of embryo-associated infectivity which cannot be readily removed by current IETS washing procedures. Also, degree of blastocyst expansion did not appear to alter association with the virus. The properties of the zona pellucida of the in vitro-produced embryo are different from those of the in vivo embryo in terms of potential BVDV transmission. **(Booth PJ, et al, Vet Rec 1999;144:150-152.)**

Bovine viral diarrhea virus (BVDV) has been isolated from washed and sonicated, in vitro-produced embryos, but the infectivity of BVDV associated with intact, developing, embryos has not been demonstrated. The objective of this study was to determine if a dose of BVDV infective for coculture cells was associated with individual, developing embryos following artificial exposure to the virus and washing. In 5 replicates, zona pellucida-intact, in vitro-produced embryos were assigned to a negative control embryo group or were incubated in 10^5 to 10^6 cell culture infective doses (50%, $CCID_{50}$) per mL of a type I, noncytopathic (strain SD-1) BVDV for 2 hours. Unexposed negative control embryos and exposed positive control embryos were washed, sonicated and assayed for BVDV using virus isolation with immunoperoxidase monolayer assay. Immediately or following cryopreservation, remaining virally-exposed, washed embryos were cocultured individually with BVDV-negative cultures of bovine uterine tubal cells in a medium free of BVDV-neutralizing activity. After 2 days in culture, uterine tubal cells and embryos (including the zona pellucida) were separated and washed. The culture medium, uterine tubal cells and embryos were then assayed for BVDV. Bovine viral diarrhea virus was not isolated from any negative control embryo group but was isolated from all positive control embryo groups. Although all uterine tubal cell populations were confirmed to be susceptible to BVDV, virus was never isolated from uterine tubal cells or embryos from post-exposure culture. In conclusion although BVDV remains associated with washed in vitro-produced embryos, the virus associated with unsonicated embryos was not infective for uterine tubal cells in vitro. **(Givens MD, et al, Vet Micro 1999;70:7-20.)**

In previous studies, bovine viral diarrhea virus (BVDV) remained associated with IVF embryos after viral exposure and washing. However, uterine tubal cells (UTC) were not infected when exposed embryos were washed and individually co-cultured with them. The objective of this study was to evaluate quantity and infectivity of embryo-associated virus and antiviral influence of a blastocyst as possible explanations for failure to infect the UTC in vitro. Morulae and blastocysts were produced in vitro and washed. A portion of the embryos were incubated for 2 h in medium containing 10^6 to 10^8 cell culture infective doses (50%, $CCID_{50}$) of a genotype I, noncytopathic BVDV per mL and then washed again. Virus isolation was attempted on sonicated negative (virus unexposed) and positive (virus exposed) control embryo groups after washing. The influence of quantity and infectivity of embryo-associated virus was evaluated by transferring exposed, washed embryo groups (2, 5, and 10 embryos/group) or sonicate fluid of exposed, washed, sonicated embryo groups (2, 5, and 10 embryos/group) to cultures containing bovine UTC in IVC medium that was free of BVDV neutralizing activity. The antiviral influence of an embryo was evaluated by adding 1 to 10^5 $CCID_{50}$ of BVDV to UTC in the presence or absence of a single unexposed blastocyst in IVC medium. After 2 d in co-culture, the UTC, IVC medium and washed embryos (when present) were tested separately for the presence of BVDV using virus isolation. Virus was isolated from sonicate fluids of all positive but no negative controls. Virus was not isolated from any UTC following 2 d of culture with virally exposed groups of intact embryos. However, virus was isolated from UTC cultured with sonicate fluids from some groups of 5 (60%) and 10 (40%) embryos. Also, infective virus remained associated with some groups of 2 (20%), 5 (40%) and 10 (60%) intact embryos after 48 h of post-exposure culture. Finally, primary cultures of UTC were more susceptible to infection with BVDV in the absence of a blastocyst ($p=0.01$). Results indicate that insufficient quantity and reduced infectivity of embryo-associated virus as well as an antiviral influence of intact IVF blastocysts may all contribute to failure of embryo-associated virus to infect UTC in vitro. **(Givens MD, et al, Theriogenology 1999;52:887-900.)**

Introduction of bovine viral diarrhea virus (BVDV) with cumulus-oocyte-complexes (COCs) from the abattoir is a concern in production of bovine embryos in vitro. Further, International Embryo Transfer Society (IETS) guidelines for washing and trypsin treatment of in- vivo-derived bovine embryos ensure freedom from a variety of pathogens, but these procedures appear to be less effective when applied to IVF embryos. In this study, COCs were exposed to virus prior to IVM, IVF and IVC. Then virus isolations from cumulus cells and washed or trypsin treated nonfertile and degenerated (NFD) ova were evaluated as quality controls for IVF embryo production. Also, the effect of BVDV on rates of cleavage and development was examined. All media were analyzed prior to the study for anti-BVDV antibody. Two approximately equal groups of COCs from abattoir-origin ovaries were washed and incubated for 1 h in minimum essential medium with 10% equine serum. One was incubated in 10_7 cell culture infective doses (50% endpoint) of BVDV for 1 h while the other was incubated without virus. Subsequently, groups were processed separately with cumulus cells present throughout IVM, IVF and IVC. Cleavage was evaluated at 4 d and development to morulae and blastocysts (MB) at 7 d of IVC. After IVC, groups of NFD ova or MB were washed or trypsin treated, sonicated and assayed for virus. Cumulus cells collected at 4 d and 7 d also were assayed for virus. Anti-BVDV antibody was found in serum used in IVM and IVC, but not in other media. A total of 1,656 unexposed COCs were used to produce 1,284 cleaved embryos (78%), 960 embryos >5-cell (58%) and 194 MB (12%). A total of 1,820 virus-exposed COCs were used to produce 1,350 cleaved embryos (74%), 987 embryos >5-cell (54%), and 161 MB (9%). Rates of cleavage (p=0.021), cleavage to >5-cell (p=0.026) and development to MB (p=0.005) were lower in the virus-exposed group (Chi-square test for heterogeneity). No virus was isolated from any samples from the unexposed group. For the exposed group, virus was always isolated from 4- and 7-d cumulus cells, from all washed NFD ova (n=40) and MB (n=57) and from all trypsin treated NFD ova (n=80) and MB (n=91). Thus, virus persisted in the system despite the presence of neutralizing antibody in IVM and IVC media, and both washing and trypsin treatment were ineffective for removal of the virus. Presence of virus in 4- and 7-d cumulus cells as well as NFD ova made these samples good indicators of virus associated with M/B. **(Stringfellow DA, et al, Theriogenology 1999;52:887-900.)**

To investigate the susceptibility of early bovine embryos to noncytopathogenic bovine viral diarrhea virus (NCP BVDV), 2- and 4-cell embryos produced in vitro from which zona pellucida had been removed by pronase treatment, and hatched blastocysts were exposed to 10_6 $TCID_{50}$/ml of NCP BVDV No. 12 strain. The virus was detected in all embryo samples immediately prior to cultivation but not in the medium. After 24-hr culture, the virus was isolated from four media and two embryo samples in four experiments in the blastocyst group, and the viral antigen was demonstrated in the cytoplasm of the embryo cells by the immunofluorescent technique. By contrast, no virus was recovered from, or viral antigen detected in samples from the 2- and 4-cell embryo group in any of the experiments, even though they were exposed to the virus after removal of the zona pellucida. These findings suggest that 2- and 4-cell embryos are unlikely to be susceptible to NCP BVDV, but that blastocysts are capable of being infected with the virus. hatched blastocyst, noncytopathogenic bovine viral diarrhea virus. **(Tsuboi T et al, J Vet Med Sci 1999;61:943-945)**

Recent studies have shown that washed, virally-exposed IVF embryos remain contaminated with bovine viral diarrhea virus (BVDV). However, a genotype I, noncytopathic strain of BVDV (SD-1) associated with intact embryos after 12 washes did not infect susceptible uterine tubal cells (UTC) in culture. The objective of this study was to evaluate the potential of 2 genotype I (SD-1 and NY-1) and 2 genotype II (CD87 and PA131) noncytopathic strains of BVDV to replicate in an in vitro embryo production system, associate with IVF embryos and remain infective. During 6 research replicates, presumptive zygotes were removed from fertilization drops, washed and transferred into primary IVC drops. These culture drops contained UTC in medium supplemented with 10% equine serum (IVC-eq) that was free of BVDV neutralizing activity. Immediately prior to addition of zygotes, the primary culture drops were inoculated with 10^4 to 10^5 cell culture infective doses (50%, $CCID_{50}$) of a strain of BVDV or maintained as a negative control. Primary culture drops were incubated for 7 d with medium added on Days 3 and 6. On Day 7, developed blastocysts were removed and washed 12 times in minimum essential medium with 10% equine serum. The UTC from primary culture drops were harvested, washed, titrated and assayed for BVDV by virus isolation (VI) using immunoperoxidase assay with anti-BVDV monoclonal antibodies. After washing, groups of 4-5 blastocysts were transferred intact into BVDV-free secondary culture drops containing UTC in IVC-eq or sonicated with sonicate fluid assayed by VI and closed one-tube reverse transcription nested polymerase chain reaction (RT-nPCR). After 3 d in secondary culture, washed embryos, IVC medium and washed UTC were tested separately for BVDV by VI. The UTC from secondary culture drops were also tested for BVDV using RT-nPCR (Table 1).

Table 1. Isolation of bovine viral diarrhea virus (BVDV) after exposure to various BVDV strains

	Neg	SD-1	NY-1	CD87	PA131
Primary culture UTC by VI (mean $CCID_{50}$)	0	5×10^5	7×10^5	9×10^3	2×10^5
Primary culture embryos by VI (P/T)	$0/6^a$	$5/6^{b,c}$	$5/6^{b,c}$	$2/6^{a,b}$	$6/6^c$
Primary culture embryos by RT-nPCR (P/T)	$0/6^a$	$6/6^b$	$6/6^b$	$5/6^b$	$6/6^b$
Secondary culture embryos by VI (P/T)	$0/12^a$	$9/15^c$	$1/13^a$	$2/12^{a,b}$	$6/12^{b,c}$
Secondary culture medium by VI (P/T)	$0/12^a$	$11/15^c$	$5/13^{b,c}$	$3/12^{a,b}$	$9/12^c$
Secondary culture UTC by VI (P/T)	0/12	0/15	0/13	0/12	1/12
Secondary culture UTC by RT-nPCR (P/T)	0/12	0/15	0/13	0/12	2/12

Neg= Negative control; $CCID_{50}$= cell culture infective doses (50%)
P= number of virus positive samples; T= total number of samples assayed for virus
^{a,b,c} Values within the same row with different superscripts differ significantly ($P<0.05$; χ^2 test)

Despite a high incidence of adherence to embryos after washing, genotype I and genotype II BVDV strains seldom infected susceptible cells in secondary culture drops. This in vitro research indicates inherent, nonspecific barriers to BVDV transmission via IVF embryos. **(Givens MD et al, Theriogenology 2000;53:319 abstr)**

Bovine IVF embryos (produced using standard techniques) in a mixture of 20% ethylene glycol, 20% ME_2SO, and 0.6% sucrose were vitrified in either unsealed standard 1/4 mL straws, modified open pulled straws or in plastic cryovials and then plunged into liquid nitrogen (LN) that was contaminated with bovine viral diarrhea virus (BVDV) by immersing an open vial containing 1 mL of cell culture suspension of BVDV (10^8 $TCID_{50}$/mL; NY-1 strain) into the same storage tank. After storage for 3 to 5 weeks, embryos were thawed, sequentially washed and batches of 3, ZP-I embryos were assayed for BVDV by virus isolation. All control embryos that were stored in sealed vials were negative. However, 16%

(5/31) of the batches of embryos stored in unsealed containers were positive for BVDV. **(Bielanski A et al, Cryobiology 2000;40:110-116)**

Bovine viral diarrhea virus (BVDV) causes congenital malformations in developing bovine fetuses. It is also fetopathic and causes abortions. The effect of different strains of BVDV representing the two biotypes and two genotypes of the virus was determined by a viability assay in an in vitro fertilization system. The viability assay was based on the measurement of bioluminescent emissions from the media surrounding day 7 embryos that were exposed to different viral strains at different developmental stages. The cytopathic type 1 and type 2 genotypes impaired embryo viability to a significantly ($P<0.05$) greater degree than the noncytopathic type 1 and type 2 genotypes. The noncytopathic type 1 and 2 genotypes, however, had no significant ($P>0.05$) effect on the viability of the embryos compared to controls. A difference was also seen between strains of the two genotypes used in this study. The in vitro fertilization method is a useful way to study the effect of viruses on developing bovine embryos. **(Brewoo JN, Shultz RD. Proceedings of the 81st Annual Meeting of the Conference of Research Workers in Animal Diseases. November 12-14, 2000. Abstract #179**

The objectives of this study were to determine the prevalence of BVDV as well as the prevalence and quantity of anti-BVDV antibodies in pooled bovine follicular fluid aspirated from ovaries of abattoir origin. Twenty samples of pooled follicular fluid collected between January and May 1999 were assayed for BVDV using standard virus isolation and reverse transcription nested polymerase chain reaction (RT-nPCR) procedures. Follicular fluid was also tested using a microtiter virus neutralization assay for antibody that would neutralize 4 representative strains of BVDV (SD-1, a genotype 1a strain; NY-1, a genotype 1b strain; CD-87, a genotype 2 strain; and PA-131, a divergent genotype 2 strain). No BVDV was detected by virus isolation, but RT-nPCR did identify the virus in one sample of follicular fluid. Automated dye-determinator nucleotide sequencing of the amplified portion of the virus indicated that it was a genotype 1 strain that was distinct from our laboratory strains. In addition to the field strain of BVDV, the positive sample contained sufficient antibody in one milliliter to neutralize $3x10_5$ cell culture infective doses (50% endpoint) of either SD-1 or NY-1 (also genotype 1). Each of the follicular fluid samples contained sufficient antibody to neutralize large quantities of each of the 4 laboratory strains that were used. Average neutralization values are found in the table.

Strain of BVDV=	SD-1	NY-1	CD-87	PA-131
Ave $CCID_{50}$ neutralized=	$6x10^5$	$5x10^5$	$2x10^5$	$1x10^5$

In this study, titers of antibodies in follicular fluid were sufficient to neutralize large quantities of the test strains of BVDV as well as the field strain that was identified. The true prevalence of BVDV in follicular fluid might be underestimated when only virus isolation is used to identify the virus, because prevalence of anti-BVDV antibodies is likely to interfere with virul detection. However, a degree of serendipitous control of BVDV might be provided by presence of antibody at these concentrations. **(Galik PK et al. Theriogenology 2001;55:376 abstr.)**

Sensitive RT-nPCR assays can be used for the rapid detection of viruses. The objective of this research was to validate an RT-nPCR assay for detection of BVDV associated with various samples collected from an IVF system.

In 12 research replicates, we maintained matured COCs as negative controls or exposed them to 1 of 4 noncytopathic strains (SD-1, NY-1, CD-87, or PA-131) of BVDV for 1 h immediately before IVF. After 4 d of IVC, we harvested groups of 5 nonfertile ova or degenerated embryos (NFD) and some associated cumulus cells and transferred developing embryos and the remaining cumulus cells into secondary IVC drops. On the seventh d of IVC, cumulus cells, groups of 5 washed NFD and groups of 5 developed, washed embryos were harvested. We also collected single developed embryos after washing, washing with trypsin, washing and cryopreservation in ethylene glycol, or washing with trypsin and cryopreservation in ethylene glycol. All washes were performed according to International Embryo Transfer Society standards. Developed embryos and NFD were sonicated prior to assay. All samples were assayed for BVDV using virus isolation and RT-nPCR.

The virus isolation and RT-nPCR assays determined that all negative control samples were BVDV-free. Virus was detected in association with all exposed cumulus cells and groups of developed embryos using both virus isolation and RT-nPCR. Results from viral assays of other exposed samples indicate enhanced sensitivity of the RT-nPCR assay (Table 1). Detection of BVDV genomes that lack infectivity may account for the increased sensitivity of RT-nPCR compared to VI. The RT-nPCR assay used in this research exhibited acceptable sensitivity, specificity, predictive value and repeatability for rapid detection of BVDV associated with the various samples obtained from an IVF system.

Table 1. Detection of bovine viral diarrhea virus by virus isolation (VI) and reverse transcription nested polymerase chain reaction (RT-nPCR) of samples collected from an IVF system

Sample assayed	Total tested	VI positive (%)	RT-nPCR positive (%)
NFD groups (from IVC day 4)	96	81 (84%)[a]	95 (99%)[b]
NFD groups (from IVC day 7)	48	23 (48%)[a]	47 (98%)[b]
Single washed embryo	59	13 (22%)[a]	40 (68%)[b]
Single washed, trypsinized embryo	59	19 (32%)[a]	36 (61%)[b]
Single washed, cryopreserved embryo	57	16 (28%)[a]	41 (72%)[b]
Single washed, trypsinized, cryopreserved embryo	59	17 (29%)[a]	41 (69%)[b]

a,b Values with different superscripts within the same row differ significantly ($P<0.01$, Pearson Chi-square test)

(Givens MD et al. Theriogenology 2001;55:378abstr and Theriogenology 2001;56(5):787-799.)

Bovine viral diarrhea virus (BVDV) can be found in cells and fluids from ovaries collected at the abattoir. On the other hand, immunoglobulins are also found in the fluid of ovarian follicles. Anti-BVDV antibodies in follicular fluid might reduce cross-contamination of COCs at the time of collection or hinder the use of virus isolation to test for the presence of virus. One objective of this study was to determine the frequency with which BVDV could be found in pooled follicular fluid collected during the periodic aspiration of COCs from abattoir-origin ovaries. A second objective was to determine the prevalence and neutralizing activity of anti-BVDV antibodies in these blended samples. We collected samples of pooled follicular fluid (n = 55) over a 20-month period as part of our routine oocyte collection activities. We assayed each sample for BVDV using virus isolation as well as reverse transcription nested polymerase chain reaction (RT-nPCR) procedures. We also tested follicular fluid for antibody that would neutralize four representative strains of BVDV (SD-1, a genotype 1a strain; NY-1,

a genotype lb strain; CD-87, a genotype 2 strain, and PA-131, a divergent genotype 2 strain). We detected no BVDV by virus isolation, but we did identify the virus by RT-nPCR in one of the 55 samples of follicular fluid. Automated dye terminator nucleotide sequencing of the amplified portion of the viral genome indicated a genotype 1 strain that was distinct from any of our laboratory strains. In addition, each of the samples of follicular fluid contained sufficient antibody to neutralize large quantities of each of the four laboratory strains that were used. Finding BVDV in just 1 of 55 samples was consistent with reports of similar studies in which the occurrence of BVDV in abattoir-origin materials ranged from 0.9 to 12%. We presumed that failure to isolate the virus was due to neutralizing antibody in the sample. Thus, the incidence of BVDV contamination of our IVF system at the level of pooling of follicular fluid was low for the 20-month period. The presence of anti-BVDV antibody in pooled follicular fluid provided a coincidental means of neutralizing BVDV when it was introduced in fluid aspirated from infected ovaries. **(Galik PK et al. Theriogenology 2002;57:1219-1227.)**

Recent studies have shown that exposed, in vitro-derived embryos remain contaminated with bovine viral diarrhea virus (BVDV) after washing. However, introduction of a Genotype II versus Genotype I strain of BVDV into an IVF system was reported to provide greater potential for transmission of disease. The primary objective of this study was to compare the potentials for different strains of noncytopathic BVDV to replicate in an IVF system, associate with IVF embryos and infect co-cultured cells via association with washed embryos. The secondary objective was to compare the effect of different strains of BVDV on embryonic development. Two Genotype I (SD-1 and NY-1) and 2 Genotype II (CD-87 and PA-131) strains of BVDV were evaluated. After IVM and IVF of oocytes, presumptive zygotes were washed and transferred into in vitro cultures containing uterine tubal cells (UTC) and medium that was free of BVDV-neutralizing activity. Immediately before addition of zygotes, the cultures were inoculated with 10(5) cell culture infective doses (50%, CCID50) of a strain of BVDV or maintained as a negative control. Cultures of zygotes were then incubated for 7 d. Embryonic development was observed on Days 3 and 7, and attempts were made to isolate BVDV from UTC and medium on Day 7. Also on Day 7, groups of intact, washed blastocysts were either transferred into virus-free secondary cultures containing UTC or sonicated with sonicate fluid assayed by both virus isolation and single-closed-tube reverse transcription nested polymerase chain reaction (RT-nPCR). After 3 d in secondary culture, hatched embryos were enumerated, and medium from the cultures, washed UTC and embryos were tested for BVDV by virus isolation. In addition, washed UTC and embryos were tested for BVDV using RT-nPCR. All strains of BVDV persisted and replicated in the embryo culture environment, but cleavage beyond the 4-cell stage, blastocyst development and hatching varied among cultures contaminated with different strains of virus. Further, the quantity of BVDV associated with washed embryos from both initial and secondary cultures varied among strains, but the variation was unrelated to difference in genotype (SD-1 and PA-131 greater than NY-1 and CD-87). Although all strains of BVDV replicated in UTC in the initial in vitro cultures and remained associated with washed blastocysts, susceptible UTC in the secondary in vitro cultures were seldom infected by any strain of virus. **(Givens MD et al. Theriogenology 2000;54:1093-1107.)**

A pathogen which has been shown to commonly contaminate in vitro bovine embryo production system is bovine pestivirus (bovine viral diarrhea virus). Three experiments were designed to evaluate the in vitro maturation (experiment I), fertilization (experiment II) and

embryo development (experiment III) of immature oocytes, inseminated oocytes and presumptive zygotes in the presence of a bovine pestivirus (non-cytopathic, nCP type 1). The virus inoculum used was derived from a persistently infected cow. In experiment I, follicular oocytes (n=1257) recovered from slaughterhouse derived ovaries were randomly assigned to either a control group (n=578) which did not become exposed to bovine pestivirus and a treatment group (n=679) which was inoculated with bovine pestivirus (2.20-3.69 log(10) TCID(50)/50 microl) at the time of commencement of in vitro maturation. Overall, there was no significant difference between the control and pestivirus inoculated oocytes in either the cumulus cell expansion rate (79+/-7.5% versus 74+/-10.7%) or the nuclear maturation rate (89+/-4.8% versus 85+/-7.4%), respectively. In experiment II, in vitro matured oocytes (n=607) were inseminated either in the absence (control; n=301) or the presence of bovine pestivirus (4-4.6 log(10) TCID(50)/50 microl; n=306). A significant (P<0.01) reduction in the overall number of fertilized oocytes with two well formed male and female pronuclei was observed in the treatment group compared to the control group (58.5+/-5.8% versus 73.3+/-3.6%, respectively). In experiment III, after in vitro maturation and fertilization, presumptive zygotes were randomly assigned to either a control group (n=139) which was not exposed to bovine pestivirus or a treatment group which was inoculated with bovine pestivirus (2.97-4.47 log(10) TCID(50)/30 microl; n=139). The zygotes were then cultured under mineral oil in an atmosphere of 88% N(2), 7% O(2) and 5% CO(2) at 39 degrees C. The morphologic appearance of the embryos was assessed 48 h after the commencement of culture, and then every 48 h up to days 7-8 after insemination. The 22% (31/139) and 3.6% (5/139) of the presumptive zygotes developed to the morula or blastocyst stage in the control and the bovine pestivirus inoculated groups, respectively (P<0.001). This study demonstrates that bovine pestivirus has a significant detrimental effect on in vitro fertilization and early in vitro embryo development. **(Kafi M et al. Anim Reprod Sci 2002;71:169-179.)**

Bovine viral diarrhea virus (BVDV) has been shown to replicate in embryo culture systems and remain associated with developing IVF bovine embryos despite washing and trypsin treatment. In this study, novel antiviral agents were evaluated for capability to inhibit replication of BVDV without affecting embryonic development. 2-[5(6)-{2-imidazolinyl}-2-benzimidazolyl]-5-(4-aminophenyl)furan (DB456) or 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) were added to tissue culture medium 199 with Earle's salts supplemented with 10% equine serum (IVC medium) at final concentrations of 1, 5, 10, and 20 µM or 0.02, 0.1, 0.2, 0.4, and 0.8 µM respectively. Primary cultures of bovine uterine tubal epithelial cells (UTC) were placed in IVC media with DB456, DB606 or no antiviral agent. Within 1 hour, a genotype I (SD-1) or genotype II (PA-131) strain of BVDV was added to the cultures of UTC. Fresh media containing equivalent antiviral concentrations was added on Days 3 and 6 of culture. We quantitated infectious virus from the media on Days 3 and 7 and in UTC lysates harvested on Day 7 by serial dilution, virus isolation, immunoperoxidase monolayer assay, and the statistical method of Reed and Muench. The concentrations of DB456 and DB606 that reduced the concentration of SD-1 or PA-131 by 99% (IC_{99}) in media and UTC lysates were determined using regression analysis with JMP software (Table). Each value is a result of 5 or 6 independent research replicates.

Agent	Virus strain	IC_{99} Day 3 media	IC_{99} Day 7 media	IC_{99} Day 7 UTC lysates
DB456	SD-1	7.25 µM	13.0 µM	9.30 µM
DB456	PA-131	9.26 µM	14.5 µM	15.5 µM
DB606	SD-1	0.27 µM	0.35 µM	0.37 µM
DB606	PA-131	0.11 µM	0.22 µM	0.12 µM

In further research, IVF bovine presumptive zygotes were cultured in IVC medium with or without DB456 (10, 20, 40 or 80 µM) or DB606 (0.15, 0.3, 0.6 or 1.2 µM) for 7 days to determine the maximum noninhibitory concentrations for bovine conceptus development. Sixty zygotes were cultured per treatment. Fresh media was added on days 2 and 6. On Day 7, embryonic development was assessed and blastocysts were harvested and stained using Hoechst 33342 to enumerate blastomeres. Numbers of developed embryos and average number of blastomeres per embryo were not significantly different between treated and control cultures except with DB456 at 80 µM (5% development). This research indicates that bovine embryo culture might be safely supplemented with antiviral agents. **(Givens MD et al. Theriogenology 2002;57:572 abstr.)**

Routine quality controls in production of bovine embryos by in vitro fertilization (IVF) should include screening all materials of animal origin for the presence of bovine viral diarrhea virus (BVDV). Using a reverse transcription nested polymerase chain reaction (RT-nPCR) assay, we detected BVDV in primary cultures of uterine tubal cells (UTC) that had been used during IVF procedures. The goal of our ensuing investigation was to determine its source and assess risks associated with the identified contaminant. Sequencing of the amplified 5' nontranslated region (NTR) of the viral genome confirmed a Genotype I BVDV contaminant. This viral contaminant was also identified by RT-nPCR in multiple samples of the same lot of fetal bovine serum (FBS) that was used in transport media by the laboratory that harvested the UTC. Both routine and enhanced roller bottle methods for virus isolation failed to detect BVDV in the FBS. Furthermore, virus neutralization assays did identify antibodies to Genotype I strains of BVDV in the FBS. After 7 days of co-incubation, neither cultured, washed UTC nor exposed, washed embryos were RT-nPCR positive for BVDV. Eight embryos produced in the contaminated system were nonsurgically transferred into eight seronegative cows. None of the embryo recipients seroconverted to BVDV. Thus, contamination of cell culture medium with BVDV did not result in transmission of the virus when IVF embryos were transferred. Failure to transmit disease was likely aided by serendipitous control from anti-BVDV antibodies in the FBS.
However, a diagnostic dilemma was created when the RT-nPCR assays used to screen for BVDV were positive, yet attempts to isolate the virus were negative. This case study illustrates that if molecular assays are to be used to confirm the pathogen-free status of IVF embryo production systems, media components of animal origin (e.g. FBS) should be screened with molecular assays for BVDV as well as traditional virus isolation techniques. **(Givens et al. Theriogenology 2002;58:1399-1407.)**

Somatic cell nuclear transfer (NT) has been developed in the sheep, cow and mouse, using fetal or adult cells as donor nuclei. However, the efficiency of the technique remains low, and early embryonic death is a major problem. Bovine viral diarrhea virus (BVDV) infections are seen worldwide and have significant economic impact due to productivity and reproductive losses. The objective of this study was to compare BVDV infected and non-infected cells for their ability to promote normal development following nuclear NT. Bovine viral diarrhea virus-infected and non-infected fetal fibroblasts were prepared for NT by serum starvation in DMEM + 0.5 % fetal bovine serum up to 5 d. These cells were then combined with enucleated oocytes by micromanipulation. Electrofusion was utilized to transfer fibroblast nuclei into the recipient oocytes. The reconstructed embryos were activated with ionomycin and 6-DMAP followed by in vitro culture for 7-8 d in CR1aa medium on a BRL cell monolayer. Expanded or hatching blastocysts were non-surgically transferred to recipient cows at Day 7. Pregnancy diagnosis was performed by ultrasound beginning Day 30. Polymerase chain reaction (PCR) and indirect immunofluorescence was performed to identify somatic cells infected by BVDV. Bovine viral diarrhea virus viral RNA was detected in NT fetuses when infected cells were used as nuclear donors (preliminary data not shown). At Days 40-55, all cloned fetuses produced from BVDV-infected donor cell lines were BVDV positive. Bovine viral diarrhea virus viral antigen was detected by immunohistochemistry and viral RNA was identified by PCR. Furthermore, pregnancy loss between 30 and 40 d gestation was significantly greater in fetuses derived from the BVDV-infected cell line, compared to non-infected cell lines ($P<0.05$). The results suggest that cell lines infected with BVDV will result in greater embryonic death and resorption of fetuses when used for NT than will non-infected cell lines. Thus, pre-screening of a cell line may be an important criterion in donor selection for somatic cell nuclear transfer as well as prevention of disease transmission. **(Shin T, et al. Theriogenology 2000;53:243 abstr.)**

The purpose of this study was to investigate the adherence of bovine viral diarrhea virus (BVDV) to bovine mature, or immature, cumulus-free oocytes and to in vitro fertilized embryos, maintained in vitro in a ligated bovine oviduct to allow for the hardening of the zona pellucida. Incubation of the oocytes and embryos in the oviduct for 5 h caused hardening of the zona pellucida as measured by resistance to pronase digestion (which increased from approximately 3 min to 7 h; $P > 0.001$). However, there was no difference between the number of infected oocytes and embryos (n = 965 in 193 samples) following experimental exposure to BVDV regardless of whether or not they were previously incubated in the oviduct ($P > 0.05$). It was concluded that the modification of the proteolytic resistance properties of the zona pellucida during in vitro oviductal incubation did not influence the adherence of BVDV to zona pellucida of oocytes or in vitro fertilized embryos. **(Bielanski AF et al. Canadian Journal of Veterinary Research 2003;67:48-51.)**

Non-cytopathogenic bovine viral diarrhea virus (NCP-BVDV) may infect primary and secondary oocytes in persistently infected cattle [Brownlie et al., Vet Rec 1997;141:335-337, Fray et al., Vet Pathol 1998;35:253-259]. In an in vitro study reported by Vanroose et al. [Bio Reprod 1998;58:857-866] BVDV was not detected in matured oocytes after exposure of mature ZP-free bovine oocytes to the virus. However, it is still not known whether the NCP-BVDV can infect immature oocytes (IO). Here we report the results of preliminary investigation of the replication of BVDV in ZP-free and ZP-damaged IO during incubation in

vitro. Duplicate experiments were performed, each involving ZP-free and ZP-damaged IO. ZP removal was performed by treatment with 3 % sodium citrate and 0.1 % pronase. Multiple piercing of ZP with microneedle was used to damage the integrity of the ZP. Both groups of IO were then exposed to BVDV-NY1 strain with a titer (10^6 $TCID_{50}$/ml). Twenty to thirty oocytes were collected immediately (0 h) and 24 h (n=40-60) after virus exposure, then washed three times with TCM-199 medium supplemented with 2 % fetal bovine serum (FBS) to remove the unbound free virus, and stored at -80^{o}C until assayed for BVDV. The FBS used was free of BVDV and anti-BVDV antibody. Prior to viral assay of ZP-damaged IO group, the oocyte cumulus cells and ZP were removed using 1 % hyaluronidase and and 0.1 % pronase. BVDV RNA of individual oocytes was measured by dilution of C-DNA with RT-PCR [Vilcek et al., Arch Virol 1994;136:309-323]. BVDV infected MDBK cells were used as positive controls. Neither the ZP-free immature oocytes nor the ZP-damaged immature oocytes showed BVDV-PCR products. MDBK cells showed the products on the cells collected at 24 h after incubation with BVDV. These results suggest that immature oocytes derived from cattle may be relatively resistant to infection with BVDV under in vitro conditions. Further study is needed to elucidate the relationship between NCP-BVDV infection and in vitro oocytes during maturation. This study was reported by OECD fellowship 2001, Cooperative Research Program. **(Tsuboi T, et al. Theriogenology 2003;59:385 abstr.)**

The objective of this study was to investigate whether or not experimentally induced changes in hardening ZP (HZP) affect adherence of BVDV to the ZP of oocytes and embryos produced in vitro. To induce HZP, the oocytes or IVF embryos in groups of 30-50 were incubated in the ligated, ampullar part of the oviduct at 38^{o}C in 5% CO_2, 5% O_2 and 90% nitrogen for 5 hours. Following incubation, a proportion of the oocytes/embryos was exposed to 1% pronase to determine HZP (the time of ZP lysis), while the remaining oocytes and embryos were incubated with 10^6 $TCID_{50}$/ml of either noncytopathic (NY-1) or cytopathic (NADL) strain of BVDV at 38^{o}C for 3 hours. Subsequently, oocytes and embryos were washed according to the methods recommended by the IETS and then tested for the presence of BVDV (virus isolation and PCR tests). At the end of the experiments, the oviductal tissues were tested by PCR and proved free of BVDV. For immature and mature oocytes and embryos not exposed to the oviduct, the ZP dissolution times were 3.6+ 0.24 (mean+ SEM, n=20), 3.8+ 0.24, and 4.0+ 1.24 minutes, respectively (Chi-square test; P>0.05). Corresponding times for those incubated in the oviduct were 393 + 47, 431+ 50 and 467+ 61 minutes, respectively (P>0.05). There was no difference between the number of virus-positive oocytes and embryos (n=965 in 193 samples) following experimental exposure to BVDV regardless of whether or not they were previously incubated in the oviduct (P>0.05). Lower, but not significant, differences in percentages of samples associated with the infectious cytopathic strain of BVDV as compared to noncytopathic strain were detected (P>0.05). It was concluded that the modification in proteolytic resistance properties of ZP during in vitro oviductal incubation did not influence the adherence of BVDV to ZP of oocytes or IVF embryos. Further studies are warranted to determine why IVF embryos are more prone to the adherence of pathogenic agents than are in vivo fertilized embryos. **(Bielanski A, et al. Reproduction, Fertility and Development 2004;16:218 (abstr.))**

Routine screening by veterinary diagnostic laboratories of 39 fetal fibroblast cell lines used in cloning research had revealed that 15 (39%) were positive for BVDV by various assays including RT-nPCR. As some were valuable transgenic cell lines, a rigorous protocol for evaluation of each line was undertaken to confirm infection with BVDV. A cryopreserved vial

of each line was thawed, medium discarded and cells incubated (38.5^{o}C in 5 % CO_2 and air) through two passages (6-10 days) in alpha-MEM supplemented with 10% equine serum. At the end of the second passage, cells were separated from medium, washed and assayed for presence of BVDV using virus isolation in 2 sequential passages in Madin Darby Bovine Kidney Cells and RT-nPCR. Available lots of fetal bovine serum and medium that had been used to culture the cells also were tested for BVDV. When the virus was detected, the R-nPCR products were sequenced and compared. Also, an attempt was made to evaluate the earliest available cryopreserved passage of any positive cell lines. Results indicated that just 5 of the 39 original cell lines tested (13%) were positive. Since cryopreserved earlier passages of 4 cell lines were available, they were assayed with the result that 2 of the 4 were not infected at the earliest passage. Further, BVDV was isolated from one lot of fetal bovine serum that was used to culture one of the cell lines. Sequence analysis verified that only 2 of these 4 cell lines were infected with the same isolate of BVDV, and one isolate was identical to the virus found in the fetal bovine serum used in medium to culture it. The discrepancy between our viral detection and that of the diagnostic laboratories is explained in part by the presumed test protocols. All BVDV-positive cells, as reported by the diagnostic laboratories, were positive by RT-nPCR. We presume that they did not separate medium from cells before assays. Thus, any noninfectious viral RNA that was in the medium (e.g. as would be expected in many lots of irradiated serum) would have been reported as positive. The only possible sources for BVDV in these cell lines were the fetuses from which they originated or fetal bovine serum used in medium. Sequence analysis confirmed that serum was the source for one cell line. The likely source for 2 other cell lines was serum, since they were not infected at earlier passages. The remaining cell lines were positive at the earliest available passages, so the fetuses from which cells were harvested could not be discounted as the source of the BVDV. This report highlights the risks of introducing BVDV in embryo technologies and the difficulties that can be encountered in attempting accurate diagnosis of the presence of infectious virus. **(Stringfellow D, et al. Reproduction, Fertility and Development 2004;16:220 (abstr.))**

Noncytopathic infections with bovine viral diarrhea virus (BVDV) can compromise research and commercial use of cultured cells. The purpose of this research was to evaluate the ability of aromatic cationic compounds to prevent or treat BVDV infections in fetal fibroblast cell lines that are used in somatic cell nuclear transfer. To evaluate preventative use of compounds, 10 cell lines were inoculated with BVDV in the absence or presence of 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606), 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772), or 2-(1-methyl-2-benzimidazolyl)-5-[4'-(2-imidazolino)-2'-methylphenyl]furan dihydrochloride (DB824). The 99% endpoints for prevention of viral replication by these treatments were 81, 6, and 14 nM. To evaluate therapeutic use of compounds, two fetal fibroblast cell lines infected with a genotype 1a strain of BVDV were cultured through four passages in the absence or presence of either 0.04 or 4 microM concentrations of DB772 or DB824. The presence and concentration of BVDV in media and cell lysates were evaluated using reverse transcription nested polymerase chain reaction and virus isolation from titrated sample. A single passage in 4 microM of either compound was sufficient to eliminate BVDV from cells without causing cytotoxicity. Our results demonstrate that in vitro infections with BVDV can be effectively prevented or eliminated by addition of aromatic cations. (**Givens MD, et al. Antiviral Res. 2004;64:113-118.)**

Culture of cell lines from fetuses or postnatal animals is an essential part of somatic cell cloning. Fetal bovine serum (FBS) is commonly used in media for propagation of these cells. Unfortunately, bovine fetuses and postnatal animals as well as FBS are all possible sources of non-cytopathic bovine viral diarrhea virus (BVDV) which is widely distributed among cattle. This study was prompted when screening of samples sent to veterinary diagnostic labs revealed that 15 of 39 fetal fibroblast cell lines used in cloning research were positive for BVDV as determined by various assays including reverse transcription-polymerase chain reaction (RT-PCR). Goals of the research were to use both virus isolation and reverse transcription-nested polymerase chain reaction (RT-nPCR) to confirm which of the cell lines were actually infected with BVDV and to assay samples of media, FBS and the earliest available passages of each cell line in an attempt to determine the source of the viral infections. Sequence analysis of amplified cDNA from all isolates was performed to provide a definitive link between possible sources of virus and infected cell lines. Only 5 of the 39 cell lines were actually infected with BVDV. Three of these five lines were not infected at the earliest cryopreserved passage, leading to the conclusion that they likely became infected after culture in media containing contaminated FBS. In fact, sequence comparison of the amplified cDNA from one lot of FBS confirmed that it was the source of infection for one of these cell lines. Since BVDV was isolated from the remaining two cell lines at the earliest available passage, the fetuses from which they were established could not be ruled out as the source of the virus. (**Stringfellow DA, et al. Theriogenology 2005;63:1004-1013.)**

Cumulus oocyte complexes (COCs were collected from abattoir-origin ovaries and washed. The cumulus cells were removed with 3 % sodium citrate solution and the zona pellucida was removed with 0.1 % pronase solution. The zona pellucida-free immature oocytes (groups of 60 to 80) were exposed for 1 hour to 10_6 $TCID_{50}$/Ml BVDV (NY-1 strain). MDBK and equine dermal cell cultures were incubated in the same way, with and without virus (positive and negative controls). Viral RNA was extracted and a single-tube fluorogenic reverse transcription (RT)-PCR-based TaqMan assay was used to quantify the BVD viral RNA. One hundred copies per reaction mixture for the NY-1 strain was determined to be the detection limit for cRNA transcripts. Under these conditions, not evidence of viral replication in oocytes was found. (**Tsuboi T, Bielanski A. Vet Rec 2005;156:546-548.)**

Bovine viral diarrhea virus (BVDV) replicates in embryo co-culture systems and remains associated with developing IVF bovine embryos, despite washing and trypsin treatment. Previous research demonstrated that 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) inhibits replication of BVDV in cultured cells. The objective of this study was to evaluate the capability of IVF embryos to develop into normal, weaned calves after exposure to antiviral concentrations of DB606 during IVC. Oocytes were obtained from cows via transvaginal, ultrasound-guided follicular aspiration. Presumptive zygotes (n = 849) that resulted from fertilization of these oocytes were cultured for 7 d in medium supplemented with 0.4 microM DB606 or medium lacking antiviral agent. All blastocysts (n = 110) were transferred individually into the uterus of a synchronized recipient. The pregnancy status of recipients was determined using transrectal ultrasonography at 2123 d after embryo transfer. Additional pregnancies as controls (n = 21) were initiated by natural breeding. Developing fetuses and resulting calves were evaluated every 2734 d. Blastocyst development, pregnancies per transferred embryo, pregnancies maintained per pregnancies

established, gestation length, gender ratio, birth weights, viability of neonates, complete blood counts, and serum chemistry profiles at 3 mo of age and adjusted 205 d weaning weights were compared for research treatments. Development to weaning after exposure to DB606 did not differ significantly from controls. In conclusion, bovine embryo cultures can be safely supplemented with antiviral concentrations of DB606; addition of DB606 agent might prevent viral transmission if BVDV were inadvertently introduced into the embryo culture system. **(Givens MD et al, Theriogenology 2006;65:344-355.)**

The effect of infection with teratogenic viruses at early stages of pregnancy is not fully understood. This study aimed to look at the effect of infection with teratogenic viruses such as bovine viral diarrhea virus (BVDV) and border disease virus (BDV), on early stage embryos at the hatched blastocyst stage. BVDV and BDV are known to cross the placenta of infected mothers and lead to congenital defects and death of developing fetuses. This study can be a good model for better understanding the effects of other teratogenic viruses such as Rubella virus in humans. **(Mabruk MJ et al, Southeast Asian J Trp Med Public Health 2006;37:113-119.)**

Because of its broad distribution among populations of cattle and its association with materials of animal origin used in embryo production, bovine viral diarrhea virus (BVDV) is a potential problem in applications of embryo technologies. While some isolates of BVDV are known to associate with both *in vivo*-derived and *in vitro*-produced bovine embryos, it has yet to be determined if the quantity of virus associated with exposed zona pellucida-intact embryos is sufficient to infect susceptible recipient cows via the intrauterine route. Techniques to detect and quantify BVDV associated with single transferable embryos are important to determine the risk of transmitting BVDV via embryo transfer. The objectives of this study were to define reproducible techniques to detect and quantify BVDV associated with single or small groups of bovine embryos contained in small aliquots of medium using virus isolation (VI) or real time quantitative polymerase chain reaction (Q-PCR) assays. *In vivo*-derived and *in vitro*-produced embryos were exposed for 2 h to approximately 10^6-cell culture infective doses (50% endpoint) per mililiter of a high affinity strain of BVDV, SD-1, and then washed according to IETS guidelines. Embryos were assayed in groups of five or two embryos, or single. There were 5 replicates of the group of five embryos, 4 of the group of two embryos, and 3 of the single embryos for the *in vivo*-derived embryos undergoing VI; 5, 4, and 2 replicates, respectively, undergoing Q-PCR, and 2, 5, and 2 replicates, respectively, for the *in vitro*-produced embryo groups undergoing VI and Q-PCR. Those to be assayed by VI were sonicated and the sonicate fluids were layered onto Madin Darby Bovine Kidney (MDBK) cells and passaged to allow for viral replication; an immunoperoxidase monolayer assay was then used for viral detection. A Roche® RNA/DNA extraction kit was used to extract RNA from virally exposed embryos, and extracted samples were assayed in duplicate Q-PCR reactions consisting of 100µL. The primers used were L1 and U3 which are specific for conserved areas of the 5 prime nontranslated regions of the viral genome of BVDV. The PCR product was detected using hybridization probes s1 and s2 as in Struder *et al*. (2002 Biologicals 40, 289–296). *In vivo*-derived groups of five or two embryos, or single embryos, were positive for BVDV 100, 50, and 30% of the time, respectively, when VI was used and 100, 75 and 100%, respectively, when Q-PCR was used. The virus was detected in all of the *in vitro*-produced embryo groups of five, or two embryos, or single embryos, 100% of the time using VI, and in 100, 80, and 100% respectively, using Q-PCR. The virus isolation technique is highly sensitive but the need to destroy embryos by

sonication to identify any embryo-associated virus precludes its use for embryos intended for transfer. Techniques for Q-PCR were sufficiently sensitive to detect and quantify 10 copies of RNA in a sample and to detect BVDV associated with single embryos. **(Waldrop J et al, Reproduction, Fertility and Development 2006;18:214-215 (abstr.))**

Bovine virus diarrhea virus (BVDV) is a pathogen of the bovine reproductive system causing reduced conception rates, abortions and persistently infected calves. Most if not all strains of BVDV are transmissible by natural mating and AI. For international trade, it is recommended that in vitro fertilized embryos be washed according to the IETS Manual. However, BVDV may not be entirely washed out, resulting in possible transmission risks to recipients. Donor cows, donor bulls and biological agents are all possible sources of contamination. The process for producing in vitro produced (IVP) embryos is complex and non-standard, and some procedures can contribute to spread of BVDV to uninfected embryos. The structure of the zone pellucida (ZP) of IVP embryos permits adherence of BVDV to the ZP. To estimate the risk of producing infected recipients and persistently infected calves from abattoir-derived IVP embryos, a quantitative risk assessment model using Microsoft Excel and Palisade @Risk was developed. Assumptions simplified some of the complexities of the IVP process. Uncertainties due to incomplete or variable data were addressed by incorporating probability distributions in the model. Model variables included: disease prevalence; the number of donor cows slaughtered for ovaries; the number of oocytes collected, selected and cultured; the BVDV status of ovaries, semen, biological compounds and its behavior in the IVP embryo process. The model used the Monte Carlo method to simulate the IVP process. When co-culture cells derived from donor cows of unknown health status were used for in vitro culture (IVC), the probability of a recipient cow at risk of infection to BVDV per oocyte selected for IVP processing averaged 0.0006. However, when co-culture free from BVDV was used, the probability was 1.2 x 10(-5). Thus, for safe international trade in bovine IVP embryos (i.e. negligible risks of transmission of BVDV), co-culture cells, if used during IVC for producing IVP embryos, should be disease-free. **(Perry GH. Theriogenology, 2007;68(1):38-55.)**

Bovine viral diarrhea virus (BVDV) infection affects cattle throughout the world. It causes significant economic losses in the cattle industry. The potential for transmission of a cytopathic biotype of BVDV by *in vivo*-derived embryos has been thought to be negligible. However, there is no study to prove non-transmission of the most common field isolate of noncytopathic biotype (NCPB) of BVDV by IVF embryos. Here we report on the preliminary outcome of embryo transfer (ET) of IVF embryos exposed *in vitro* to type-1 (NY-1) and type-2 (P-131) genotypes of NCPB of BVDV. For this experiment, IVF embryos were generated using standard methods which briefly involve: maturation of cumulus–oocyte complexes in TCM medium, fertilization of oocytes with BVDV-free semen, and culture of zygotes to the blastocyst stage in SOF medium without somatic cells. Day 7 blastocysts were exposed for 1 h to NY-1 or P-131 (10^3–10^7 $TCID_{50}$ mL^{-1}) BVDV strains before being washed (without trypsin) as recommended by IETS. Two embryos were transferred on each occasion. Embryo recipients were virus-free and anti-BVDV antibody-free prior to ET. The recipients remained individually in isolation premises after ET. In total, 126 ET procedures were performed resulting in 57 pregnancies and 34 calves born free of the infectious virus and BVDV antibodies (5 pregnancies are still pending). In total, 23 pregnancies were lost after 30 days. Exposure of embryos to type-2 BVDV resulted in a loss of 46% (17/37) of pregnancies after 30 days post-ET and 20 recipients seroconverted to BVDV.

Within seroconverted and pregnant animals (*n* = 14), only 2 recipients maintained pregnancy and delivered uninfected calves at term. In contrast, exposure of embryos to type-1 caused 30% (6/20) of the pregnancy losses after 30 days and did not cause any seroconversion in ET recipients. After washing, 33% (3/9) and 38% (17/44) single embryos from the infected pool of IVF embryos tested positive for the BVDV. In conclusion, under these experimental conditions, a proportion of recipients was apparently infected after receipt of BVDV-exposed embryos. However, all of the calves that survived to term were BVDV-free and anti-BVDV antibody free. **(Bielanski A, Algire J, Lalonde A. Reproduction, Fertility and Development, 2008;20;156 abstr.)**

The primary objective of this study was to determine the percentage of individual, preimplantation, *in vitro*-produced bovine embryos which maintained association with virus despite washing following artificial exposure to a high affinity strain of bovine viral diarrhea virus (BVDV). Another objective of this study was to determine the quantity of virus associated with these embryos. A total of eighty-seven zona pellucida-intact, Day 7, *in vitro*-produced bovine embryos were exposed for 1 h to 2 • 10_6 cell culture infected doses per mL to the 50 percent endpoint ($CCID_{50}$ mL^{-1}) of a type 1 noncytopathic strain of BVDV (SD-1). Following exposure, the embryos were washed according to International Embryo Transfer Society standards for *in vitro*-produced bovine embryos; they then underwent sonication, RNA extraction, and freezing at –80°C until assayed for virus. A real-time quantitative polymerase chain reaction (QPCR) was run in duplicate on each of the 87 embryos. Forty-two percent (39/87) of the embryos assayed were determined to be positive for virus. The quantity of virus associated with the embryos averaged 0.55 viral copies per 5 µL (SD = 0.89 copies/5 µL, SEM = 0.14 copies/5 µL). Assessment of data using tolerance intervals (*P* = 0.05) indicates that 90% of contaminated embryos were associated with ≤2.40 viral copies per 5 µL while 99% of contaminated embryos were associated with ≤3.44 viral copies per 5 µL. These findings show that there is a low level of virus associated with *in vitro*-produced embryos but virus is associated with a significant number of exposed embryos. In conclusion, this study indicates that the potential for transmission of BVDV via embryo transfer of *in vitro*-produced embryos is small given the amount of virus that was found to associate with individual embryos. (**Gard JA, et al. Reproduction, Fertility and Development, 2008;20:157 abstr.)**

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The objective was to determine the average amount of bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced bovine embryos following recommended processing procedures for embryos. In vivo-derived and in vitro-produced bovine embryos at 7d post-fertilization were exposed (for 2h) to 2 x 10(5-7) cell culture infective dose (CCID(50))/mL of SD-1 (a noncytopathic, Type 1a strain of BVDV), and then washed according to International Embryo Transfer Society (IETS) guidelines prior to testing. Of the 87 in vivo-derived embryos tested, 27% were positive for virus by quantitative polymerase chain reaction (qPCR). The range in amount of virus associated with 99% of the contaminated embryos was <or=6.62+/-1.57 copies/5 microL; 90% of the contaminated embryos had <or=4.64+/-1.57 viral copies/5 microL of embryo-associated virus, using tolerance intervals (P<0.05). The SEM was 0.33 and the mean of averages was 1.12/5 microL. Of the 87 in vitro-produced embryos, 42% were positive for virus. The range in amount of virus associated with 99% of the contaminated embryos was <or=3.44+/-0.89 copies/5 microL; 90% of the contaminated embryos had <or=2.40+/-0.89 viral copies/5 microL of embryo-associated virus using tolerance intervals (P<0.05; S.E.M. was 0.14 and the mean of averages was 0.55/5 microL). Therefore, although many embryos were positive for virus, there were limited numbers of copies, thereby posing doubt regarding their potential for contamination following embryo transfer.
Gard JA, Givens MD, Marley MS, Galik PK, Riddell KP, Stringfellow DA, Zhang Y, Edmondson MA. Bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced preimplantation bovine embryos following artificial exposure. Theriogenology. 2009;71(8):1238-44.

The objective of this study was to perform a comprehensive risk assessment on infectious disease transmission in the system of in vitro embryo production via somatic cell nucleus transfer (SCNT) technology using bovine viral diarrhea virus (BVDV) as a model. The risks of BVDV transmission in each step of the SCNT embryo production procedure, from donor cells to preimplantation SCNT embryo culture, were carefully examined using a sensitive real-time polymerase chain reaction assay. The identified primary source of BVDV transmission in SCNT embryo production was donor cell infection, most likely caused by contaminated fetal bovine serum in the culture medium. The risk of disease transmission through contaminated oocytes from an abattoir was relatively low, and it can be greatly minimized by cumulus cell

removal and adequate oocyte washing procedures. Of the 200 cumulus-oocyte complexes (COCs) and more than 1500 cumulus cell-free oocyte (CFO) samples collected from multiple sources over a course of 7 months, only 2.5% of the COCs were BVDV positive, and all of the CFOs (100%) were BVDV negative. To evaluate the risk of BVDV introduction during in vitro SCNT m embryo culture, 324 SCNT embryos were produced from 18 different cell lines using oocytes from 26 different batches collected over a course of 9 months. The embryos were cultured in vitro for 7 days and then tested for BVDV. All of the 324 SCNT embryos (100%) were negative, indicating that the embryo culture system is virtually risk-free for BVDV transmission. Based on these results, a standard operational protocol (SOP) for SCNT embryo production was proposed to greatly minimize the risk of BVDV transmission through the SCNT embryo production system. This SOP could be a starting point to produce a SCNT system that is virtually risk-free for disease transmission in general.
Gregg K, Chen SH, Sadeghieh S, Guerra T, Xiang T, Meredith J, Polejaeva I. Experimental risk assessment of bovine viral diarrhea virus transmission via in vitro embryo production using somatic cell nucleus transfer. Theriogenology. 2009 Jul 1;72(1):99-110.

The purpose of this study was to determine whether or not embryos derived from in vitro fertilization of oocytes from persistently infected (PI) cattle would contain infectious virus.Three in vitro embryo production treatment groups were assessed: 1) oocytes and uterine tubal cells (UTC) free of bovine viral diarrhoea virus (BVDV) (negative control), 2)oocytes free of BVDV fertilized and cultured in media containing UTC obtained from PI heifers, and 3) oocytes from PI heifers fertilized and cultured in media containing UTC free of BVDV. The developmental media, UTC and embryos (individual or groups of five) were assayed for virus.Virus was not isolated from any samples in treatment group 1.As shown in previous studies, a proportion of embryo samples were positive for BVDV in treatment group 2. In treatment group 3, the virus associated with the oocytes contaminated the developmental media and infected susceptible co-culture cells used during fertilization and culture. In addition, 65% (11/17) of the degenerated ova from treatment group 3 had infectious virus associated with them. While none of the ova developed into transferable embryos, the study did confirm that use of oocytes from PI cows could lead to amplification of BVDV and cross contamination during in vitro embryo production. **Marley MS, Givens MD, Galik PK, Riddell KP, Stringfellow DA. Amplification of bovine viral diarrhoea virus introduced into an in vitro embryo production system via oocytes from persistently infected cattle. Reprod Domest Anim. 2009;44(3):532-5.**

The objective was to use the bovine viral diarrhea virus (BVDV) as a model to assess the risk of infectious disease transmission in the system of in vitro embryo production and transfer via somatic cell nuclear transfer (SCNT) technology. The risks of BVDV transmission in the SCNT embryo production were previously evaluated. In that in vitro study, following standard operating procedures (SOP), including pre-nuclear transfer donor cell testing, oocyte decontamination and virus-free cell and embryo culture conditions, SCNT embryos produced were free of detectable viral RNA. The current study focused on the evaluation of the potential risk of disease transmission from SCNT embryos to recipients, and the risk of producing persistently infected animals via SCNT embryo transfer. Blood samples were collected from 553 recipients of SCNT embryos and 438 cloned calves and tested for the presence of BVDV viral RNA via a sensitive real time PCR method. All samples tested were

negative. These results, in conjunction with the previous in vitro study, confirmed that the established SCNT embryo production and transfer system is safe and presents no detectable risk of disease transmission.

Gregg K, Gosch G, Guerra T, Chen SH, Xiang T, Broek D, Bruner B, Polejaeva I. Large scale in vivo risk assessment of bovine viral diarrhea virus (BVDV) transmission through transfer of bovine embryos produced via somatic cell nuclear transfer (SCNT). Theriogenology 2010 Oct 15;74(7):1264-70. Epub 2010 Aug 12.

Bovine viral diarrhoea virus (BVDV), a member of the Pestivirus genus, is one of the most important pathogens of dairy cattle; it can cause several clinical syndromes, ranging from subclinical to severe disease. The objectives of the current studies were to assess the effects of two biotypes of BVDV on sperm attachment to the zona pellucida (ZP) of oocytes and on fertilization rate in bovine in vitro fertilization (IVF). In two experiments, sperm at two concentrations (10^5 and 10^6/mL) and oocytes were incubated with 10^6 $TCID_{50}$/mL cythopatic (CP) or noncythopatic (NCP) BVDV. In the first experiment, with the lower sperm concentration (10^5/mL), male and female gametes were infected with CP or NCP BVDV, whereas in the second experiment, the sperm concentration was 10^6/mL, and sperm and oocytes were also infected with CP or NCP BVDV. The number of sperm attached to the ZP and the fertilization rate were evaluated with fluorescence microscopy on the ZP of fertile and infertile oocytes. In the first experiment, compared to the control group (n = 97), oocytes infected with CP BVDV and incubated at the lower (10^5/mL) sperm concentration positively affected sperm attachment (n = 123) to the ZP of fertile oocytes ($P < 0.05$). In comparison with the control group (n = 115), sperm infected with CP BVDV negatively affected sperm binding (n = 93) to the ZP of infertile oocytes ($P < 0.05$). In the second experiment (10^6 sperm/mL), for both fertile and infertile oocyte groups, sperm attachment in the control group was very high and deemed uncountable. However, in treated groups, the number of sperm attached to the ZP was countable. Only sperm infected with CP BVDV negatively affected sperm binding capacity (n = 81) to the ZP of fertile oocytes ($P < 0.05$). Although CP and NCP BVDV significantly reduced the fertilization rate of oocytes incubated with a higher sperm concentration, with the lower sperm concentration, only NCP BVDV significantly diminished fertilization rate with contaminated sperm and oocytes ($P < 0.05$). In conclusion, this study supported the detrimental impacts of sperm or ooctyes infected with CP or NCP BVDV on sperm attachment to the ZP of bovine oocytes and on fertilization rate during bovine IVF

Garoussi MT, Mehrzad J. Effect of bovine viral diarrhoea virus biotypes on adherence of sperm to oocytes during in-vitro fertilization in cattle. Theriogenology. 2011 Apr 1;75(6):1067-75. Epub 2011 Jan

To ensure the freedom of embryos from pathogenic agents prior to embryo transfer (ET), a specific sanitary washing procedure has been recommended by the International Embryo Transfer Society (IETS). In the present study, the efficacy of removing the bovine viral diarrhea virus (BVDV) from cumulus-free matured oocytes at the stage of extruded first polar body (N = 240) was evaluated, using the IETS-recommended 10 sequential wash procedure, after exposure in vitro to BVDV type 2 (strain PA-131, 1 × 10(5.2) TCID(50)/mL for 1 h). In general, the percentage of contaminated oocytes was reduced ($P < 0.03$) after the first two washes. Nevertheless, after 10 washes, approximately 20% of oocytes still

remained infectious or contaminated with virus, as detected by the virus isolation test (VI) and quantitative reverse transcription PCR (qRT-PCR) of viral RNA (on average 13 copies/oocyte). Similarly, a higher percentage of positive washing fluid samples were detected in the first three washes (50-100%). The six subsequent washes had lower but variable proportions of fluid samples contaminated with infectious virus. We concluded that the standard washing procedure may not render all oocytes free from the infectious virus adhered to the zona pellucida (ZP), and application of an additional method of oocyte disinfection was warranted to ensure nontransmission of BVDV to recipients by embryos derived from infected oocytes.

Lalonde A, Bielanski A Efficacy of the International Embryo Transfer Society (IETS) washing procedure for rendering oocytes matured in vitro free of bovine viral diarrhea virus (BVDV). Theriogenology. 2011 Jul 15;76(2):261-6

The aim of this study was to study the effect of Bovine Viral Diarrhea Virus on the reproductive female tract by means of analyzing the ovarian follicular population of persistently infected (PI) heifers, and evaluating the performance of oocytes procured form those heifers in in vitro fertilization procedures. Seven BVDV PI Aberdeen Angus and British crossbred heifers ranging from 18 to 36 months of age were spayed and their ovaries used for viral isolation, microscopic examination, and in vitro fertilization procedures. Bovine Viral Diarrhea Virus was detected from the follicular fluid and sera of all PI heifers. Microscopic examination of the ovaries from PI heifers showed a significant drop in the number of follicles cortical regions, compared with controls. A comparative analysis of the stages of follicular development showed a significant decrease in the number of primordial and tertiary follicles in the cortical regions of ovaries from PI heifers. Viral antigen was detected by immunohistochemistry, and was widely distributed throughout the ovarian tissues. There were differences in the rate of cleavage and embryo development between oocytes obtained from the ovaries of control animals and PI heifers. Furthermore, two developed embryos obtained from oocytes from one of the PI heifers were positive to BVDV, as well as two media from in vitro fertilization (IVF) procedures. The results of this study demonstrate that BVDV PI heifers exhibit alterations in follicular population through of the early interaction between the virus and germ cell line affecting directly the mechanisms involved in the ontogenesis of the ovary.

González Altamiranda EA, Kaiser GG, Mucci NC, Verna AE, Campero CM, Odeón AC. Effect of Bovine Viral Diarrhea Virus on the ovarian functionality and in vitro reproductive performance of persistently infected heifers. Vet Microbiol.2013 Aug 30;165(3-4):326-32

As production of in vitro (IVP) bovine embryos steadily increases, the sanitary risk associated with IVP embryos remains a concern. One of the greatest concerns is how BVDV may be transmitted through IVP embryos. The objective of this study was to evaluate the effects caused by BVDV-1, BVDV-2 and Hobi-like virus exposure during in vitro maturation on embryo development and viral infection. Abittior-derived oocytes were randomly assigned for in vitro maturation with serial concentrations of BVDV-1 (3.12 x 10(2) - 2.50 x 10(3) TCID50/100 muL), BVDV-2 (6.25 x 10(1) - 5.20 x 10(2) TCID50/100 muL) or Hobi-like virus (1.90 x 10(2) - 1.58 x 10(3) TCID50/100 muL) for 22-24 h. After maturation, oocytes were fertilized and embryo cultured following standard in vitro procedures. Embryo development was evaluated and percentage of respective, positive BVDV degenerated and viable embryos

were evaluated by RT-qPCR. No concentration of BVDV-1 altered embryo development as measured by cleavage and blastocyst rates, compared to negative control group. However 100% of degenerated embryos and 50-100% of viable embryos tested positive for BVDV-1, depending on the viral concentration. BVDV-2 exposed oocytes had higher cleavage rates than the negative control group (60.2-64.1% vs 49.8%; P = 0.003-0.032). However, no difference was detected for blastocyst rates. In aadition, 100% of degenerated embryos and 20-50% of viable embryos tested positive for BVDV-2. Hobi-like virus treated oocytes had reduced cleavage rates for the three highest viral concentrations (33.3-38.0% vs 49.8% for negative controls; P </= 0.0010.014). Blastocyst rates were only reduced in the 7.9 x 10(2) Hobi-like virus concentration (6.9 +/- 0.9% vs 15.1 +/- 1.6%; P = 0.009), when calculated by oocyte number. 50-80% of degenerated embryos tested positive for Hobi-like virus. No viable embryos from the Hobilike virus treated oocytes tested positive. These results suggest that IVP embryos from BVDV1 and -2 infected oocytes develop normally, but carry the virus. However, Hobi-like virus infected oocytes had reduced cleavage and cause pre-implantation embryo loss, but viable embryos did not carry the virus. **da Silva Cardoso Pinto V., M.F. Alves, M. de Souza Nunes Martins, A.C. Basso, J.H. Tannura, J.H.F. Pontes, M.S. Lima, T. Garcia da Silva, L.H. Okuda, E. Stefano, A. Romaldini, D.R. Arnold and E.M. Pituco 2017. Effects of oocytes exposure to bovine diarrhea viruses BVDV-1, BVDV2 and Hobi-like virus on in vitro-produced bovine embryo development and viral infection. Theriogenology 97, 67-72. doi:10.1016/j.theriogenology.2017.04.028**

Structural changes in the zona pellucida (ZP) of bovine oocytes seem to modulate their interaction with various viral agents, facilitating the viral infection in in vitro production systems. To evaluate the susceptibility of bovine oocytes to noncytopathogenic bovine viral diarrhea virus (ncp-BVDV), cumulus–oocyte complexes were exposed to 107 tissue cultureinfective doses (TCID50)/mL of an ncp-BVDV strain during IVM (in vitro maturation). After that, cumulus cells and the ZP were removed by hyaluronidase and pronase treatment, respectively, and the percentages of oocytes with polar body were analyzed as a sign of nuclear maturation. After passage through cell culture, the virus was isolated from granulosa cells, ZP-free mature oocytes, and ZP-intact mature oocytes. These results were confirmed by reverse transcription–polymerase chain reaction. After consecutive washes, the virus remained associated with ZP-free oocytes, maintaining its replication and infectivity in permissive cells. Based on these findings, it is concluded that the classical viral isolation procedure has a predictive value to detect BVDV associated with ZP-free oocytes and that it was novelty demonstrated that both washing and trypsin treatment of oocytes were ineffective to remove BVDV infection. **González Altamiranda E.A., G.G. Kaiser, G.L. Ríos, M.R. Leunda and A.C. Odeón 2016. Interaction of bovine viral diarrhea virus with bovine cumulus–oocyte complex during IVM: Detection in permissive cells. Theriogenology 86, 1999-2003. doi:https://doi.org/10.1016/j.theriogenology.2016.06.020**

BVDV and BHV-1

The objective was to determine the effect of cryopreservation by conventional slow controlled cooling (0.5 degrees C/min) and by vitrification on the presence of bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) infectivity associated with frozen-thawed Day 7 bovine embryos. In this study, Day 7 embryos generated by in vitro fertilization (IVF) were exposed in vitro for 1.5h to BVDV (N=393) and BHV-1 (N=242) and

subsequently tested before and after cryopreservation for the presence of infectivity. Exposure of embryos to viral agents resulted in 72% of them infected prior to cryopreservation. Stepwise exposure of embryos to cryoprotectants, as well as their removal, substantially reduced the proportion of contaminated embryos (46% vs. 72%, P<0.05). Overall, both freezing methods reduced the percentage of infectious embryos compared with that of embryos similarly exposed to viruses but not cryopreserved (31% vs. 72%, respectively; P<0.001). The percentage of embryos with infectious viruses was not significantly higher after vitrification than after slow cooling (38% vs. 22%). In addition, after cryopreservation, a higher percentage (P<0.002) of embryos exposed to BHV-1 (42%) remained infectious than did embryos exposed to BVDV (24%). In conclusion, cryopreservation reduced the proportion of infected embryos but did not render all of them free from infectious pathogens.

Bielanski A, Lalonde A. Effect of cryopreservation by slow cooling and vitrification on viral contamination of IVF embryos experimentally exposed to bovine viral diarrhea virus and bovine herpesvirus-1. Theriogenology. 2009;72(7):919-25.

Bluetongue virus serotype 8

In 2006 and 2007, Bluetongue virus serotype 8 (BTV-8) caused devastating outbreaks in Northern Europe; the outbreaks were controlled in 2008 and 2009 by an international vaccination policy. Remarkably, BTV-8 differs from other serotypes in that it spread transplacentally (De Clercq K et al. 2008 Transboundary and Emerging Diseases 55, 352-359). Apart from the transplacental spreading, a significant increase in the incidence of abortions was reported in Belgium (Meroc E et al. 2009 Transboundary and Emerging Diseases 56, 39-48). The aim of the present study was to investigate the susceptibility of bovine-hatched, in vitro-produced blastocysts to BTV-8. A total of 1390 immature bovine oocytes were matured and fertilized in vitro. Presumed zygotes (n = 1148) were denuded 24 h post-insemination and cultured in 50-µL droplets of modified synthetic oviduct fluid (SOF) medium with 10% fetal calf serum (tested negative for BTV antibodies) at 39.0°C in 5% CO2, 5% O2, and 90% N2. At 7 days post-insemination (dpi), blastocysts were grouped to enhance hatching. For virus incubation, BTV-8 Bel 2006/2 from Veterinary and Agrochemical Research Centre (VAR, Brussels, Belgium) was used. At 8.5 dpi, hatched embryos were placed in 800µL of minimum essential medium (MEM) containing 103.8 50% tissue culture infectious doses (TCID50) of BTV-8 and incubated for 1 h at 39°C in an atmosphere of 5% CO2 in air. At the same time, 2 groups of hatched control embryos were incubated under the same circumstances in 800 µL of SOF and 800 µL of MEM, respectively. After infection, all embryos were washed according to IETS guidelines with the exception that they were not zona pellucida intact and cultured in new SOF. At 48, 60, 72, and 96 h post-infection (hpi), one-fourth of the embryos of each group were fixed in 4% paraformaldehyde for 12 to 24 h and subsequently stained for BTV-8 with double immunofluorescent staining using a BTV-8 monoclonal antibody (8A3B.6, ID-Vet, Montpellier, France). All control embryos (CTRL and MEM) were negative for BTV-8 virus antigen at all time points. At 48 hpi, only 1 out of 7 infected embryos was positive for virus antigen (in all its cells). At 60 hpi, all remaining embryos (n = 6) were negative, whereas at 72 hpi and 96 hpi all embryos had 25% to 100% BTV-8-positive cells (n = 6 at 72 hpi and n = 7 at 96 hpi). Furthermore, 1 embryo at 72 hpi and 2 embryos at 96 hpi showed morphological signs of degeneration. This study has showed for the first time that hatched in

vitro-produced blastocysts are susceptible for BTV-8 virus infection and replication in vitro. The relatively long time between virus infection and detection of viral antigen is in accordance with the slow replication cycle of the virus. Further research is ongoing to investigate the importance of BTV-8 infection in early embryonic death.

Vandaele L, Wesselingh W, De Clercq K, Nauwynck H, and Van Soom A. Susceptibility of bovine-hatched blastocysts to bluetongue virus serotype 8 infection. Reproduction, Fertility and Development. 2010; 22(1): 254 (abstr.).

Bluetongue virus serotype 8 (BTV-8), which caused an epidemic in ruminants in central Western Europe in 2006 and 2007, seems to differ from other bluetongue serotypes in that it can spread transplacentally and has been associated with an increased incidence of abortion and other reproductive problems. For these reasons, and also because BTV-8 is threatening to spread to other parts of the world, there is a need for more information on the consequences of infection during pregnancy. The aim of the present study was to investigate whether hatched (i.e. zona pellucida-free) in vitro produced bovine blastocysts at 8-9 days post insemination are susceptible to BTV-8 and whether such infection induces cell death as indicated by apoptosis. Exposure of hatched in vitro produced bovine blastocysts for 1 h to a medium containing 103.8 or 104.9 TCID50 of the virus resulted in active viral replication in between 25 and 100% of the cells at 72 h post exposure. The infected blastocysts also showed growth arrest as evidenced by lower total cell numbers and a significant level of cellular apoptosis. We conclude from this in vitro study that some of the reproductive problems that are reported when cattle herds are infected with BTV-8 may be attributed to direct infection of blastocysts and other early-stage embryos in utero.

Vandaele L, Wesselingh W, De Clercq K, De Leeuw I, Favoreel H, Van Soom A, Nauwynck H. Susceptibility of in vitro produced hatched bovine blastocysts to infection with bluetongue virus serotype 8. Vet Res. 2011 Jan 24;42(1):14.

Chlamydia abortus

The objectives of this study were to determine (i) whether *Chlamydia (C.) abortus* would adhere to the intact zona pellucida (ZP-intact) of early in vitro produced bovine embryos; (ii) whether the bacteria would adhere to the embryos (ZP-free) after in vitro infection; and (iii) the efficacy of the International Embryo Transfer Society (IETS) washing protocol.

The experimentation was made twice. For each replicate 100 (8-16-cell) bovine embryos produced in vitro were randomly divided into 10 batches. Height batches (4 ZP-intact and 4 ZP-free) of 10 embryos were incubated in a medium containing 4 x 10^7Chlamydia/ml of AB7 strain. After incubation for 18 h at 37 °C in an atmosphere of 5% CO_2, the embryos were washed in accordance with the IETS guidelines. In parallel, two batches (1 ZP-intact and 1 ZP-free) of 10 embryos were subjected to similar procedures but without exposure to *C. abortus* as a control group. The 10 washing fluids from each batch were collected and centrifuged for 1 h at 13,000xg. Each batch of washed embryos and each wash pellets were tested using PCR.

C. abortus DNA was found in all ZP-intact and ZP-free batches of 10 embryos after 10 successive washes. For ZP-intact infected embryos, *Chlamydia* DNA was also detected in all 10 wash baths for two batches (2/8) of embryos, whereas for ZP-free infected embryos, *Chlamydia*-DNA was detected in all 10 wash baths for 6/8 batches of embryos. In contrast,

none of the embryos or their washing fluids in the control batches was DNA positive. The bacterial load for batches of 10 embryos after the 10 wash baths was significantly higher for batches of ZP-free embryos (20.7 ± 9 x 10^3 bacteria/mL) than for batches of ZP-intact embryos (0.47 ± 0.19 x 10^3 bacteria/mL).These results demonstrate that *C. abortus* adheres to the ZP as well as the early embryonic cells of in vitro produced bovine embryos after in vitro infection, and that the standard washing protocol recommended by the IETS fails to remove it. **Pellerin, J.L., Oseikria, M., Moreno, D., Rodolakis, A., Vorimore, F., Laroucau, K., Bruyas, J.F., Roux, C., Michaud, S., Larrat, M. and Fieni, F., 2019. Risk of Chlamydia abortus transmission via embryo transfer using in vitro produced early bovine embryos. Theriogenology, 126, pp.114-120.**

Coxiella burnetii

Coxiellaburnetii, an obligate intracellular bacterium of worldwide distribution, is responsible for Q fever. Domestic ruminants are the main source of infection for humans. The objectives of this study were to determine (i) whether *C. burnetii* would adhere to the intact zona pellucida (ZP-intact) of early *in vitro* produced bovine embryos; (ii) whether the bacteria would adhere to or infect the embryos (ZP-free) after *in vitro* infection; and (iii) the efficacy of the IETS washing protocol.

One hundred and sixty, 8 to 16-cell bovine embryos produced *in vitro,* were randomly divided into sixteen batches of 10 embryos. Twelve batches *(8 ZP-intact and 4 ZP-free)* were incubated in a medium containing *C. burnetii* CbB1 (IASP, INRA Tours). After 18 h of incubation at 37°C and 5% CO_2 in air, the embryos were washed in 10 successive baths of a phosphate buffer saline (PBS) and 5% FCS solution in accordance with the IETS guidelines. In parallel, four batches (2 ZP-intact and 2 ZP-free) were subjected to similar procedures but without exposure to *C. burnetii* to act as controls. The 10 washing fluids from each batch were collected and centrifuged for 1 hour at 13,000 x g. The embryos and wash pellets were tested using C-PCR.

C. burnetii DNA was found in all ZP-intact and ZP-Free embryos after 10 successive washes. It was also detected in the first 4 washing fluids for ZP-intact embryos and in the 10^{th} wash fluid for two of the four batches of ZP-free embryos. In contrast, none of the embryos or their washing fluids in the control batches were DNA positive.

These results demonstrate that *C. burnetii* adheres to and/or penetrates the early embryonic cells as well as the ZP of *in vitro* bovine embryos after *in vitro* infection, and that the standard washing protocol recommended by the IETS for bovine embryos, failed to remove it. The persistence of these bacteria after washing makes the embryo a potential means of transmission of the bacterium during embryo transfer from infected donor cows to healthy recipients and /or their offspring. Further studies are required to investigate whether enzymatic and/or antibiotic treatment of bovine embryos infected by *C. burnetii* would eliminate the bacteria from the ZP and to verify if similarly results are obtain with *in vivo*-derived embryos.

Alsaleh A, , Fieni F*, Moreno D, Rousset E, Tainturier D, Bruyas JF, Pellerin JL. Risk of *Coxiella burnetii* transmission via embryo transfer using *in vitro* early bovine embryos. Theriogenology 2014, 81:849-853.

Enzootic hemorrhagic disease

Infectious viruses bind more tenaciously to the ZP of in vitro than in vivo derived embryos. In contrast to in vivo derived embryos, trypsin treatment of IVP embryos is not completely effective for viral elimination from zonae. Our working hypothesis was that treatment of IVP embryos with protease (type XIV, Sigma) could effectively remove EHDV-2 without sereverly compromising embryonic viability. Bovine oocytes were matured (IVM), fertilized and cultured in completely defined in vitro conditions. For virus exposure, oocytes were incubated with EHDV-2 (10_6TCID$_{50}$/mL) during IVM. Presumptive zygotes (Pzs) were washe d5x in PBS + 0.3 % polyvinal alcohol (PVA), 1 x in 0.1 % protease XIV (4 units per ml) or 0.25 % trypsin and an additional 5x in PBS-PVA. Embryos exposed to EHDV-2 were taken at the morula (M) stage for virus isolation. PZs were able to withstand protease treatment for 45 seconds without compromising ($p>0.05$, chi-square) the proportion that cleaved (C, 67.7 %) and became blastocysts (B, 18.5 %) compared to controls (C, 43.4 %; M, 77.3 %; B 25.8 %). Exposure to EHDV-2 prevented cumulus expansion and reduced ($p<0.05$) development (C, 43.4%; M, 14.2 %) when compared to controls (C, 76.8%; M 47.0 %). Proportions of infected embryos were reduced after a 45 sec protease treatment (20.0 %) versus positive controls (63.6 %) and a 60 second trypsin treatment (66.7%). Data show EHDV-2 exposure during IVM to be detrimental to embryonic development. Also, IVP zygotes can withstand exposure to protease type XIV in conditions that reduced (p,0.05) infectious EHDV-2 associated with their zonae pellucidae. **(Dinkins MB et al. Biol Reprod 1999; 60 suppl 1:abstract 268)**

Photoinactivation was employed to eliminate EHDV-2 from in vitro produced bovine embryos experimentally exposed to this virus. Immature oocytes were matured, fertilized, and cultured in chemically defined conditions. All treatments were performed on zygotes. Developmental potential of zygotes and cell numbers of resulting hatched blastocysts were assessed after exposure to a 1 mW helium neon laser (633 nm, red) for 1, 5, 10, and 15 min; the photosensitive chemicals hematoporphyrin (15 microM) and hypericin (1 and 10 microM) for 15 min; a combination of 10 microM hypericin and laser light for 1, 3, or 5 min; and a combination of 15 microM hematoporphyrin and laser light for 1, 2, or 3 min. There were no significant differences among proportions of embryos developing or cell numbers after treatment with or without exposure to laser light alone for up to 10 min. No differences were observed after exposure of zygotes to photosensitive chemicals alone. Exposure to 10 microM hypericin and 5 min of laser light or 15 microM hematoporphyrin and 2 min of laser light compromised zygote developmental potential. After exposure to 10(6) TCID50/mL EHDV-2 for 90 min groups of 10 zygotes were exposed to 10 microM hypericin or 15 microM hematoporphyrin and laser light to inactivate the virus. Hematoporphyrin was effective with 3 min light exposure at reducing the percentage of EHDV-2 contaminated zygote pools (16.7%) as compared to EHDV-2 exposed pools without treatment (88.9%) but hematoporphyrin + 1 min light was ineffective. Hypericin + 3 min light provided an intermediate effect (55.6%). **(Dinkins MB et al. Theriogenology 2001;55:1639-1655)**

Infectious viruses bind more tenaciously to the zonae pellucidae of in vitro produced bovine embryos than to zonae of in vivo derived embryos. Currently, the International Embryo Transfer Society recommends that all in vivo derived embryos be subjected to a rigorous washing procedure in combination with exposure to trypsin to remove viruses adherent to the zonae. In contrast to in vivo derived embryos, this method is not effective for disinfecting in vitro produced embryos. Our hypothesis was that a more potent, non-specific protease from Streptomyces griseus (S. griseus) would provide a more effective treatment

for virus removal from in vitro produced bovine embryos. Bovine oocytes were matured, fertilized, and cultured in completely defined in vitro conditions. Zygotes were washed according to the procedure outlined by the International Embryo Transfer Society, replacing trypsin with the experimental protease. Experimental incubations were with 0.1% (4 units/ml) protease for 0, 30, 45, 60 and 75s intervals. Embryos were able to withstand exposure to this enzymatic treatment for only 45s before their developmental potential was significantly reduced; 60s exposure was detrimental ($P<0.05$). Oocytes were exposed to epizootic hemorrhagic disease virus serotype 2 (EHDV-2, 10(6) TCID(50)/ml) during in vitro maturation. Resulting zygotes were washed according to the International Embryo Transfer Society procedure and either exposed to trypsin or protease. Exposure to EHDV-2 prevented cumulus expansion and markedly reduced embryonic development ($P<0.05$). There were no differences in development among virus exposed groups receiving no treatment or treatment with trypsin or protease. However, proportions of infected embryos were reduced after protease treatment versus positive controls and trypsin treated embryos. **(Dinkins MB et al. Anim Reprod Sci 2001;65:205-213)**

Foot-and-mouth disease virus

The objective was to determine if FMD virus interacts with IVP bovine embryos. Six trials were performed in which developed (n=256) or degenerated (n=260) 7 d embryos were incubated with FMDV (2 x 10_7 $TCID_{50}$/ml) for 1, 2 or 4 h, washed according to IETS standards for in vivo derived embryos, ground and assayed for virus on cell culture. In addition PCR and cell culture were used to assay first and second washes and pooled 8_{th} - 10_{th} washes. FMDV was found in all 1_{st} and 2_{nd} washes except one 2_{nd} wash, but no virus was found in any pooled 8_{th} - 10_{th} washes. FMDV was isolated from 3/3 groups of developed and 2/3 groups of degenerated embryos after 1 h viral exposure; FMDV was isolated from 0/2 groups of developed and 1/2 groups of degenerated embryos after 2 h of viral exposure; FMDV was isolated from 1 group of developed and 1 group of degenerated embryos after 4 h of viral exposure. The authors concluded that 7 d IVP bovine embryos can retain FMDV after washing. (**Marquant-Le Guienne B et al. Theriogenology 1998;50:109-116**)

The trade of livestock or their products between nations requires informed decisions regarding the risks they pose of carrying infectious agents such as foot and mouth disease virus (FMDV). Although the transmission pathway for FMDV varies, a matter of recent concern in the USA is that it might gain entry via imported cloned embryos. To examine this, a quantitative risk assessment model was developed to determine the scenarios (with mathematical probabilities) that could lead to the introduction and maintenance of FMDV via the importation of cloned bovine embryos into the USA. Using @RISK software and by setting the Monte Carlo simulation at 50,000 iterations, the probability of introducing FMDV into the USA via cloned bovine embryos was estimated to be $3.1X10^{-7}$. Given the current cloning protocol, and assuming the importation of 250-1,700 (mean = 520) cloned embryos per year, the expected number of infected embryos ranges from $1.1X10^{-7}$–$4.4X10^{-4}$ (mean = $1.6X10^{-4}$) per year. Based on our critical pathway analysis, the risk that FMD virus could enter the USA by this route is extremely low. **Asseged B, Tameru B, Nganwa D, Fite R, Habtemariam T (2012) A quantitative assessment of the risk of introducing foot and mouth disease virus into the United States via cloned bovine embryos. Rev Sci Tech 31: 761-775.**

Campylobacter fetus (C. fetus)

Cumulus-oocyte complexes were matured for 24h. and then incubated for 18h. with swim-up prepared sperm with or without 1 x 10_8 *C. fetus* (sub-sp. *venerealis*) organisms/ml. Thereafter the cumulus cells were removed and presumptive zygotes were co-cultured for 8 days with bovine oviduct epithelial cells. Antibiotics were not added to the culture media. From 224 oocytes in the infected group there were 119 (53%) that cleaved and 41 (18%) that developed to blastocyst, whereas from the 212 oocytes in the control group 125 (59%) cleaved and 51 (24%) became blastocysts. These results were not significantly different and thus it appeared that the presence of *C.fetus* had no detrimental effect on early embryonic development (**Bielanski et al, Theriogenology 1994;41:163 abstr)**

The objectives of this study were to examine the effect of *Campylobacter fetus* subsp. *venerealis* (Cfv) on the in vitro fertilization (IVF) of bovine ova and to detect Cfv associated with IVF produced embryos. Cumulus-oocyte complexes were matured for 24 h in TCM-199 and were fertilized with swim-up spermatozoa prepared in modified Tyrode's medium supplemented with BSA (6mg/ml), and heparin (15 µg/ml)with or without 1 X 10_8 Cfv organisms/ml. Thereafter, zygotes were cultured for 8 days in TCM-199 medium containing oviductal epithelial cells. In comparing ova inseminated with spermatozoa either treated or untreated with Cfv, no significant differences were observed in the percentages of: 1. Ova fertilized (72% vs. 76%); 2. Fertilized ova which cleaved (52% vs. 60%); or 3. Embryos that developed to the blastocyst stage (31% vs. 41%). Motile Cfv were observed microscopically in the fertilization medium 48 h after insemination. A high proportion of cumulus-free uncleaved and cleaved oocytes washed 10 times 24 h after insemination tested positive for the presence of Cfv. After 7 days of in vitro culture, however, embryos were free from the organism as determined by culture. However, in some embryos the presence of Cfv DNA was detected using polymerase-chain-reaction technology. (**Bielanski et al, Reprod Dom Anim 1994;29:488-493**)

Leptospira spp.

Leptospira borgpetersenii serovar *hardjo* type hardjobovis could not be isolated from IVF embryos that were generated from ova harvested from experimentally infected donor heifers and cultured in commercial Menezo B2 medium containing low levels of penicillin and streptomycin. Recipient heifers (n=14) to which embryos cultured in Menezo B2 medium were transferred, did not develop antibody titers to *Leptospira*, and this organism was not isolated from the resulting fetuses (n=5). Similarly, the organism could not be isolated from IVF embryos that were produced from oocytes exposed in vitro to *Leptospira* and cultured in Menezo B2 medium or TCM-199 medium supplemented with penicillin and streptomycin.
In contrast, leptospires were isolated from IVF embryos that were produced from in vitro exposed oocytes cultured in TCM-199 medium free of antibiotics. The sequential washing procedure that was used with medium free of antibiotics did not render infected embryos free of the pathogenic microorganisms. Oocytes exposed to *Leptospira* in vitro were not transferred into the recipients. The presence of serovar *hardjo* in the IVF system had no detrimental effect on fertilization rates or on embryonic development to the blastocyst stage. The study showed

that it was possible to obtain transferrable embryos from oocytes recovered from infected donors and from oocytes exposed to *Leptospira* in vitro. The authors suggest that the use of culture medium supplemented with antibiotics would be advisable to prevent risk of transmission. (**Bielanski and Surujballi, Theriogenology 1996;46:45-55**)

The association of *Leptospira borgpetersenii* serovar *hardjo* type *hardjobovis* with bovine IVF embryos after artificial exposure was evaluated with scanning electron microscopy (SEM) and transmission electron microscopy (TEM). After exposure of morulae with intact ZP to the organism for 24 h, they were washed using procedures conforming to IETS standards and examined by SEM. Typical leptospires were observed with SEM on the surface and in pores of the ZP. With TEM, cross and longitudinal sections of leptospires were observed in the matrix and channels of the ZP. Also, they were observed in the perivitelline and intercellular spaces, on the vitellus and in the embryonic cells. Damage to membranes and cytoplasm were observed in some embryos that were penetrated by the leptospires. (**Bielanski A et al. Can J Vet Res 1998;62:234-236**)

Lumpy skin disease

Lumpy skin disease (LSD) is an important transboundary animal disease of cattle with significant economic impact because of the implications for international trade in live animals and animal products. LSD is caused by a *Capripoxvirus*, LSD virus (LSDV), and results in extensive hide and udder damage, fever and pneumonia. LSDV can be shed in semen of infected bulls for prolonged periods and transmitted venereally to cows at high doses. This study examined the effects of LSDV in frozen-thawed semen on in vitro embryo production parameters, including viral status of media and resulting embryos. Bovine oocytes were harvested from abattoir-collected ovaries and split into three experimental groups. After maturation, the oocytes were fertilized in vitro with frozen-thawed semen spiked with a high (HD) or a lower (LD) dose of LSDV, or with LSDV-free semen (control). Following day 7 and day 8 blastocyst evaluation, PCR and virus isolation were performed on all embryonic structures. After completing sufficient replicates to reach 1,000 inseminated oocytes, further in vitro fertilization (IVF) runs were performed to provide material for electron microscopy (EM) and embryo washing procedures. Overall, in vitro embryo yield was significantly reduced by the presence of LSDV in frozen-thawed semen, irrespective of viral dose. When semen with a lower viral dose was used, significantly lower oocyte cleavage rates were observed. LSDV could be detected in fertilization media and all embryo structures, when higher doses of LSDV were present in the frozen-thawed semen used for IVF. Electron microscopy demonstrated LSDV virions inside blastocysts. Following the International Embryo Transfer Society washing procedure resulted in embryos free of viral DNA; however, this may be attributable to a sampling dilution effect and should be interpreted with caution. Further research is required to better quantify the risk of LSDV transmission via assisted reproductive procedures.

Annandale, C.H., Smuts, M.P., Ebersohn, K., Du Plessis, L., Thompson, P.N., Venter, E.H. and Stout, T.A., 2019. Effect of using frozen–thawed bovine semen contaminated with lumpy skin disease virus on in vitro embryo production. ***Transboundary and emerging diseases*****, *66*(4), pp.1539-1547. https://doi.org/10.1111/tbed.13179**

Mycoplasma spp

Frozen-thawed bovine semen contaminated with *Mycoplasma bovis (M. bovis)* or *Mycoplasma bovigenitalium (M. bovigenitalium)* at either a high (10_6 CFU/mL) or low (10_4 CFU/mL) concentration was used for bovine oocyte insemination. The resulting embryos were washed 10 times as recommended by the International Embryo Transfer Society (IETS) prior to isolation of agent. A total of 1494 oocytes was inseminated with contaminated sperm cells and 855 oocytes with uninfected control semen. There was a significantly higher proportion of embryos that developed to the blastocyst stage in control than in the mycoplasma exposed groups ($P<0.05$). Isolation of motile spermatozoa by swim-up procedure prior to insemination did not render sperm cells free of *Mycoplasma* spp. Although *M. bovis* was isolated from all washed embryos after the high exposure level, it was found in only 60% of the samples after the low exposure level. In contrast, *M. bovigenitalium* was isolated from 70 and 12% of washed embryos exposed to the high and low levels of microorganism, respectively. Using scanning electron microscopy, both microorganisms were detected in association with the surface of zona pellucida-intact embryos and with sperm cells. These results indicate that mycoplasmas present in semen can be transmitted through the IVF system and infect embryos. Furthermore, the experiments showed that supplementation of culture media with standard antibiotics and washing embryos as recommended by IETS were not effective in rendering IVF embryos free from *M. bovis* and M. bovigenitalium. **(Bielanski A et al, Theriogenology 2000;53:1213-1223.)**

Several *Mycoplasma* species may adversely affect bovine spermatozoa viability and embryo development. *Mycoplasma mycoides* ssp. *mycoides* largecolony (LC) has been isolated from naturally aborted bovine fetuses and from bull semen. The objective of this study was to evaluate whether *M. mycoides* ssp. *mycoides* LC contaminated bovine ejaculates could (i) impair in vitro fertilizing ability of bull spermatozoa, (ii) impair embryo development, and (iii) evaluate potential spread by reproductive technologies. In the present study, spermatozoa of 10 fertile bulls were contaminated with *M. mycoides* ssp. *mycoides* LC, at a final concentration of 1.5 million CFU/ml and incubated for 60 min before evaluating spermatozoa motility and acrosome reaction inducibility with calcium ionophore. In addition, in vitro contaminated semen of a bull previously shown to have a good in vitro fertilizing ability, was used in an IVF procedure. Embryo development stage on Day7 of culture was evaluated. Spermatozoa and embryos at morula and blastocyst stages were routinely processed for transmission electron microscopy observation. Both mean total and progressive motility decreased ($P < 0.01$) upon spermatozoa incubation with *Mycoplasma*. Onehour incubation with calcium ionophore increased the percentage of acrosomereacted spermatozoa, although *Mycoplasma* contamination reduced calcium ionophore treatment efficacy ($P < 0.05$). Ultrastructurally, *Mycoplasma* microorganisms appeared as moderately electrondense sphereshaped particles, adhering to cell membranes. Sperm midpiece sections showed numeric aberrations of the central singlets such as nine + zero or nine + one of the axonemal complex. Further morphological abnormalities included partial or total absence of dinein arms and radial fibers, with lack of the bridge and the central ring in 35.00 +/ 4.20% of contaminated cells, whereas these abnormalities were not observed in uninfected ones. The IVF trials showed that

twofour cell blocks were higher (P < 0.05) in the infected group. Ultrastructure of Day7 contaminated embryos showed *Mycoplasma* particles adhering and infiltrating the outer layer of the zona pellucida. Our investigations suggest that *M. mycoides* ssp. *mycoides* LC contaminating the bovine ejaculate induced adverse effects on in vitro spermatozoafertilizing ability and embryonic development. Some satisfactory quality transferable embryos could be produced in contaminated IVF systems. This could imply a potential transmission of this microorganism through reproductive technologies. **(Sylla L et al, Anim Reprod Sci. 2005 Jan;85:81-93.)**

Neospora caninum

Embryos were produced by in vitro fertilization of IVM oocytes that had originated from an abattoir. After fertilization, presumptive zygotes were cultured for 7 days on monolayers of vero cells in 0.5 mL of synthetic oviductal fluid containing approximately 10_4/mL tachyzoites (field strain). Cultures were examined daily for cytopathology and development and compared to unexposed control cultures. Some of the resulting ZP-I embryos and ZP-free blastocysts were washed briefly, fixed in 3 % glutaraldehyde and examined by scanning and transmission electron microscopy (SEM & TEM). Also, some ZP-I morula and blastocysts that were cultured with tachyzoites were placed in groups of 10 on filters that were flushed with 500 mL of phosphate-buffered saline and then washed 10 times (each at 1:200 dilution). After washing, groups of 2 embryos were placed on monolayers of vero cells (both exposed and control groups). After 10 days of culture, control and exposed culture samples were tested for *N Caninum* DNA using PCR. No morphological differences were noted in embryos developed in the presence of tachyzoites. During the culture period motile parasites were noted as was progressive degeneration of vero cells. Exposure of hatched blastocysts to tachyzoites resulted in collapse of the embryos and degeneration of the trophoblastic cells within 3 to 5 days. *N caninum* was observed by TEM in trophoblastic cells of 2 or 6 hatched blastocysts and SEM revealed single or small groups of parasites on the surface of some unwashed ZP-I embryos (2 of 35). *N caninum* was not detected by light microscopic exam or by PCR in any samples collected from cultures containing embryos that had been thoroughly washed as described above. The study demonstrated that the ZP protects from *N caninum* invasion. **(Bielanski A et al. Vet Rec 2002;150:316-318)**

In this abstract was reported the embryonic development of preimplantation embryos and their sanitary status after experimental exposure to tachyzoites of *Neospora caninum* followed by processing the embryos according to the sanitary recommendations of the IETS. In vitro derived embryos were produced using the standard methodology. Presumptive zygotes were transferred to culture dishes with monolayers of Vero cells in SOF culture medium containing tachyzoites (10_4/ml, from aborted fetus) and incubated for 7 days under silicone oil at 38.5_oC in 5 % co_2, 5 % oxygen and 90 % nitrogen. The resulting embryos were examined with a scanning electron microscope (SEM) and a transmission electron microscope (TEM). In a further experiment, embryos at morula and blastocyst stages after incubation with tachyzoites as described above were placed onto an embryo “Em Con” filter in groups of 10 and washed with 500 ml of PBS followed by an additional 10 sequential washes in 2 ml of PBS (each at 1:200 dilution). Washed embryos in groups of two were placed onto Vero cell monolayers grown in tissue culture plates. Cultures were examined daily for cytopathic effect and the presence or absence of tachyzoites. Finally, control and experimental culture samples were tested for presence of the *N caninum* DNA by PCR. No observable morphological effect was

detected in ZP-I embryos developed in the presence of *N caninum*. The percentages of cleaved zygotes and blastocysts resulting from culture of experimental and uninfected control groups were 56 % (n=216) and 28 % (n=34) compared to 59 % (n=168) and 29 % (n=29), respectively (P>0.05, chi-square test). Exposure of hatched blastocysts to tachyzoites resulted in degeneration of trophoblastic cells and death of embryos. *Neospora caninum* was not detected by microscopic examination nor by PCR in any samples collected from cultures containing embryos after the application of multiple washing. Scanning electron microscopy revealed a single or small groups of parasites on the surface of some unwashed ZP-I embryos. **(Bielanski et al. Theriogenology 2002;57:568 abstr.)**

Here, we studied the potential of Neospora caninum tachyzoites to infect heifers when administered in utero by artificial insemination via contaminated semen. Eighteen primiparous cyclic heifers were hormonally synchronized and artificially inseminated. Nine of them, which were inseminated with semen containing 10_7 live N. caninum NC1 isolatetachyzoites, reacted with seroconversion and a specific IFNgamma response. Moreover, N. caninum DNA was demonstrated by a nestedPCR in the blood of all nine heifers and in brain, lungs, liver and uterine horn of several of them. In contrast, nine heifers inseminated with tachyzoitefree semen developed no antibody or IFNgamma responses, and no parasite DNA was detected in blood or organs. At necropsy, viable embryos were detected in one and six of the infected and noninfected heifers, respectively. No specific Neospora DNA was detected in any of the embryos. This study provides evidence that intrauterine inoculation via contaminated semen cause N. caninum infection in cattle. **Serrano E et al, Vet Parasitol 2006;135:197-203.**

Neospora caninum, an intracellular protozoon, causes encephalomyelitis in dogs (Bjerkas I et al. 1984 Zentralblat fur Parasitenkunde 70, 271-274). For the past decade, neosporosis has been a main cause of abortion in dairy cattle worldwide (Anderson M et al. 2000 Anim. Reprod. Sci. 60-61, 417-431; Dubey JP 2003 Korean J. Parasitology 41, 1-16). Vertical transmission has been indicated as an important way of spreading neosporosis (Hall CA et al. 2005 Vet. Parasitology 31, 231-41); thus, we investigated whether the protozoon could be transferred by embryo production techniques. Blood samples were collected from 92 dairy cows with history of reproductive failure and abortion within the previous 90 days at 7 dairy farms in Tizayuca, Mexico. For serology evaluation, a commercial indirect ELISA kit (Civtest Bovis Neospora, Laboratories Hipra S.A, Girona, Spain), yielded 46.74% (43/92) positive results, 46.74% (43/92) negative results, and 6.52% (6/92) suspicious to N. caninum infection. Thirteen positive cows were chosen for uterine flush (UF), ovum pickup (OPU), and a blood sample collection. Lymphocytes from blood and cells within the UF and OPU collection fluids were collected after centrifugation and DNA was extracted. All samples were tested for the presence of N. caninum by PCR, using primers and protocols that amplified a 275-bp fragment of the genomic region (5-GGGTGAACCGAGGGAGTTG-3 and 5-CCTCCCAATGCGAACGAAA-3). The N. caninum vaccine (Bovilis® NeoGuard, Intervet, Santiago Tianguistenco, Mexico) was used as a positive control and water as a negative control. Uterine flush could not be obtained from 1 cow. From 13 cows seropositive to N. caninum, only 38% were positive to PCR from blood lymphocytes. In contrast, PCR amplification was obtained from OPU cell sediment in 92.31% (12/13) and in 33.33% (4/12) of UF. Of these 12 OPU- and 4 UF-positive samples, only 5 and 3 of their corresponding blood lymphocytes were positive. Our results using uterine and follicular fluid were contradictory to those

published by Moskwa et al. (2008 Vet. Parasitology 158, 370-375) where oocytes and embryos were evaluated. These results indicate that N. caninum is present in the ovary and uterine lumen of the cows, suggesting a possible risk of neospora transmission during oocyte and embryo collection and transfer techniques.

Marques AF, Ortiz CG, Lima MR, Zanella EL, Rangel L, Morales E, and Gutierrez CG. Detection by polymerase chain reaction of Neospora caninum from ovum pickup and uterine flushing fluids from dairy cattle in Mexico. Reproduction, Fertility and Development. 2010; 22(1): 252 (abstr.).

Trichomonas fetus

Trichomonas fetus (*T. fetus*), a world-wide distributed parasitic protozoan, is a cause of infertility and abortion. There is no documented information on the susceptibility of bovine embryos to the parasite. Here we report on the embryonic development of preimplantation embryos and their sanitary status after exposure to *T. fetus* during fertilization. Briefly, in vitro matured COC were fertilized with approximately $1X10^6/ml^{-1}$ motile frozen-thawed sperm cells obtained after the swim up procedure in 50 µl of modified Tyrode medium (TALP). In the experimental group, *T. fetus* at approximately $1X10^5/ml^{-1}$ (ATCC strain) was added to the fertilization droplets. After 18 h, presumptive zygotes were freed of cumulus cells, transferred to 500 µl of modified synthetic oviductal fluid (SOF) and incubated for 7 days under silicone oil at 38.5^oC in 5 % CO_2, 5 % oxygen and 90 % nitrogen [Holm et al. Theriogenology 1999;52:683-700]. Cultures were examined daily for the presence or absence of motile parasites. After multiple washing (trypsin not included), as recommended by the IETS, the resulting embryos were examined with a light microscope, a scanning electron microscope (SEM) and a transmission electron microscope (TEM) for the presence of *T. fetus*. The percentages of cleaved zygotes, blastocysts and hatched embryos resulting from culture of experimental and uninfected control groups were 57 % (n=140), 35 % (n=38) and 46 % (n=13) compared to 60 % (n=151), 32 % (n=29) and 45 % (n=13) respectively (P>0.05, Chi-square test). No motile parasites were observed in SOF culture drops beyond 72 h post IVF. Exposure of hatched blastocysts (n=25) to *T. fetus* did not result in degeneration of trophoblastic cells or death of embryos. *Trichomonas fetus* was not detected in embryonic cells of ZP-intact or hatched embryos by TEM (n=10). However, SEM revealed a single or small groups of parasites on the surface of unwashed, as well as washed, ZP-intact (n=15) and ZP-free (n=15) embryos. It is concluded that *T. fetus* has no effect on the development of IVF embryos and the potential risk of transmission of trichomoniasis is unlikely due to the limited survival of the parasite in IVF culture conditions. **(Bielanski A, et al. Theriogenology 2003;59:381 abstr.)**

Tritrichomonas foetus, a world-wide distributed parasitic protozoan is a cause of infertility and abortion. There is no documented information on the susceptibility of bovine embryos to the parasite. To determine the effect of T. foetus on fertilization and embryonic development of preimplantation bovine embryos, we added approximately 10^4/ml or 10^6/ml T. foetus (Belfast strain) to sperm cells and oocytes prior to in vitro fertilization (IVF) or to presumptive zygotes 24 hours post-fertilization. Light and scanning electron microscopy (SEM) revealed that exposure of oocytes or embryos at any stage of development to T. foetus caused rapid adhesion of the trichomonads to the embryonic intact zona pellucida (ZP) and to trophoblastic cells of hatched blastocysts. Treatment of contaminated embryos with 0.25% trypsin for 3 minutes did not render them free from T. foetus. Motile parasites

were not observed after 18 hours incubation in IVF medium, or after 72 hours in synthetic oviductal fluid (SOF) embryo culture medium. The percentages of cleaved zygotes, blastocysts and hatched embryos resulting from culture of experimental and uninfected control groups of embryos were not different ($P > 0.05$). Tritrichomonas foetus was not detected in embryonic cells of ZP-intact or hatched embryos when examined by transmission electron microscopy (TEM). In conclusion, T. foetus has no detrimental effect on the fertilization and development of IVF embryos and the potential risk of transmission of trichomonosis is unlikely, due to the limited survival of the parasite in IVF culture conditions. **(Bielanski A, et al. Theriogenology 2004;61:821-829.)**

Mycobacterium avium ssp. paratuberculosis

Over a 5-year interval, experiments were conducted to determine if *Mycobacterium avium* ssp. *paratuberculosis* (Map) is associated with in vivo and in vitro fertilized (IVF) embryos and whether it can be transmitted by embryo transfer. The present studies included: collection of embryos from five asymptomatic, naturally infected donors and transfer to uninfected recipients; collection of oocytes from two naturally infected donors with overt clinical signs; exposure of in vivo and IVF embryos to Map and transfer to uninfected recipients; and the inoculation (transfer) of ‘‘clean’’ IVF embryos to the uterine lumen of infected cows. The presence of Map was confirmed in the uterine horns of all asymptomatic, infected donors. None of the tested embryos, which were not used for embryo transfer, or unfertilized ova (two per batch), were positive for Map, as determined by culture (n = 19) or by PCR (n = 13). However, all in vivo fertilized embryos exposed to Map in vitro (and subsequently sequentially washed) tested positive for Map, by both culture (12 batches) and PCR (15 batches), whereas IVF embryos treated in the same manner tested positive on culture (51%, 18/35 batches) and by PCR (28%, 20/71 batches). Transferring both in vivo embryos and IVF embryos potentially contaminated with Map into 28 recipients resulted in 13 pregnancies and eight calves born without evidence of disease transmission to either the recipients or the offspring over the following 5-year period. In samples collected from one of the clinically infected animals, two of seven (28%) cumulus oocyte complexes (COC) and follicular fluid tested positive by PCR and 10/10 cumulus oocyte complexes on culture for Map. From the second clinically infected cow, three of five batches of IVF embryos (n = 20) were positive on PCR and two of four batches containing unfertilized oocytes and embryos were positive on culture. Only 10% of embryos reached the morula and blastocyst stage 10 days after fertilization. In conclusion, Map is unlikely to be transmitted by embryo transfer when the embryos have been washed as recommended by the International Embryo Transfer Society. **(Bielanski A et al, Theriogenology 2006;66:260-266.)**

The aim of the project was to ascertain if *Mycobacterium avium* subsp. *paratuberculosis* (Map) could be cultured from frozen-thawed in vitro produced (IVP) embryos derived from cows with subclinical Johne’s disease (JD). Straws of 109 IVP embryos were obtained from 267 cumulus–oocyte-complexes (COCs) collected from 12 clinically normal cows in which antibodies against Map were detected in blood by an enzyme-linked immunosorbent assay (ELISA). These embryos were processed, washed using the standard protocol as described by the International Embryo Transfer Society (IETS) and frozen in a commercial IVP embryo laboratory. Of the 12 donor cows, 11 had histopathological or bacteriological evidence of

infection at post-mortem inspection. The frozen embryos were thawed and the contents of the straws were cultured using the radiometric mycobacterial culture method. No Map was detected in any of the 109 embryos or freezing media. This suggests that the use of in vitro produced and cryopreserved embryos derived from cows with subclinical JD poses very low, if any, risk of spreading infection to susceptible animals. **(Perry GH et al, Theriogenology 2006;66:1267-1273.)**

Miscellaneous infections (and mixed agents) or multiple microorganisms

A total of 29 cows were submitted to various samplings. They were of undetermined origin and age, and at any stage of their reproductive cycle. Sampling included blood from peripheral veins (10ml), immature oocytes, follicular fluids, oviductal cells for serological (Brucellosis, Leucosis, IBR/IPV, BVD/MD) or bacteriological or viral analysis (BHV-1; BVD/MDV). Non-cleaved ova (n = 78) or embryos (n = 66) up to the blastocyst stage were obtained by total in vitro procedures. Among the 29 cows, some were serologically positive as follows: Brucellosis, 1; Leucosis, 2; IBR/IPV, 5; BVD/MD 21. None were BVD/MD virus positive from blood samples. No bacterial or viral contamination was found from uncleaved ova or from IVF embryos. These observations were interpreted as being consistent with a possible lack of risk of contamination from IVF embryos originating from oocytes collected at the abattoir (**Guerin et al, Bull Acad Vet France 1988;61:513-520**).

Frequency of virus contamination of materials from 5 IVF embryo production laboratories in Canada was investigated. The laboratories obtained ovaries and other culture materials from their local commercial abattoirs. Samples of embryos, follicular fluid and oviductal cells were tested for BHV-1 and BVDV. Of 85 samples containing 759 IVF embryos, 2 (2.4%) were positive for BHV-1, but all were negative for BVDV. Of 85 samples of follicular fluid, 10 (11.8%) were positive for BHV-1 and 4 (4.7%) were positive for BVDV. Of 80 samples of oviductal cells, 5 (6.2%) were positive for BHV-1 and one (1.2%) was positive for BVDV. At least some samples from 4 of the 5 laboratories were found contaminated with one or both viruses (**Bielanski et al, Theriogenology 1993;40:531-538**).

Efficacy of the IETS Manual 10 x washing procedure, plus or minus pre-washing antibiotic treatment, for the removal of bacteria (*Streptococcus agalactiae*, *Actinomyces pyogenes* and *Escherichia coli*) from IVF bovine embryos at the blastocyst stage (8-9d post insemination) was studied. Groups of 1-5 embryos were contaminated by placing them in medium (TCM 199 + 10% calf serum) containing bacterial cultures at 10_1 to 10_9 CFU/ml and incubating for 18 hr after which they were examined microscopically for viability and washed 10 x. Following bacterial contamination and prior to washing some groups of embryos were cultured for 2hr at 39°C in medium containing 50µg/ml gentamycin. After washing, embryos and samples (0.1ml) of medium were cultured by appropriate methods to detect bacteria. Irrespective of the level of contamination that embryos were exposed to, the antibiotic treatment effectively removed all viable bacteria from them, and bacteria were never isolated from samples of any of the washing fluids in the antibiotic groups. Without antibiotic, however, the 10 x washing procedure failed to remove the bacteria from the embryos when the level of exposure was more than 10_6 (*S.agalactia*), 10_7 (*A.pyogenes*) and 10_3 (*E.coli*). Bacteria were also recovered from wash samples up to the seventh, fifth and tenth washes after the embryos had been exposed to over 10_6 (*S.agalactiae*), 10_6 (*A.pyogenes*) and 10_3 (*E.coli*) without subsequent antibiotic treatment. The

recovery of bacteria from groups of embryos exposed to bacterial suspensions, then washed 10x by the IETS Manual recommended procedure, proved that that procedure is ineffective for IVF embryos unless it is used in conjunction with antibiotics (**Otoi et al, J Vet Med Sci 1992;54:763-765**).
Oocytes were fertilized and cultured in vitro while exposed to *Streptococcus agalactiae*, *Escherichia coli*, *Actinomyces pyogenes* or *Staphylococcus aureus*. The rates of cleavage and development to the blastocyst stage were not different between the groups exposed to bacteria and control groups, ie 51.4 - 62.6% vs. 52.5 - 58.4% and 4.6 - 14.4% vs. 7.5 - 15.6% respectively. Bacteria were not removed effectively from matured oocytes by washing alone and antibiotics had to be added to the in vitro culture media for removing or killing them (**Otoi et al, J Reprod Develop 1992;38:61-66**).

Oocytes and cumulus-oocyte complexes (COCs) were exposed in vitro to *E.coli* (serogroup 09:K99) and standard washing procedures were evaluated to determine their effectiveness for its removal. Removal of *E.coli* was complete (ie no bacteria in the washing fluid) after the seventh wash of cumulus cell-free oocytes, but with COC the number of bacteria isolated from the washing fluid declined up to the eighth wash and thereafter there was no further decline. It was concluded that standard washing procedures are not effective for removing *E.coli* adhering to COCs (**Otoi et al, J Mammalian Ova Res 1992;9:21-26**).

Using fluorescently labelled virus particles and fluorescence dequenching techniques it was shown that 3 different enveloped viruses, namely Sendai, influenza and Semliki Forest viruses, are able to fuse with bovine sperm cells in a similar manner to that observed following fusion of such viruses with cultured cells. Fusion was influenced by environmental pH and was mediated by virus receptors as indicated by the fact that the viruses failed to fuse with sperm cells from which the receptors had been removed by neuraminidase treatment. Preliminary (unpublished) results have shown that human sperm cells behave the same as bovine ones with these viruses. Fusion of enveloped viruses with sperm cells may enable the latter to carry the virus genome and introduce it into ova at fertilization (**Nussbaum et al, Exp Cell Res 1993;206:11-15**).

The mass production of embryos from ova harvested at commercial abattoirs may present problems for the control of pathogenic microbes in the IVF system. In this abstract we report microbial contamination detected in our IVF system over a 4 year period. Ovaries, collected at an abattoir, were washed twice in saline supplemented with oxytetracline HCL (1mg/ml). In the laboratory, ovaries were washed 3X then oocytes retrieved by slicing the ovarian surface with a razor blade in a beaker with 100 ml medium. Subsequently, contents were filtered and oocyts washed out with 20 ml fresh medium. The oocytes were washed 3X both before and after maturation and again after fertilization, and cumulus cells were removed by vortexing. The maturation, fertilization and coculture media were supplemented with gentamicin (25 µg/ml) or kanamycin (50 µg/ml). Fertilization and coculture were carried out in 50 µl media containing oviductal cells. All media were filtered (22 µm Millex-GV) and the ova/embryos were processed under a laminar flow hood.
In two cases the contamination was detected during the maturation of ova, and in other cases during the 8d of coculturing embryos. In most of these cases microbes were not observed microscopically in the IVF media, yet embryonic development (0-34%) was suppressed; subsequently microorganisms were cultured from these samples. Although the

source of contaminants remains unknown, it could be that in some, microbes were associated with the oviductal cells from donor cows. It is concluded that in addition to rigorous washing of ova/embryos after each step of the IVF procedure, a broad spectrum of antibiotics in media is required to prevent microbial growth and losses in IVF produced embryosembryos. When embryonic development is suppressed, culture of the media would be advisable.

Table 1. Microbes isolated from the IVF system and number of culture positive cases

	Genera	Number	Medium
Gram +	*Corynebacterium*	1	IVC
	Streptococcus	3	IVC
	Staphylococcus	3	IVC
	Micrococcus	2	IVM
Gram -	*Pseudomonas*	12	IVC
Fungi	Yeast	6	IVC
	Total	27/215 (13%)	

(Bielanski A and Stewart B, Theriogenology 1996;45:269 abstr)

In an effort to produce IVF embryos from an infertile cow, *Stenotrophomonas maltophilia* was isolated from contaminated cultures of all zygotes resulting from insemination with previously untested semen from a single bull. The organism was resistent to the antibiotics that had been used in IVM, IVF, and IVC. *Stenotrophomonas maltophilia* and a few colonies of *Pseudomonas putida* also were isolated from the pelleted semen. (**Stringfellow JS et al, Theriogenology 1997;46:382 abstr**)

Unsuccessful IVF resulted from bacterial contamination that was traced to semen from 4 different native Korean bulls. Original semen samples and contaminated cultures contained *Pseudomonas aeroginosa*, *Acinetobacter calcoaceticus*, or *Flavobacterium* spp. (**Lee et al, Theriogenology 1997;46:375 abstr**)

Semen from 5 native Korean breed bulls (A,B,C,D,E) was used in IVF. Previously, lots of semen from bulls A,B,D,and E had been identified as contaminated with a variety of bacteria (*Pseudomonas aeruginosa, Staphylococcus sciuri, Enterobacter cloacae, Acinectobacter cacloaceticus, Flavobacterium* spp. and *Pantoea agglomerans).* After IVF, cumulus were removed from presumptive zygotes by pipetting or vortexing. Viability and cleavage was assessed at 44 h and development was assessed at 7 d. When cumulus was removed by pipetting, only Bull-C-fertilized embryos developed. Embryos from all other bulls started to degenerate and culture medium was noticeably turbid at 44 h. When cumulus was removed by vortexing, good rates of development (16 to 32 %) to morulae and blastocyst were observed in zygotes derived from oocytes fertilized by both contaminated and uncontaminated semen. (**Kim IH et al. Theriogenology 1998;50:293-300**)

Genetic diseases

A DNA test, based on the polymerase chain reaction (PCR), for deficiency of uridine monophasphate synthase (DUMPS) was developed. DUMPS deficiency is usually lethal at

about 40 days of gestation. Oocytes from cows heterozygous for DUMPS were fertilized in vitro with spermatozoa from a DUMPS-carrier bull and cultured in vitro until the morula or blastocyst stage. Heminested PCR was performed and the genotype of 12 embryos was unambiguously determined by AvaI digestion of PCR products. Two embryos homozygous for the defective DUMPS allele (R405<right arrow>STOP) were detected. Of the remaining embryos, 4 were homozygous and 6 were heterozygous for the normal allele. The observed distribution was close to the expected ration of 1:2:1. The results supported the monogenic recessive inheritance of the defect. (**Schwenger B et al, J Reprod Fertil 1994;100:511-514**)

Quality control testing

Over a four-year period, 339 in vitro production batches were screened for the presence of BVDV and BHV-1, and 151 batches were screened for the presence of bacteria. For each batch of materials (of abattoir origin) (mean number of oocytes = 250), the following samples were examined: 1) pooled follicular fluid, 2) maturation medium, 3) washed and ground-up degenerated embryos, and 4) last three washes (pooled). Bovine viral diarrhea virus and BHV-1 were isolated from 0.88 % and 1.47 % of the batches, respectively (i.e. 3/339 and 5/339, respectively). Bovine viral diarrhea virus was never isolated from follicular fluid (possibly due to antibody). When BVDV was found in maturation medium (2/3), degenerated embryos and/or washes were also contaminated. In one case, BVDV was only found in degenerated embryos. Bovine herpesvirus-1 was detected in maturation medium (3/5 positive batches) and in degenerated embryos for the other 2 positive batches (washes were also positive in one of the latter cases).
Bacteria were isolated (*Staphylococcus* spp., *Bacillus* spp., *Corynebacterium* spp., etc) from 103 batches out of the 151 total analyzed (68.2 %). From 72.8 % of the bacteria-positive samples only follicular fluid was contaminated at a low level (90.3 % of samples contained less than 100 colony forming units). Ovaries had been rinsed and disinfected, but contamination was hypothesized to have occurred during the follicle puncturing process. For 10.7 % of the batches (11/103), follicular fluid and degenerated embryos were both contaminated. Bacteria in follicular fluid had no negative effect on development rate in vitro except when *Escherichia coli* was found in follicular fluid (3 cases). In summary, when materials were of abattoir origin, 99.1%, 99.4 %, and 92.7 % of the embryo batches were free of BVDV, BHV-1 and bacteria, respectively. **(Marquant-Leguienne B et al, Theriogenology 2000;53:321 abstr)**

Goats

Caprine arthritis-encephalitis virus (CAEV)

The objective of this study was to investigate the in vitro susceptibility of primary goat granulosa cells (such as those present during IVM) to caprine arthritis encephalitis virus (CAEV) infection. Primary cultures of caprine granulosa cells were established and exposed (multiplicity of infection of 1) to CAEV-pBSCA (an infectious molecular clone) and CAEV-3112 (a field isolate). Cultures were incubated for 6 days and then supernatants were harvested, frozen and later titrated in goat synovial membrane cell cultures. Monolayers were also fixed stained and examined for cytopathic effect. Cytopathic effect was noticed for both

viruses (greater for the field virus). Titers of supernatant were $10^{4.75}$ and $10^{5.25}$ $TCID_{50}$/mL for CAEV-pBSCA and CAEV-3112, respectively. Thus, the granulosa cells were susceptible to infection and high titers of virus were produced. **(Lamara A et al, Theriogenology 2000;53:320 abstr)**

Recent reports demonstrated the susceptibility of epithelial cells from different organs to caprine arthritis-encephalitis virus (CAEV) both in vitro and in vivo. Since granulosa cells (GC) are of epithelial origin and currently used for in vitro oocyte maturation, we addressed the question whether these cells are susceptible or resistant to CAEV infection. GC were isolated from goats from certified CAEV-free herds. PCR analysis on GC DNA using CAEV specific primers confirmed the absence of CAEV infection and immunocytochemistry using specific K813 anti-cytokeratin monoclonal antibodies confirmed the epithelial nature of GC. These cells were then inoculated with CAEV using two strains: the CAEV-pBSCA molecular clone and the CAEV-3112 French field isolate. Cytopathic effects (CPE) were observed on cell culture monolayers inoculated with both CAEV strains. Expression of CAEV proteins was shown both by immunocytochemistry using anti-p24 gag specific antibodies and by immunoprecipitation using an hyperimmune serum. Supernatant of infected cells were shown to contain high titers (ranging 10(5) tissue culture infectious doses 50 per ml: TCID(50) per ml) of infectious cytopathic viruses when assayed onto the indicator goat synovial membrane (GSM) cells. Our findings demonstrate the large cell tropism of CAEV and suggest that GC could serve as a reservoir for the virus during the sub-clinical phase of infection. Furthermore, given the high seroprevalence of CAEV in the all industrialised countries and the large number of ovaries derived from unknown serological status animals used for in vitro goat embryo production, one can conclude that these feeder cell cultures might be a potential source of early transmission of CAEV to goat embryos. **(Lamara A et al. Virus Res 2001;79:165-172)**

Caprine oviduct epithelial cells (COEC) are commonly used in in vitro goat embryo production protocols to stimulate early embryonic development. These feeder cells are usually collected from slaughterhouses from unknown serological status animals for caprine arthritis-encephalitis virus (CAEV) infection which is frequent in many regions of the world. Tissues derived from this source may be contaminated with CAEV and the use of such material in in vitro fertilisation systems may contribute to transmission of this pathogen to the cultured embryos and dissemination via embryo transfer (ET). The aim of this study was to determine the permissiveness of COEC to CAEV replication in vitro. Cells were isolated from goats from certified CAEV-free herds and then were inoculated with two CAEV strains: the molecularly- cloned isolate of CAEV (CAEV-pBSCA) and the French field isolate (CAEV-3112). Cytopathic effects (CPE) were observed on cell culture monolayers inoculated with both CAEV strains. Expression of CAEV proteins was shown both by immunocytochemistry using anti-p24 gag specific antibodies and by immunoprecipitation using a hyperimmune serum. The CAEV proteins were correctly and properly processed by artificially-infected COEC and the titers of virus released into the supernatant reached 10(6) TCID(50)/ml 6 days post-inoculation. Although the macrophage lineage cells are the main centre of infection in the virus-positive animal, these findings suggest that epithelial cells may be important in the viral life cycle probably as a reservoir allowing the viral persistence, dissemination and pathogenesis. These results suggest also that the use in in vitro fertilisation systems of co-culture feeder cells that support efficient replication of CAEV to high titers could represent a serious risk for permanent transmission of

virus to the cultured embryos and to the surrogate dam involved. **(Lamara A et al. Virus Res 2002;87:69-77)**

The aim of this study was to determine whether oocytes taken from ovarian follicles in 123 naturally infected goats were carrying the proviral CAEV genome. Examination of DNA isolated from 190 batches of oocytes with intact cumulus cells and 190 batches of oocytes whose cumulus cells had been removed, taken from follicles of the same ovaries, demonstrated that 42/190 batches of oocytes with intact cumulus cells had the proviral CAEV genome, whereas none of the 190 batches of oocytes without cumulus cells were positive for the provirus. To confirm that the proviral genome was present in the cumulus cells and not in the oocyte cells, 586 oocytes from 56 different ovaries, were separated from their cumulus cells. The DNA was then extracted from each fraction and examined. The purity of the oocyte fraction was verified by searching for granulosa cell-specific mRNA, using RT-PCR; this was negative in all the batches of oocytes in which the cumulus cells had been removed. PCR analysis demonstrated that none of the oocytes without cumulus cells were positive, whereas 22/56 of the batches with cumulus cells were found to be positive. This study clearly demonstrates that despite being surrounded by infected cumulus cells, the oocytes are not infected, and that the enzymatic and mechanical technique for removing the cells surrounding the oocyte, as used in this study, is effective, thus enabling CAEV-free oocytes to be obtained from infected goats. **(Ali Al Ahmad MZ, et al. Theriogenology. 2005;64:1656-1666).**

Zonapellucidafree embryos at 816 cell stage were cocultured for 6 days in an insert over a mixed cell monolayer infected with CAEVpBSCA. Embryos were washed and transferred to an insert on CAEV indicator goat synovial membrane cells for 6 h, then they were washed and cultivated in B2 Menezo for 24 h, finally, embryo cells were dissociated and cultivated on a feeder monolayer for 8 days. After 5 weeks, multinucleated giant cells typical of CAEV infection were observed in indicator GSM cell monolayers. In the acellular medium, the early embryonic cells produced at least $10^{3.25}$ $TCID_{50}$/ml over 24 h. The monolayer of cultivated embryonic cells developed cytopathic lesions within 8 days, and CAEV RNA, CAEV proviral DNA and protein p28 of the capsid were detected. All of these results clearly demonstrate that caprine early embryonic cells are susceptible to infection with CAEV and that infection with this virus is productive. **(Ali Al Ahmad MZ et al, Virology 2006;353:307-315.)**

For each of the five fertilization trials of the experiment, frozen semen was prepared for in vitro capacitation at a concentration of 1 × 10(7) spz/ml and divided into three groups. One group was used as a control, while the two others were inoculated with 100 µl/ml of either culture medium from non-infected cells (placebo group) or cell culture medium containing virus at a concentration of 10(5) TCID(50)/ml (infected group). A total of 789 oocytes were used for IVF. For each of the five trials a group of oocytes were used as a non-infected control and were found to be caprine arthritis-encephalitis virus (CAEV) free. The other oocytes were divided in two equal batches. Oocytes in the first batch were in vitro fertilized with CAEV infected sperm (infected group) and the second batch were fertilized with CAEV non-infected sperm (placebo and control groups). After IVF, the zygotes of each group were washed 12 times. The CAEV genome was not detected (using RT-PCR) in the washing media of either the control or placebo groups from each trial. In contrast, the first three washing

media from the infected group were consistently found to be positive for the CAEV genome (5/5), whereas subsequent washing media were CAEV-free ($P < 0.05$). Zygotes obtained using all semen groups tested negative for both the provirus and genome of CAEV. These results clearly show that the first four washes were sufficient to remove viral particles from CAEV infected fertilization media and that CAEV-free embryos can be produced by IVF using spermatozoa infected in vitro by CAEV. (**Fieni F et al. Can caprine arthritis encephalitis virus (CAEV) be transmitted by in vitro fertilization with experimentally infected sperm? Theriogenology 2012;77: 644-651)**

For each of the five fertilization trials of the experiment, frozen semen was prepared for in vitro capacitation at a concentration of 1 × 10(7) spz/ml and divided into three groups. One group was used as a control, while the two others were inoculated with 100 µl/ml of either culture medium from non-infected cells (placebo group) or cell culture medium containing virus at a concentration of 10(5) TCID(50)/ml (infected group). A total of 789 oocytes were used for IVF. For each of the five trials a group of oocytes were used as a non-infected control and were found to be caprine arthritis-encephalitis virus (CAEV) free. The other oocytes were divided in two equal batches. Oocytes in the first batch were in vitro fertilized with CAEV infected sperm (infected group) and the second batch were fertilized with CAEV non-infected sperm (placebo and control groups). After IVF, the zygotes of each group were washed 12 times. The CAEV genome was not detected (using RT-PCR) in the washing media of either the control or placebo groups from each trial. In contrast, the first three washing media from the infected group were consistently found to be positive for the CAEV genome (5/5), whereas subsequent washing media were CAEV-free ($P < 0.05$). Zygotes obtained using all semen groups tested negative for both the provirus and genome of CAEV. These results clearly show that the first four washes were sufficient to remove viral particles from CAEV infected fertilization media and that CAEV-free embryos can be produced by IVF using spermatozoa infected in vitro by CAEV. **Fieni F, Pellerin JL, Roux C, Poulin N, Baril G, Fatet A, Valas S, Chatagnon G, Mermillod P, Guignot F. Can caprine arthritis encephalitis virus (CAEV) be transmitted by in vitro fertilization with experimentally infected sperm? Theriogenology. 2012 Feb;77(3):644-51.**

The transmission of CAEV from male goats has not been well studied and the target cells that support viral replication are not well characterized. Epididymal epithelial cells (EECs) are important and play a key role in the fertility and motility of spermatozoa. During their transit, spermatozoa incorporate several EEC-produced proteins into their plasma membranes to stabilize them and prevent premature acrosomal reaction. This intimate interaction between spermatozoa and EECs may increase the likelihood of the infection of semen with CAEV if epididymal tissue is productively infected and sheds the virus into the duct. The aim of this study was to examine whether goat EECs are susceptible to CAEV infection in tissue culture. Cells were isolated from epididymides obtained from goats that were sampled from a certified-CAEV-free herd. Cultured cells were then inoculated with a molecularly-cloned isolate of CAEV (CAEV-pBSCA). Inoculated cells developed cytopathic effects (CPE), showing numerous multinucleated giant cells (MGC) in cell-culture monolayers. Expression of CAEV proteins was detected by immunofluorescence using an anti-p28, Gag-specific antibody. The culture medium of inoculated cells was shown to contain high titers (10(6) tissue culture infectious doses 50 per ml (TCID50/ml)) of

infectious, cytopathic virus when assayed using indicator goat synovial membrane (GSM) cells. Our findings clearly demonstrate that cells of the buck genital tract are targets of CAEV and are thus a potential reservoir that sheds infectious CAEV into the semen of infected animals. These data suggest the use of sperm from CAEV-free goat males for artificial insemination in genetic selection programs to minimize CAEV dissemination.
Lamara A, Fieni F, Chatagnon G, Larrat M, Dubreil L, Chebloune Y. Caprine arthritis encephalitis virus (CAEV) replicates productively in cultured epididymal cells from goats. Comp ImmunolMicrobiol Infect Dis. 2013 Jul;36(4):397-404

Coxiella burnetii

Previous work demonstrated that after infection of in vivo derived caprine embryos, Coxiella burnetti (C. burnetii) showed a strong tendency to adhere to the zona pellicida (ZP). To investigate the risk of C. burnetii transmission via embryo transfer of in vitro-produced goat embryos the aim of this study was, (i) to evaluate the ability of C. burnetii to adhere to the intact zona pellicida of in vitro-produced goat embryos and to determine by confocal microscopy the location of the bacteria, (ii) to test the efficacy of IETS recommended rules for the washing of bovine embryos to eliminate C. burnetii. One hundred ZP-intact caprine embryos, produced in vitro, at the 8 to 16 cell stage, were randomly divided into 11 batches of eight to nine embryos. Nine batches were incubated for 18 h with 10(9)Coxiella/ml of CbB1 strain (IASP, INRA Tours). The embryos then were recovered and washed in batches in 10 successive baths following the IETS guidelines. In parallel, two batches of embryos were subjected to similar procedures but without exposure to C. burnetii, to serve as the control group. One of the nine batches of infected embryos and one of the two non-infected control batches were separated to perform immunolabeling to locate the bacteria. C. burnetii DNA was detected by C-PCR in all eight batches of infected embryos after 10 successive washings. However, bacterial DNA was not detected in the embryo control batch. The first five washing media of the infected group were consistently found to be positive and Coxiella DNA was detected in the wash bath up to the 10th wash for two batches. After immunolabeling, the observation of embryos under confocal microscopy allowed C. burnetti to be found on the external part of the zona pellucida without deep penetration. This study clearly demonstrates that C. burnetii, after in vitro infection at 10(9)Coxiella/ml, stick strongly to the external part of the zona pellucida of in vitro produced caprine embryos without deap penetration and that the 10 washings protocol recommended by IETS to eliminate the pathogenic agents of bovine embryos is unable to eliminate these bacteria from in vitro-produced goat embryo. **Pellerin J.L., A. Alsaleh, P. Mermillod, J.M.G. Souza-Fabjan, A. Rodolakis, E. Rousset, L. Dubreil, J.F. Bruyas, C. Roux and F. Fieni 2018. Attachment of Coxiella burnetii to the zona pellucida of in vitro produced goat embryos. Theriogenology 106, 259-264. doi:10.1016/j.theriogenology.2017.10.033**

Horses

Equine arteritis virus

The objectives of the study were to investigate the effect of low level contamination of IVM medium by equine arteritis virus (EAV) and to evaluate 10X washing protocol for removal of EAV from in vitro matured equine oocytes. Ovaries were removed via colpotomy and oocytes collection began within 1 h. Oocytes (n=11) were divided into virus exposed (n=8)

and unexposed (n=3) groups. Maturation for both was the same except one group was matured in medium with 10^5 pfu/0.1 ml of Bucyrus strain of EAV (ATCC# VR 796). After maturation oocytes were washed using the protocol recommended by IETS for in vivo derived embryos. After washing oocytes were sonicated and assayed by plaque assay. Washs were also assayed for infectious virus by titration in 96-well plates. No virus was detected in the control sonicate fluids or washes. Virus was detected in washes 1,2,3, and 5 for the exposed group. One plaque at the zero dilution was observed in cell cultures of sonicate fluids of the exposed oocytes. (**Sherod J et al. Theriogenology 1998;49:260 abstr.**)

The presence of Equine Arteritis Virus (EAV) in the reproductive tracts of clinically infected mares has not been well characterized. To our knowledge there has been no research conducted to determine the presence or absence of EAV in infected reproductive tract tissues of the mare. Neither has there been any research looking at the risks of viral transfer using currently applied assisted reproductive techniques. The objective of this preliminary study was to document the presence or absence of EAV in the reproductive tissues of experimentally infected mares.

Seven known sero-negative mares were challenged with Kentucky 84 field strain of EAV (5 ml) of stock virus at 10^4 pfu/ml by intra-nasal inoculation. Animals were housed under quarantine, and monitored twice daily for signs of infection. Serum was drawn pre-challenge, and post-challenge at d 19 and d 28.

Aspiration procedures were performed under standing restraint, with detomodine HCL and phenylbutazone given for tranquilization and analgesia. Follicular fluid was sampled from the dominant follicle in five of seven mares on days 0, 1, 3, and 6 post challenge (day zero = challenge) using transvaginal ultrasound guided follicular aspiration. Each sample was placed on ice until the end of the procedure and then frozen (-80 ^oC) for later assay. Two mares served as non-aspirated controls.

On day 6 post challenge, mares underwent gonadectomy via a colpotomy approach (n = 7). The resulting ovaries and oviductal tissues were placed on ice and transported to the laboratory for further separation. Oviductal and ovarian tissues for histopathologic analysis were taken from each pair of ovaries. Reproductive tract tissue samples from five of seven mares were frozen for later virus isolation attempts. In addition, oocytes from each pair of ovaries (n= 5 pair) were collected and frozen in groups of five for later assay.

Serum neutralization tests were performed on post challenge sera from all exposed animals. Results indicate 6/7 animals sero-converted by day 19. Virus isolation performed on oviductal tissues displayed an average titer of 4.9 x 10^3 pfu/ml in 4 of 5 animals (1/5 negative). Virus in low levels was isolated from follicular fluid aspirates in 4 of 5 animals (1/5 negative). No virus was recovered from follicular fluid samples taken on day zero, or day one; 2 of 4 were positive on day 3; and 4 of 5 were positive on day 6.

Histopathological findings of ovarian and oviductal tissues were typical of post challenge EAV infections (d 6) in 6 of 7 animals. Lesions were of variable severity and included small vessel intimal layer disruption, adherence of polymorphonuclear neutrophils (PMN's) and monocytes to the vascular intimal with a perivascular mononuclear cell infiltrate. These results indicate that ovarian tissues and follicular fluid from EAV infected mares can contain low levels of infectious virus. The presence of virus in tissues and aspirates therefore presents a route of contamination for assisted reproduction techniques and for culture systems. **(Holyoak GR et al. Proceedings for the Society for Theriogenology, Vancouver, British Columbia, Canada September 2001)**

Recent preliminary work conducted in this lab has shown the presence of infectious equine arteritis virus (EAV) in the reproductive tissues and follicular aspirates of experimentally infected mares. Increasing use of assisted reproductive technologies involving the mare, specifically in vitro oocyte maturation and culture utilizing follicular fluid combined with intraoviductal transfer of those cultured oocytes, could pose a risk for horizontal transmission of EAV. To our knowledge the effect of contaminated oocytes and /or holding medium on a sero-negative recipient has not been characterized for any equine viral disease to date. The purpose of this experiment is to determine the potential for disease transmission when contaminated oocytes are utilized in assisted reproduction techniques such as GIFT.

Eleven sero-negative mares ranging in age from 3 to 20 years with unknown reproductive histories were used. The mares'estrous cycles were synchronized using a two-injection protocol of synthetic prostaglandin F2α. Four days after the second injection, ovaries from three mares displaying estrus were obtained by gonadectomy. Within one hour after excision all visible follicles were incised and scraped. Following reported techniques, the collected oocytes (n = 10) were matured for 18 hours. An inoculum of 1 ml stock virus (K-84 strain EAV $H10_4$ pfu/ml) was added to the culture media of the pooled oocytes and allowed an additional 2 hours of culture before transfer of the oocytes into recipient mares. Oocytes (n = 6) were either washed by gentle pipetting in warmed PBS and then placed in warmed holding medium prior to intraoviductal transfer; or directly transferred from stock virus solution (n = 2). A single oocyte in approximately 30 µl of fluid was transferred into the oviduct of each donor mare through a standard standing flank laparotomy. Pipette tips were rinsed into separate dishes of holding medium to confirm that the oocyte had been transferred. Recipient mares were treated with antibiotics (penicillin) daily for 5 days, and an oral anti-inflammatory/analgesic (phenylbutazone) as needed for comfort. Recipient mares were housed separately under quarantine and monitored twice daily for signs of infection. Blood samples were collected by jugular venipuncture into 10 ml evacuated blood tubes for serological testing on day 16 and 24 after oocyte transfer. Buffy coat blood samples for virus isolation were collected daily and aliquots were frozen (-80 °C) until assayed. Serum neutralization antibodies to EAV were assayed, using a micro-neutralization test and monolayer cultures of RK-13 cells in the presence of 10% guinea pig complement. Serum neutralization assays detected antibodies in 5 of 8 animals at day 19 post-transfer. Buffy coat samples from days 06, 08, 10, and 12 post oocyte transfer were sent to the University of Kentucky for virus isolation and quantitation. EAV was isolated from 5 / 8 of the recipient mares.

These initial and preliminary results indicate that there is a potential for experimentally contaminated and "washed" oocytes to transmit EAV during intraoviductal transfer procedures. The washing protocols utilized in this experiment followed those previously reported and did not follow those recommended by the International Embryo Transfer Society for in vivo produced embryos. Further study in this area is warranted. (**Holyoak GR et al. Proceedings for the Society for Theriogenology, San Antonio, Texas, November 2000, page 135)**

Equine infectious anemia virus

Deriving horse oocytes in the USA is hampered by the lack of abattoirs processing horse carcasses which could provide abundant quantities of ovaries from slaughtered mares. Therefore, several cloning industries in the USA are attempting to import cloned horse embryos from Canada. Like any agricultural commodity, cloned embryos pose a risk of introduction of exotic animal diseases into the importing country. Under such circumstances, risk assessment could provide an objective, transparent, and internationally accepted means for evaluating the risk. This quantitative risk assessment (QRA) was initiated to determine the risk of introduction of Equine infectious anemia virus (EIAV) into the USA via cloned horse embryos imported from Canada. In assessing the risk, a structured knowledge base regarding cloning in relation to Equine infectious anemia (EIA) was first developed. Based on the knowledge base, a scenario tree was developed to determine conditions (with mathematical probabilities) that could lead to the introduction and maintenance of EIAV along the cloning pathway. Parameters for the occurrence of the event at each node were estimated using published literature. Using @Risk software and setting Monte Carlo simulation at 50,000 iterations, the probability of importing an EIAV-infected cloned horse embryo was 1.8 × 10(-9) (R = 1.5 × 10(-12) to 2.9 × 10(-8)). Taking into account the current protocol for equine cloning and assuming the yield of 5 to 30 clones per year, the possible number of EIAV-infected cloned horse embryos ranged from 2.0 × 10(-10) to 9.1 × 10(-5) (Mean = 1.4×10(-6)) per year. Consequently, it would take up to 1.5 × 10(7) (R = 1.6 × 10(4) to 5.1 × 10(10)) years for EIAV to be introduced into the USA. Based on the knowledge base and our critical pathway analysis, the biological plausibility of introducing EIAV into USA via cloned horse embryos imported from Canada is extremely low.
Asseged BD, Habtemariam T, Tameru B, Nganwa D.The risk of introduction of equine infectious anemia virus into USA via cloned horse embryos imported from Canada.Theriogenology. 2012 Jan 15;77(2):445-58. doi: 10.1016/j.theriogenology.2011.08.019.

Deriving horse oocytes in the USA is hampered by the lack of abattoirs processing horse
Review
Prevention and regulation of equine infectious anemia virus (EIAV) disease transmission solely depend on identification, isolation, and elimination of infected animals because of lack of an effective vaccine. Embryo production via the somatic cell nuclear transfer (SCNT) technology uses oocytes collected mainly from untested animals, which creates a potential risk of EIAV transmission through infected embryos. The current review examines the risk of EIAV disease transmission through SCNT embryo production and transfer. Equine infectious anemia virus is a lentivirus from the family Retroviridae. Because of a lack of direct reports on this subject, relevant information gathered from close relatives of EIAV, such as human immunodeficiency virus (HIV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), and small ruminant lentiviruses (SRLVs), is summarized and used to predict the biological plausibility of EIAV disease transmission through transfers of the equine SCNT embryos. Based on published information regarding interaction of oocytes with lentiviruses and the sufficiency of oocyte and embryo washing procedures to prevent lentivirus transmission from in vitro-produced embryos of various species, we predicted the risk of EIAV transmission through SCNT embryo production and transfer to be very small or absent. **Gregg K, Polejaeva I. Risk of equine infectious anemia virus disease transmission**

through in vitro embryo production using somatic cell nuclear transfer. Theriogenology. 2009;72(3):289-99.

Pigs

Hog cholera virus (HCV) (classic swine fever virus)

The objective of this study was to investigate the susceptibility of in vivo- and in vitro-produced (IVP) porcine embryos to classical swine fever virus (CSFV). IVP zona pellucida (ZP)-intact porcine embryos (n = 721) were co-cultured with CSFV for 120 h. After washing according to the International Embryo Transfer Society guidelines (without trypsin) and transferring embryos to CSFV-susceptible porcine kidney cells (PK15 cell line), no virus was isolated. However, when 88 IVP ZP-intact porcine embryos were co-cultured with CSFV for only 48 h before being transferred to PK15 cells, virus was isolated in three of six replicates. Similarly, 603 in vivo-produced porcine embryos were co-cultured with CSFV for 120 h. Subsequently, CSFV was isolated in eight of 50 groups (16%) and the ability of these to form a blastocyst was significantly reduced when compared with the control group (68.2 +/- 19.9% vs 81.9 +/- 9.7%; p < or = 0.001). In contrast, the development of CSFV-exposed IVP porcine embryos was not affected when compared with control embryos (19.1 +/- 10.8% vs 18.9 +/- 10.6%; p > or = 0.05). After removal of the ZP of IVP embryos and subsequent co-culture with CSFV, the virus was isolated from all groups of embryos. These data suggest that virus replication had occurred in the embryonic cells. In conclusion, data indicate that in vivo- and in vitro-produced ZP-intact porcine embryos differ in their susceptibility to CSFV. Hatched or micro-manipulated embryos may increase the risk of transmission of CSFV by embryo transfer, which has to be confirmed by in vivo tests under isolation conditions. **(Schuurmann E, et al. Reprod Domest Anim. 2005;40:415-421.)**

Multiple viruses (including EMCV, PCV2, PPV PRRSV and BVDV)

The objective of this study was to explore approaches to decontaminate embryos either contaminated naturally or under experimental conditions with different viruses. Embryos were obtained from in vitro maturation and fertilisation of porcine oocytes. After 7 days of development, morula and blastocyst stages were exposed for 1 h to the following viruses: encephalomyocarditis virus (EMCV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), and bovine viral diarrhea virus (BVDV) at an infectivity of 100 TCID50/mL. Embryos samples were treated with different washing procedures, which all included the following standard washing solutions: PBS+0.4% BSA (five times for 10 s), Hank's+0.25% trypsin (two times for 60-90 or 120-150 s, or one time of 5 min), Hank's+0.1 mg/mL DNase 1+20 U/mL RNase One (one time of 30 min) and PBS+0.4% BSA again (five times for 10s). Two new approaches were used to improve trypsin treatment, 0.1% hyaluronidase (one time for 5 min) instead of trypsin and a pre-incubation with oviductal cells. Therefore, in the first experiment, oocytes received standard maturation treatments and in the second, they were also co-incubated with oviductal cells for the last 3 h of maturation. The effectiveness of the different washing

techniques in removing viruses was evaluated by polymerase chain reaction (PCR) analysis. In the first experiment, trypsin treatment did not eliminate PRRSV, PPV, PCV, and EMCV from contaminated embryos. Surprisingly, treatment with hyaluronidase eliminated all tested viruses. In the second experiment, all viruses tested were removed from the oocytes following the different enzymatic treatments. In conclusion, in vitro embryo decontamination was more effective following exposure to oviductal secretions and hyaluronidase eliminated more virions than trypsin in washing techniques. **Bureau M, Dea S, Sirard MA. Theriogenology 2005;63:2343-2355.**

Porcine circovirus

The purpose of this study was to investigate the possibility of rendering oocytes and embryos free of porcine circovirus type 2 (PCV2). Groups of cumulus oocytes complexes, cumulus free oocytes, and embryos 3 to 5 days post breeding were exposed to PCV2 (10^5 $TCID_{50}$/mL) prior to disinfection by washing and different combinations of enzymatic treatments. The study suggests that under the in vitro conditions used, standard washing procedures with, or without, trypsin or incorporating pronase or hyaluronidase and DNase/RNase in the treatment was not effective in rendering oocytes and embryos free from PCV2 nucleic acid. Since the virus is noncytopathic in cell culture and for embryonic cells, it appears that there is a possibility of introducing viral contamination through oocytes collected from infected pigs into the in vitro fertilization system with subsequent potential of producing in vitro fertilized embryos associated with PCV2. **Bielanski AF, et al. Canadian Journal of Veterinary Research 2004;68:222-225.**

A total 69 reproductive tract and 15 additional serum and follicular fluid samples from PCV infected pigs were examined. The overall percentages of positive samples in the serum, follicular fluid, uterine washes, oviductal cells, and oocytes detected by PCR were 75% (63/84) , 21.6%, 31.34%, 62.23% (22/59), and 11.2% respectively. Overall viral DNA was detected in some part of reproductive tract in 63% (53/84) of investigated. Of the animals with DNA-PCV negative serum, 30.09% (8/21) had a viral antigen associated with reproductive samples. A total of 120 embryos were collected from 6 infected animals. None of the samples containing a single embryo or group of 2-3 embryos tested positive for PCV-DNA (after washing + trypsin treatment). Development of the embryos ranged from 4 cells to hatched blastocyst which corresponded well with the time of breeding. One of the gilts slaughtered on day 6 post-breeding yielded 7 hatched blastocysts, all negative for PCV2. Another gilt slaughtered on 28 day of gestation yielded 12 fetuses of which 7 tested negative for PCV2 DNA. Pigs inoculated experimentally with infected semen showed antibodies to PCV present in serum and viral DNA in blood approximately 2 weeks after insemination. All collected tissues from the reproductive tracts of these pigs were negative by PCR. One pig had 3 fetuses with evidently normal development . Collected samples of fetal spleen and kidneys and amniotic fluids tested negative for the presence of viral PCV DNA. **Bielanski A, et al. Vet Rec 2004;155:597-598.**

This study was aimed at assessing the capability of semen experimentally infected with porcine circovirus type 2 (PCV2) to produce porcine blastocysts PCR positive for PCV2. Embryos were obtained from in vitro maturation (IVM) and in vitro fertilization (IVF) of

porcine oocytes or by parthenogenesis. Sperm suspension was exposed to PCV2b and utilized for IVF. PCV2 spiked semen did not reveal any reduction in sperm viability or motility but its ability to produce infected blastocysts was irrelevant as only one out of 15 blastocysts obtained by IVF were PCV2b; however two blastocysts were PCV2a positive. Furthermore, the presence of PCV2 was demonstrated also in embryos obtained by parthenogenesis (one out of 17 was PCV2b and one PCV2a positive). Even if PCV2 firmly attaches to the surface of spermatozoa, experimentally spiked sperm were not effective in infecting oocytes during IVF and in producing PCR positive embryos. The infected blastocysts we obtained derivedmost probably from infected oocytes recovered at the abattoir. **Galeati G et al.** ***Animal Science journal 2015 doi: 10.1111/asj.12465***

In vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) are important breeding techniques for livestock. High-quality MII oocytes produced from in vitro maturation (IVM) are required for the two techniques listed above. The ovaries used for IVM operations are primarily acquired from commercial abattoirs, and the pathogen status of slaughtered animals becomes an unavoidable issue. Our previous monitoring data showed that porcine circovirus type 2 (PCV-2) is the main pathogen present in ovaries from abattoirs. However, the characteristics and effects of PCV-2 infection in oocyte maturation and in vitro production (IVP) of embryos are unclear, and currently there are no relevant studies. Therefore, the aim of this study was to determine the PCV-2 infection pattern and determine whether it affects oocyte in vitro maturation and IVP embryo development. More than five hundred ovaries and five thousand oocytes were utilized in the present study. Polymerase chain reaction (PCR) was used to detect PCV-2 DNA in ovaries, follicular fluid (FF), oocytes, cumulus cells and IVP embryos. The effects of viral infections on the rate of oocyte maturation and IVP embryo development were evaluated. We also analyzed the number of copies of the virus in the IVM and IVP process by absolute quantitative fluorescence PCR. Our study showed that the prevalent virus subgenotype in ovaries was PCV-2a. PCV-2a infection did not significantly affect ovarian/oocyte morphology and maturation. Moreover, virus infection did not have a significant effect on the development of the IVP embryos except for a reduction in IVF blastocyst cell numbers. Further tests showed that the viral copy numbers fluctuated at different stages between the IVP embryos and culture medium. For the first time, this study identified the infection pattern of naturally sourced PCV-2 in the course of oocyte maturation and embryo development. **Weng, X.G., Liu, Y., Zhou, S.H., Zhang, Y.T., Shao, Y.T., Xu, Q.Q. and Liu, Z.H., 2019. Evaluation of porcine circovirus type 2 infection in in vitro embryo production using naturally infected oocytes. Theriogenology, 126, pp.75-80.**

Porcine reproductive and respiratory syndrome virus (PRRSV)

The pathogenesis of porcine reproductive and respiratory syndrome virus (PRRSV) infection in ovary was studied in sexually mature, cycling, nonsynchronized gilts infected with the PRRSV 16244B, a virulent field strain. Previous studies have shown that PRRSV can be isolated from ovaries and is transplacentally passed from gilts to the fetuses. The cause of infertility following PRRSV infection is not known. In this study, we identified the tropism of PRRSV in ovarian tissue from experimentally infected gilts in samples collected between 7 and 21 days postinfection (DPI). Tissues were collected and examined by virus isolation, in

situ hybridization (ISH), immunohistochemistry (IHC), and double labeling to identify PRRSV-infected cell types. PRRSV was isolated in ovarian follicles at 7 days DPI. The IHC and ISH indicated that PRRSV-positive cells in ovaries were predominantly macrophages, which were numerous in atretic follicles. No evidence of infection and/or perpetuation of PRRSV in ova was observed, indicating that the female gonad is an unlikely site of persistence. No alteration of the normal ovarian architecture that would support a possible role of PRRSV infection in porcine female infertility was observed. **(Sur JH et al. Vet Pathol 2001;38:58-66)**

It is known that porcine pre-implantation embryos before the morula stage are refractory to infection with pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) (Bolin *et al.* 1981 Am. J. Vet. Res. 42, 1711–1712; Prieto *et al.* 1996 Theriogenology 46, 687–693, respectively). The effects of PRV and PRRSV on embryonic cells of morulae and blastocysts are unknown. Therefore, the objectives of the present study were to (1) assess the effects of PRV and PRRSV exposure on further embryo development, and (2) determine whether PRV and PRRSV are able to replicate in embryonic cells. Zona pellucida (ZP)-intact morulae (6 days post-insemination, 6 dpi) and early blastocysts (7 dpi) were microinjected subzonally with approximately 3 nL of 109 TCID50/mL PRV (strain 89v87, second passage in swine testicle cells) or 108.6 TCID50/mL PRRSV (Lelystad virus strain, 13th passage in swine alveolar macrophages). Control embryos were microinjected under the same circumstances with phosphate-buffered saline (PBS). Hatched blastocysts (8 dpi) were exposed for 1 h at 39C to 105 TCID50/mL of PRV or PRRSV of the same strains used for injecting earlier embryonic stages. Control hatched blastocysts were incubated with PBS. Each group of morulae and blastocysts consisted of approximately 20 embryos. Embryonic development was assessed every 12 h. At 48 h post injection, the percentage of infected embryos and the percentage of viral antigen positive cells per embryo were determined by immunofluorescence. Subzonal microinjection of ZP-intact morulae and blastocysts with PRV inhibited *in vitro* development in comparison to the controls. Moreover, under direct immunofluorescence, PRV antigen-positive cells were detected in association with the embryos. Exposure of hatched blastocysts to PRV inhibited further embryo development; the majority (16/20) of the embryos degenerated 24 h after incubation. Perivitelline microinjection of ZP-intact morulae and blastocysts with PRRSV and incubation of hatched blastocysts with PRRSV did not inhibit *in vitro* development in comparison to the controls. No PRRSV antigen positive cells were detected in association with the embryos. Based on these results, it can be deduced that embryonic cells of morulae and blastocysts are susceptible to PRV infection but refractory to PRRSV infection. Another argument substantiating insusceptibility of embryos to certain viral pathogens is the demonstration of the lack of virus receptors at a given embryonic cell stage. Therefore, the expression of sialoadhesin, the receptor that mediates the internalization of PRRSV in cells, was investigated in hatched blastocysts (*n*=10). By indirect immunofluorescence using monoclonal antibody 41D3 directed against porcine sialoadhesin, no positive signals were detected. The result of this experiment strengthens the statement that embryonic stages up to the hatched blastocyst stage are refractory to PRRSV infection. **Mateusen B et al, Reproduction, Fertility and Development 2006;18:214 (abstr.)**

Somatic cell nuclear transfer (SCNT) technology has become a powerful tool for reproductive biology to preserve and propagate valuable genetics for livestock. Embryo production through SCNT involves enucleation of the oocyte and insertion of a somatic donor cell into

the oocyte. These procedures lead to a few small openings on the zona pellucida that may elevate risk of viral infection for the produced SCNT embryos. The oocytes used for SCNT are mainly obtained from abattoirs where viral contamination is almost inevitable. Therefore, a systematic evaluation of risk of disease transmission through SCNT embryo production is necessary prior large scale implementation of this technology in the livestock industry. The objective of the current study was to evaluate the risk of disease transmission via SCNT embryo production and transfer by testing for the presence of porcine reproductive and respiratory syndrome virus (PRRSV) throughout the process of SCNT embryo production. The presence of PRRSV in each step of SCNT embryo production, from donor cells to pre-implantation SCNT embryo culture, was carefully examined using a real-time PCR assay with a sensitivity of five copies per-reaction. All 114 donor cell lines derived from pig skin tissue over a period of 7 years in our facility tested negative for PRRSV. Out of the 68 pooled follicular fluid samples collected from 736 ovaries, only four (5.9%) were positive indicating a small amount of viral molecule present in the oocyte donor population. All 801 Day 7 SCNT embryos produced in four separate trials and over 11,571 washed oocytes obtained in 67 batches over 10 months tested negative. These oocytes were collected from multiple abattoirs processing animals from areas with high density of pig population and correspond to a donor population of over 5828 individuals. These results indicate that the oocytes from abattoirs were free of PRRSV infection and therefore could be safely used for in vitro embryo production. Additionally, the established SCNT embryo production system, including donor cell testing, oocytes decontamination, and pathogen free embryo reconstruction and culturing, bears no risk of PRRSV transmission.

Gregg K, Xiang T, Arenivas SS, Hwang E, Arenivas F, Chen SH, Walker S, Picou A, Polejaeva I. Risk assessment of porcine reproductive and respiratory syndrome virus (PRRSV) transmission via somatic cell nuclear transfer (SCNT) embryo production using oocytes from commercial abattoirs. Anim Reprod Sci. 2011 May;125(1-4):148-57. Epub 2011 Apr 21

Pseudorabies virus

It is known that porcine pre-implantation embryos before the morula stage are refractory to infection with pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) (Bolin *et al.* 1981 Am. J. Vet. Res. 42, 1711–1712; Prieto *et al.* 1996 Theriogenology 46, 687–693, respectively). The effects of PRV and PRRSV on embryonic cells of morulae and blastocysts are unknown. Therefore, the objectives of the present study were to (1) assess the effects of PRV and PRRSV exposure on further embryo development, and (2) determine whether PRV and PRRSV are able to replicate in embryonic cells. Zona pellucida (ZP)-intact morulae (6 days post-insemination, 6 dpi) and early blastocysts (7 dpi) were microinjected subzonally with approximately 3 nL of 109 TCID50/mL PRV (strain 89v87, second passage in swine testicle cells) or 108.6 TCID50/mL PRRSV (Lelystad virus strain, 13th passage in swine alveolar macrophages). Control embryos were microinjected under the same circumstances with phosphate-buffered saline (PBS). Hatched blastocysts (8 dpi) were exposed for 1 h at 39C to 105 TCID50/mL of PRV or PRRSV of the same strains used for injecting earlier embryonic stages. Control hatched blastocysts were incubated with PBS. Each group of morulae and blastocysts consisted of approximately 20 embryos. Embryonic development was assessed every 12 h. At 48 h post injection, the percentage of infected embryos and the percentage of viral antigen positive cells per embryo were determined by immunofluorescence. Subzonal microinjection of ZP-intact morulae and blastocysts with PRV inhibited *in vitro* development in

comparison to the controls. Moreover, under direct immunofluorescence, PRV antigen-positive cells were detected in association with the embryos. Exposure of hatched blastocysts to PRV inhibited further embryo development; the majority (16/20) of the embryos degenerated 24 h after incubation. Perivitelline microinjection of ZP-intact morulae and blastocysts with PRRSV and incubation of hatched blastocysts with PRRSV did not inhibit *in vitro* development in comparison to the controls. No PRRSV antigen positive cells were detected in association with the embryos. Based on these results, it can be deduced that embryonic cells of morulae and blastocysts are susceptible to PRV infection but refractory to PRRSV infection. Another argument substantiating insusceptibility of embryos to certain viral pathogens is the demonstration of the lack of virus receptors at a given embryonic cell stage. Therefore, the expression of sialoadhesin, the receptor that mediates the internalization of PRRSV in cells, was investigated in hatched blastocysts (*n*=10). By indirect immunofluorescence using monoclonal antibody 41D3 directed against porcine sialoadhesin, no positive signals were detected. The result of this experiment strengthens the statement that embryonic stages up to the hatched blastocyst stage are refractory to PRRSV infection. **Mateusen B et al, Reproduction, Fertility and Development 2006;18:214 (abstr.)**

Chlamydophila/Chlamydia

Chlamydial infections of the genital organs cause reproductive failure in female pigs, and the uterus is recognized a target tissue for an infection. In contrast, information on the effect of chlamydiae on the porcine oviduct is patchily and inconclusive, although the bacteria are known to cause severe tubal defects in humans and laboratory animals. The aim of this study was to examine the segments ampulla (A), isthmus (I) and utero-tubal junction of the left (n = 20) or both (n = 22) oviducts, and uteri (U) from 42 culled repeat breeder pigs for chlamydiae using ompA-PCR, partial ompA gene sequencing, immunohistochemistry (IHC) and microscopy of tissue specimens for histopathology. As revealed by PCR, among a total of 26 chlamydia-positive females, 19 were tested positive in one or more segments of one or both oviducts, 14 were found positive in the uterus, and concomitant infections of both organs were observed in 7 of them. Sequencing of 33 PCR products revealed the following chlamydial species: Chlamydophila (Cp.) psittaci (n = 18), Cp. abortus (n = 2), Chlamydia (C.) suis (n = 10), and C. trachomatis (n = 3). Immunopositive staining was observed within the surface epithelium (in A, I, U), stromal tissue (in I, U) and muscular layer (in A, I, U). A total of 24 females had inflamed oviductal segments (in A and/or I) and 36 inflamed uteri. However, there was no relationship between histopathology and results of PCR or IHC. In conclusion, chlamydiae were found to infect oviducts and uteri of pigs. Further studies are required to clarify whether chlamydial infection causes specific histopathology and alters tubal function. **Kauffold J et al, Theriogenology 2006;66:1816-1823.**

Sheep

Maedi-Visna Virus (MVV)

The aim of this study was to examine the Maedi-Visna virus (MVV) infection status of oocytes, cumulus cells, and follicular fluid taken from 140 ewes from breeding flocks. MVV proviral-DNA and MVV RNA were detected using nested-PCR and RT-PCR MVV gene

amplification, respectively in the gag gene. Nested-PCR analysis for MVV proviral-DNA was positive in peripheral blood mononuclear cells in 37.1% (52/140) of ewes and in 44.6% (125/280) of ovarian cortex samples. The examination of samples taken from ovarian follicles demonstrated that 8/280 batches of cumulus cells contained MVV proviral-DNA, whereas none of the 280 batches of oocytes taken from the same ovaries and whose cumulus cells has been removed, was found to be PCR positive. This was confirmed by RT-PCR analysis showing no MVV-viral RNA detection in all batches of oocytes without cumulus cells (0/280) and follicular fluid samples taken from the last 88 ovaries (0/88). The purity of the oocyte fraction and the efficacy of cumulus cell removal from oocytes was proved by absence of granulosa cell specific mRNA in all batches of oocytes lacking the cumulus cells, using RT-PCR. This is the first demonstration that ewe cumulus cells harbor MVV genome and despite being in contact with these infected cumulus cells, the oocytes and follicular fluid remain free from infection. In addition, the enzymatic and mechanical procedures we used to remove infected-cumulus cells surrounding the oocytes, are effective to generate MVVfree-oocytes from MVV-infected ewes. (**Cortez Romero C et al, Theriogenology 2006;66:1131-1139.)**

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The objective of this study was to determine whether MVV can be transmitted by ovine embryos produced in vitro and whether the zona pellucida (ZP) provides any protection against MVV infection. Zona pellucida (ZP)-intact and ZP-free embryos, produced in vitro, at the 8-16 cell stage, were cocultured for 72h in an insert over an ovine oviduct epithelial cell (OOEC)-goat synovial membrane (GSM) cell monolayer that had been previously infected with MVV (K1514 strain). The embryos were then washed and transferred to either direct contact or an insert over a fresh GSM cell monolayer for 6 h. The presence of MVV was detected using RT-PCR on the ten washing fluids and by the observation of typical cytopathic effects (CPE) in the GSM cell monolayer, which was cultured for 6 weeks. This experiment

was repeated 4 times with the same results: MVV viral RNA was detected using RT-PCR in the first three washing media, while subsequent baths were always negative. Specific cytopathic effects of MVV infection and MVV-proviral DNA were detected in GSM cells that were used as a viral indicator and cocultured in direct contact or as an insert with MVV-exposed ZP-free embryos. However, no signs of MVV infection were detected in cells that were cocultured with exposed ZP-intact or non-exposed embryos. This study clearly demonstrates that (i) in vitro, ZP-free, early ovine embryos, which had been exposed to 10(3) TCID(50)/m MVV in vitro, are capable of transmitting the virus to susceptible GSM target cells, and that (ii) the IETS recommendations for handling in vivo produced bovine embryos (use of ZP-intact embryos without adherent material and performing ten washes) are effective for the elimination of in vitro MVV infection from in vitro produced ovine embryos. The absence of interaction between ZP-intact embryos and MVV suggests that the in vitro produced embryo zona pellucida provides an effective protective barrier.
Romero CC, Pellerin JL, Poulin N, Cognié Y, Chebloune Y, Pépin M, Fieni F. Maedi-Visna virus was detected in association with virally exposed IVF-produced early ewes embryos. Theriogenology. 2010 Sep 1;74(4):682-90

Rat

Antiviral treatments (rat embryos)

BACKGROUND: Previous guidelines for HIV-infected pregnant women have recommended zidovudine (ZDV) monotherapy during the second and third trimesters of pregnancy to prevent fetal HIV infection. New guidelines suggest that women should continue or be offered combination antiretroviral therapy (including protease inhibitors) during pregnancy. Nevertheless, little animal or human toxicity data underlie these recommendations. METHODS: We used an in vitro rat whole embryo culture system to assess the embryo toxicity of various nucleoside analogues, namely, ZDV, dideoxyinosine (ddI), and 2', 3'-dideoxycytidine (ddC), and the HIV-1 protease inhibitor, indinavir, both alone and in combination. RESULTS: Although human fetal concentrations of these compounds are unknown, no gross abnormalities were detected after incubation with these agents, either alone or in combination at concentrations that would be expected to be achievable in human maternal serum (1-50 microM). ZDV in combination with ddC at >100 microM, resulted in severe growth retardation and morphologic abnormalities not seen with either agent singly. CONCLUSIONS: We conclude that the combination of ZDV/ddC results in severe concentration-dependent embryo toxicity. No growth retardation or gross morphologic abnormalities were found for any of the agents, either singly or in combination, at clinically relevant concentrations. **(Fujinaga M et al. Teratology 2000;62:108-114)**

Human

Human (Hepatitis B&C, HIV, Rubella)

The Human Fertilisation and Embryology Authority requires all sperm donors to be screened for human immunodeficiency virus (HIV), hepatitis B and C and their semen quarantined for six months. No guidelines exist for screening prior to in vitro fertilisation or intracytoplasmic sperm injection. We prospectively analysed the prevalence of these viruses in our patients. Screening detected one case of HIV (0.13%), four of hepatitis C (0.5%) and 14 new cases of hepatitis B (1.7%). The prevalence of hepatitis B and HIV in our antenatal population at this time was 1.4% and 0.8%, respectively. Knowledge allows measures to be taken to reduce the risk of transmission to partner, fetus, new born baby, or by cross-contamination during embryo cryostorage and enables couples to make an informed decision regarding proceeding with treatment. Detection of infection in one Partner should no longer preclude fertility treatment. **(Hart R et al. BJOG 2001;108:654-656)**

OBJECTIVE: We sought to assess the fertilization, implantation, and ongoing pregnancy rates with a minimal precycle and ongoing cycle monitoring protocol for in vitro fertilization and embryo transfer. STUDY DESIGN: Retrospective review was conducted of 103 consecutive cycles of fresh in vitro fertilization and embryo transfer from 1996 to 1998. Precycle screening included semen analysis without strict morphologic analysis, and hysterosalpingography-sonohysterography within the last year. Serum prolactin, serum thyroid-stimulating hormone, reactive plasma reagin, human immunodeficiency virus, rubella titer, blood type, hepatitis B surface antigen, and hepatitis C antibody testing was performed on all patients within 3 months of cycle initiation. Women > or =37 years old underwent clomiphene challenge testing. The monitoring protocol included the following: baseline transvaginal ultrasonography after 12 to 14 days of midluteal gonadotropin-releasing hormone agonist down-regulation to assess endometrial thickness and adnexal appearance, transvaginal ultrasonography for follicle evaluation at 7 and 10 days, serum estradiol assay if > or =20 follicles, quantitative beta-human chorionic gonadotropin 12 to 14 days after pre-embryo transfer, repeat quantitative beta-human chorionic gonadotropin 3 to 5 days later, and transvaginal ultrasonography for intrauterine gestational sac confirmation 7 to 9 days after the initial positive pregnancy test result. The dose of gonadotropin used remained constant unless the sonogram obtained on day 7 indicated a suboptimal response (<3 follicles each, with an average diameter of 10 to 12 mm) or hyperresponse (> or =15 follicles with an average diameter of 10 to 12 mm). RESULTS: The per embryo implantation rate (fetal cardiac activity) was 13.1%, and the live birth rate per 100 pre-embryo transfers was 31.5 for patients < or =40 years old. The average number of pre-embryos transferred was 3.1. The singleton pregnancy rate was 71%, and there were no multiple gestations greater than twins. The mean number of oocytes fertilized was 66%. There was 1 case of failed fertilization with intracytoplasmic sperm injection. There were two other cases of failed fertilization. One case of severe ovarian hyperstimulation occurred in spite of cryopreservation of all embryos. CONCLUSIONS: In vitro fertilization and embryo transfer can be accomplished with minimal precycle testing and ongoing cycle monitoring without compromising fertilization, implantation, and ongoing pregnancy rates. This results in reduced overall costs for couples. **(Strawn EY,Jr et al. Am J Obstet Gynecol 2000;182:1623-1628)**

OBJECTIVE: To investigate the incidence of microorganism contamination in in vitro fertilizationembryo transfer (IVFET) and to determine the sources of microorganism. METHODS: Two thousand one hundred and seventyfour cycles of in vitro fertilization from January 1999 to June 2003 were evaluated retrospectively and bacterial cultures were

performed in 61 semen samples from asymptomatic men with normal semen parameters and in 34 follicle fluid samples from infertility women through oocyte picking up procedures. RESULTS: Microorganisms were found in 11 cases. The incidence of their contamination in IVF culture system was 0.51% and the most common microorganisms were *Escherichia coli* and fungi. Microorganisms were detected in 97% of unprocessed semen, 10% in processed semen, 6% in semen mixed with media and 9% in follicle fluid. CONCLUSIONS: The incidence of microorganism contamination was 0.51% and the most common microorganisms were Escherichia coli and fungi. Semen may have the potential to contaminate IVF culture system. **Zhu GJ et al, Zhonghau Fu Chan Ke Za Zhi 2004;39:382-384.**

Acquired immunodeficiency syndrome (AIDS) is a major public health problem worldwide. This study was performed to explore the feasibility of vertical transmission of human immunodeficiency virus-1 (HIV-1) gag gene via oocyte. The recombinant plasmid (pIRES2-EGFP-gag) was injected into mouse ovaries to transfect germ cells. Induction of superovulation and then animal mating were performed to collect oocytes and two-cell embryos. Positive FISH signals for HIV-1 gag DNA were detected in the nuclei of oocytes and embryos, and in chromosomes of mature oocytes, indicated integration of the gene into the oocyte genome and gene replication in the embryo. HIV-1 gag cDNA positive bands detected by RT-PCR in oocytes and embryos indicated successful gene transcription, while positive immunofluorescence signals for HIV-1 gag protein indicated successful translation in both oocytes and embryos. The HIV-1 gag gene was transmitted vertically to the next generation via oocytes and it retained its function in replication, transcription and translation following at least one mitotic division in embryos.
Gao YS, Huang TH, Wang D, Xie QD, Kang XJ. In vivo study on vertical transmission of the HIV-1 gag gene via mouse oocytes. Curr HIV Res. 2009;7(5):562-8.

(Human) - "By immunocytochemistry and in situ hybridization at the electron microscopy level, and by the PCR technique, we have shown that HIV-1 binds and enters normal sperm, that viral particles their antigens, and nucleic acid are present in sperm from HIV-1 infected men: and that such sperm can transfer HIV-1 like particles to normal human oocytes. We also present evidence that galactosylceramide-like compound is present in the sperm membrane and could function as an alternative receptor for HIV. (**Baccetti B, et al. J Cell Biol 1994;127:903-914**)

Rabbits

Genetically valuable New Zealand White rabbits were contaminated by different types of pathogenic agents such as parasite (*Encephalitozoon cuniculi*), a bacteria (*Clostridium perfringens*) and a virus (rotavirus). To regain the SPF status of this contaminated breeding unit, embryos from the infected does were transferred into SPF recipient females. Does (n=32) received ovarian stimulation and were sacrificed 62 to 70 hours post mating to recover compact morulae. After laparotomy, uterine horns were excised. Retrograde flush of uterine horns was performed with DPBS added with 10 % FCS. After collection, quality of embryos was evaluated to select only embryos suitable for cryopreservation and further transfer. Only zona pellucida intact embryos were washed. All embryos with cellular

inclusions inside their mucin coat were discarded. Selected embryos from each donor were washed 10 successive baths of DPBS to dilute contaminants as much as possible Embryos were cryopreserved in 1.5 M DMSO in DPBS added with 20 % FCS in 0.15 ml transparent straws. All the surgical embryo transfers were performed in SPF conditions. Recipient rabbits (n=32) received IV injection of 75 IU hCG to induce pseudo pregnancy. At 60 h after injection, thawed embryos were introduced into fallopian tube (5 to 9 morulae per oviduct). Health screenings were performed on sanitised rabbits 24-26 weeks after birth to follow Federation of European Laboratory Animal Science Association guidelines. Eight hundred and ninety-three embryos were collected, among which 92 % (821) had an intact zona pellucida and were selected for cryopreservation (ave of 25.7 per donor, 821/32). To preserve the genetic value of the breed, 40 % of frozen embryos were kept in liquid nitrogen. From 478 thawed embryos, 97.5 % were recovered (499) and 89.5 % showed good quality mucin layer and zona pellucida (417) and were transferred into 36 recipients (10 to 18 embryos per recipient). After transfer, 72 % of recipients delivered (26/36) and 24.9 % (104/147) of transferred embryos developed to full term into live pups. One year later, health screenings performed on sanitised rabbits confirmed the recover of the SPF health status. In conclusion, we demonstrated ET of washed cryopreserved embryos is an effective method to eliminate different types of pathogenic agents in the rabbit species. This method is suitable for rabbit embryos which are enclosed in a mucin coat provided embryos with mucin attached cells are eliminated to avoid possibility of disease transmission. **(Ectors FJ et al. Proceedings, 18e Reunion AETE, Rolduc, 06-07 September 2002, page 164)**

Mice

Mouse Minute Virus (MMV)

In the present study, the risk of transmission of mouse minute virus (MMV) to recipients of murine embryos arising from in vitro fertilization (IVF) of oocytes with MMV-exposed spermatozoa and to resulting pups was evaluated. Also, the time of seroconversion of recipients and pups was investigated. To achieve this goal, IVF of oocytes with cryopreserved spermatozoa from the inbred C3HeB/FeJ mouse strain was performed and resulting embryos transferred to suitable Swiss recipients. Three groups were investigated: 1) oocytes or the developing embryos were continuously exposed to 10(4) $TCID_{50}$ MMVp/ml in the fertilization (HTF), culture (KSOM) and embryo transfer (M2) media (positive control), 2) oocytes and spermatozoa were exposed to MMVp in the HTF medium only and transferred after a standard washing procedure with 10 washing steps in virus-free KSOM and M2, and 3) oocytes and spermatozoa were exposed to virus-free HTF, KSOM and M2 (negative control). To detect antibodies to MMV in recipients and progeny, serological analyses were performed by ELISA on d14, 21, 28, and 42, and on d42 and 63 post embryo transfer, respectively. The presence of MMV in the washing drops was analyzed by PCR and an in vitro infectivity assay while organs of some recipients and pups were analyzed by PCR. Using 10(4) $TCID_{50}$ MMVp/ml in the fertilization medium only, the present results demonstrate that 10 washing steps in the IVF-ET procedure are sufficient to remove the virus to a non-infectious dose, producing MMV-free seronegative recipients and pups. As such, there is minimal risk of transmission of MMV to recipients and pups if spermatozoa become contaminated with such viral loads. **(Mahabir E, et al. Biology of Reproduction, 2008. 78(1):53-8.**

The risk of transmission of mouse minute virus (MMV) to recipients of murine embryos arising from in vitro fertilization (IVF) of cumulus-enclosed oocytes (CEOs) or without cumulus cells (CDOs) in the presence of MMV-exposed (10(4) TCID(50) [mean tissue culture infective dose]/ml MMVp [prototype strain of MMV]) spermatozoa was evaluated. Also, the time after embryo transfer to detection of MMV antibody and the presence of MMV DNA in the mesenteric lymph nodes of recipients and pups were investigated. All mice were MMV free, but two seropositive recipients and four seropositive pups were found in the group with CDOs. With regard to the CEOs, two of 11 holding drops and five of 11 groups of embryos were MMV positive using PCR, while neither holding drops nor embryos carried infectious MMVp, as evidenced by the in vitro infectivity assay. From IVF with CDOs, five of 14 holding drops and four of nine groups of embryos were MMV positive, while one of 14 holding drops and no embryos carried infectious MMVp. When 10(5) cumulus cells were analyzed 5 h after exposure to 10(4) TCID(50)/ml MMVp, cells had an average titer of 10(4) TCID(50)/ml MMVp. The present data show that, in contrast to CDOs, 2-cell embryos from CEOs did not transmit infectious MMVp to the holding drops and to recipients. This observation is due to the presence of cumulus cells during the IVF process that reduce entry of MMV into the zona pellucida and absorb some of the virus. These data further confirm the efficacy of the IVF procedure in producing embryos that are free of infectious virus, leading to virus-free seronegative recipients and rederived pups.

Mahabir E, Bulian D, Needham J, Schmidt J. Lack of transmission of mouse minute virus (MMV) from in vitro-produced embryos to recipients and pups due to the presence of cumulus cells during the in vitro fertilization process. Biol Reprod. 2009;81(3):531-8.

Mouse minute virus and & Mouse Hepatitis Virus

The aim of this study was to determine the susceptibility of murine embryonic stem (mESCs) to mouse hepatitis virus (MHV-A59) and mouse minute virus (MMVp) and the effect of these viruses on germline transmission (GLT) and the serological status of recipients and pups. When recipients received 10 blastocysts, each injected with 10(0) TCID(50) MHV-A59, three out of five recipients and four out of 14 pups from three litters became seropositive. When blastocysts were injected with 10(-5) TCID(50) MMVp, all four recipients and 14 pups from four litters remained seronegative. The mESCs replicated MHV-A59 but not MMVp, MHV-A59 being cytolytic for mESCs. Exposure of mESCs to the viruses over four to five passages but not for 6 h affected GLT. Recipients were seropositive for MHV-A59 but not for MMVp when mESCs were cultured with the virus over four or five passages. The data show that GLT is affected by virus-contaminated mESCs.

Mahabir E, Reindl K, Mysliwietz J, Needham J, Bulian D, Markoullis K, Scherb H, Schmidt J. Impairment of germline transmission after blastocyst injection with murine embryonic stem cells cultured with mouse hepatitis virus and mouse minute virus. Transgenic Res. 2009;18(1):45-57.

HIV-1 (AIDS)

Acquired immunodeficiency syndrome (AIDS) is a major public health problem worldwide. This study was performed to explore the feasibility of vertical transmission of human immunodeficiency virus-1 (HIV-1) gag gene via oocyte. The recombinant plasmid (pIRES2-

EGFP-gag) was injected into mouse ovaries to transfect germ cells. Induction of superovulation and then animal mating were performed to collect oocytes and two-cell embryos. Positive FISH signals for HIV-1 gag DNA were detected in the nuclei of oocytes and embryos, and in chromosomes of mature oocytes, indicated integration of the gene into the oocyte genome and gene replication in the embryo. HIV-1 gag cDNA positive bands detected by RT-PCR in oocytes and embryos indicated successful gene transcription, while positive immunofluorescence signals for HIV-1 gag protein indicated successful translation in both oocytes and embryos. The HIV-1 gag gene was transmitted vertically to the next generation via oocytes and it retained its function in replication, transcription and translation following at least one mitotic division in embryos.
Gao YS, Huang TH, Wang D, Xie QD, Kang XJ. In vivo study on vertical transmission of the HIV-1 gag gene via mouse oocytes. Curr HIV Res. 2009 Sep;7(5):562-8.

P. pneumotropica and P. ureae

P. pneumotropica and *P. ureae* were isolated from the uteri and oviducts of donors and the seminal vesicles and testes of donor males. Morphologically intact embryos collected from infected donors were of two types. Either they were recovered from the oviducts in an advanced stage of degeneration or they appeared normal but failed to develop in culture. Antibiotic (kanamycin) in the culture media did not improve results even though both species of *Pasteurella* showed in vitro sensitivity to it. Over a four month period, 984 4-cell, 8-cell, and early morulae stage embryos from infected donors were cultured in a modified Brinster's medium. Overall normal development for all 984 embryos cultured was 14.4% (+9.8%) with the remaining embryos undergoing rapid degeneration after short periods in culture. New non-infected mice introduced into the same colony produced viable embryos that developed normally when placed in the same culture system. Seventy-eight percent (+20.4%) of three hundred 4-cell, 8-cell, and early morulae stage embryos from these donors developed to the hatched blastocyst stage in culture. However, after these mice had been in the infected colony for a period of one month their embryos failed to develop normally and *P. pneumotropica* and *P. ureae* were subsequently isolated from their tissues. Both species of Pasteurella reported in this case have been found to cause infertility and abortion in mice..." (**Hagele WC, and Bielanski A, Theriogenology 1987;27:235 abstr**)

Neospora caninum

Vertical transmission of *Neospora caninum* was evaluated in BALB/c mice using an *N caninum*-specific PCR assay as a means of detecting parasite transmission to offspring. BALB/c mice were infected with the NC-1 isolate of *N caninum* during pregnancy (days 8-15). Transmission of parasite, detected by PCR, was determined in 2- to 23-day-old offpring. When dams were infected on days 13-15 of gestation, transfer of parasites was detected in only a portion of the litter. Infection between days 8 and 12 of gestation resulted in a high frequency of parasite transmission (every offspring from all litters was infected). In young pups (2- to 4-day-old), the predominant sites of infection were the lungs and the brain. In older pups (7- to 23-day-old) the predominant site of infection was the brain. **(Liddell S, et al, J Parasitology 1999;85:550-555)**

Ecotropic murine leukemia viruses (MuLV)

Mouse strains carrying endogenous ecotropic murine leukemia viruses (MuLV) are capable of expressing infective virus throughout life. Risk of transplacental transmission of MuLV raises concerns of embryo infection and induction of pathogenic effects, and postnatal MuLV infection may lead to tumorigenesis. METHODS: Endogenous ecotropic MuLV-negative SWR/J embryos were implanted into Akv-infected viremic SWR/J mice, into spontaneously provirus-expressing AKR/J mice, and into noninfected SWR/J control mice; virus integration and virus expression were investigated at 14 days' gestation. Tumor development was monitored over 18 months. RESULTS: Of 111 embryos, 20 (18%) recovered from Akv-infected SWR/J mice, which had developed normally, were infected. New proviruses were detected in 10 of 111 (9%) embryos from Akv-infected SWR/J mice, and in 2 of 60 (3%) embryos from AKR/J mice; none expressed viral protein. Of 127 embryos recovered from Akv-infected SWR/J mice, 16 (13%) were dead; 4 of 5 (80%) were infected and expressed viral protein. Of 71 embryos from AKR/J mice, 11 (15%) were dead, and 2 of 2 had virus integration; virus expression was not detected. Numbers of dead embryos recovered from experimentally infected, viremic SWR/J mice and from spontaneously endogenous MuLV-expressing AKR/J mice were significantly higher, compared with numbers from nonviremic SWR/J control mice, and embryo lethality was significantly associated with prenatal provirus expression. Postnatal inoculation of Akv induced lymphoblastic lymphomas in 15 of 24 (61%) SWR/J mice within mean +/- SD latency of 14 +/- 2.4 months. Only 3 of 39 (8%) control mice developed lymphomas ($P < 0.005$). CONCLUSION: Embryos in MuLV-viremic dams are readily infected, and inappropriate prenatal expression of leukemogenic endogenous retroviruses may play a critical role in embryo lethality and decreased breeding performance in ecotropic provirus-positive mouse strains. **(Hesse I et al, Lab Anim Sci 1999;49:488-489)**

Mouse Hepatitis Virus (MHV)

The aim of this study was to estimate the risk of MHV transmission by the in vitro fertilization and embryo transfer (IVF-ET) procedure. In addition, resistance to infection of zona-intact and laser-microdissected oocytes was compared. For this purpose, infectious Mouse Hepatitis Virus (MHV), a common viral pathogen in mouse facilities, was utilized. Oocytes having an intact or laser-microdissected zona pellucida were incubated for fertilization in media containing MHV-A59 and resulting embryos were transferred to the oviduct of specific pathogen free (SPF) Swiss recipients. The oocytes were divided into three experimental groups: 1) zona-intact oocytes continuously exposed to MHV in fertilization (HTF), culture (KSOM) and embryo transfer (M2) media, 2) zona-intact oocytes exposed to MHV in HTF medium and transferred after a standard washing procedure with virus-free KSOM and M2 and 3) laser-microdissected oocytes exposed to MHV in HTF medium and transferred after a standard washing procedure with virus-free KSOM and M2. Respective serum samples of embryo recipients and their offspring were tested for MHV-antibodies using ELISA. In experiment 1, 10 out of 14 embryo recipients seroconverted to MHV and only their offspring (8 of 19) received maternal antibodies. In experiment 2 and 3 MHV antibodies were detected neither in the recipients nor in the offspring. These results indicate for the first time that even if the zona pellucida is partially disrupted by laser microdissection, the transmission of MHV-A59

can be avoided by correctly performed washing steps in the IVF-ET procedure. (**Peters DD, et al. Biol Reprod 2006; 74:246-52.)**

Bison

Brucellosis

Reproductive procedures for cattle were adapted to American bison (*Bison bison*) to evaluate the potential preservation of germ plasm from bison infected with *Brucella abortus* without transmission of pathogen to the recipient or offspring. Two of 4 experimentally inoculated bison bulls excreted *Brucella abortus* in the semen. Four healthy calves were produced from non-infected, unvaccinated bison cows by natural breeding with a bison bull excreting *Brucella abortus* in the semen. There was no seroconversion of the cows or their calves. Twenty viable embryos obtained after superovulation of infected bison were washed according to IETS standards in medium with 100µg/ml of streptomycin and transferred to 9 recipients. Two culture negative bison calves were produced after embryo transfer. *B abortus* was not transmitted to recipient cows or calves. It was concluded that these limited data indicate that embryo manipulation procedures and natural breeding in bison may facilitate preservation of valuable germ plasm from infected bison while reducing risk of transmission of brucella to recipients and progeny. **(Robinson CD, et al, J Wildlife Diseases 1998;34:582-589)**

EMBRYO DISINFECTION

Biosecurity

Reviews In taking the bovine species as a model, the aim of the present paper is to provide an overview of the biohazards and biosecurity issues related to transfers of in vivo derived and in vitro produced embryos. Sound scientific data have been elaborated by the researchers along the last decades which have been able to generate appropriate regulatory measures. There are risks at stakes here reported, to associate pathogens to embryos both in vivo collected or in vitro produced but there are here described, efficient means to mitigate them to a negligible level. Those have been identified and assessed through an active participation of the International Embryo Transfer Society/ Health And Safety Advisory Committee, then acting as an ad hoc committee to the World Animal Health Organization (OIE). This has allowed the OIE to introduce those measures as recommendations into its Terrestrial Animal Health Code giving the international world efficient and workable proven guidelines. The basic principles rely on the critical and ethical role of the embryo transfer or the embryo production teams under the leadership of one veterinarian. Several decades of experience with close to 10 million of embryos transferred since the beginning of this century have demonstrated that providing that those rules are strictly followed, biosecurity is a real added value to these technologies

M Thibier Biosecurity, its added value to Embryo Transfer. *Acta Scientiae Veterinariae*, 38 (Supl 2) s649-s659, 2010

This paper uses cattle as a model to provide an overview of the hazards involved in the transfer of in vivo-derived and in vitro-produced embryos. While scientific studies in recent decades have led to the identification of pathogens that may be associated with both in vivo- and in vitro-derived embryos, those studies have also been the basis of appropriate disease control measures to reduce the risks to a negligible level. These disease control measures have been identified and assessed by the International Embryo Transfer Society's (IETS) Health and Safety Advisory Committee, the expert body that advises the World Organisation for Animal Health (OIE) on matters related to the safety of embryo transfer. Through the OIE's processes for developing and adopting international standards, the disease control measures identified by the IETS have been incorporated into the Terrestrial Animal Health Code. The basic principles rely on the crucial ethical roles of the embryo collection team and embryo transfer team, under the leadership of approved veterinarians. Decades of experience, with nearly 10 million embryos transferred, have demonstrated the very significant biosecurity advantage that embryo transfer technology has when moving germplasm internationally, provided that the international standards developed by the IETS and adopted by the OIE are strictly followed.

M. Thibier Embryo transfer: a comparative biosecurity advantage in international movements of germplasm Rev. sci. tech. Off. int. Epiz., 2011, 30 (1), 177-188

Trypsin treatment

Embryos were collected nonsurgically from superovulated cows (n = 19) 7.5 d after insemination. Grade 1 and Grade 2 embryos were washed 12 times in modified Dulbecco's phosphate buffered saline (PBS) containing 0.4% bovine serum albumin (BSA), or in a sequence of five washes in BSA-PBS (without Ca^{++} and Mg^{++}), two in 0.25% trypsin in Hank's solution (without Ca^{++} and Mg^{++}), and five in PBS-BSA medium. Within 30 min after washing, embryos were either transferred nonsurgically into recipient cows, 7 to 8 d post estrus, or cryopreserved and transferred later. Frozen-thawed embryos from five of the donors were cultured for 72 h in vitro and their development was evaluated. Pregnancy rates did not differ ($P>0.1$) between recipient cows receiving control-washed and trypsin-washed embryos transferred fresh [25/49 (51.0%) vs 27/48 (56.3%)]. However, pregnancy rates were higher ($P <0.05$) for frozen-thawed embryos treated with trypsin before cryopreservation than for frozen-thawed, control-washed embryos [15/22 (68.2%) vs 10/26 (38.5%)]. Survival and development of embryos in vitro after cryopreservation did not differ between embryos subjected to the control and trypsin-wash procedures. These results suggest that exposure of bovine embryos to trypsin for 2 to 3 min during washing did not have a detrimental effect on embryonic development, but may have enhanced viability of the embryos which were cryopreserved (**Echternkamp et al, Theriogenology 1989;32:131-137**).

The transfer of 329 bovine embryos that had been trypsin-treated according to IETS recommendations resulted in a 64% pregnancy rate compared to a 55% pregnancy rate for 121 control embryos (**Hasler and Reinders, Proc Am Emb Trans Assoc Meet 1988;pp91-93**).

In a series of experiments trypsin treatment of bovine embryos was carried out according to IETS recommendations. Control embryos were handled similarly except for non exposure to

the trypsin solution. In experiment I embryos were transferred fresh after trypsin treatment. Transfer of 32 trypsin-treated embryos resulted in a pregnancy rate at 60 d of 53.1% compared to 41.4% from the transfer of 29 control embryos. In experiment II embryos were frozen after trypsin treatment. After thawing they were evaluated for in vitro development after 48 hrs in culture at 38°C. Fifty-six point seven per cent of 30 trypsin-treated embryos developed compared to 50% of 28 control embryos. In experiment III embryos were trypsin treated and then frozen. After thawing they were transferred and the pregnancy rate determined at 60 d. Transfer of 65 treated embryos gave a pregnancy rate of 49.2% compared to 54.1% from the transfer of 61 control embryos. (**Bondioli et al, Proc Am Emb trans Assoc Meet 1988;85-89**).

In vitro produced embryos (blastocysts) at 7-8d after insemination were washed 12 x in Dulbecco's PBS with 20% FBS or in a sequence of washes, ie 3 in DPBS with 20% FBS, 2 in DPBS with 0.3% BSA, 2 in 0.25% trypsin in Hanks solution without Ca^{++} and Mg^{++} (for a total time in trypsin of 60-90 seconds) and 5 in DPBS with 20% FBS. Some embryos were also kept in trypsin for 5-10 minutes during washing to evaluate the effects of longer exposures on in vitro development. After washing embryos were used fresh or they were frozen. Fresh and frozen - thawed embryos were either cultured for 72 h., to evaluate their in vitro development, or they were transferred non-surgically to recipient cows. Development (ie hatching) in vitro of the fresh embryos after 60-90 seconds trypsin treatment was not significantly different from that of controls, ie 37/45 (82.2%) versus 33/43 (76.7%) respectively. Similarly, trypsin treatment for 60-90 seconds had no significant effect on development in vitro of the frozen-thawed embryos: 18/41 (43.9%) versus 21/45 (46.7%) respectively for the trypsin and control groups. When the trypsin exposure time was increased to 5 minutes, 11/12 (91.7%) of fresh embryos hatched in culture, but further increase to 10 minutes exposure led to a significant ($P < 0.05$) reduction in hatching, ie 7/13 (53.8%). Pregnancy rates for embryos transferred fresh following trypsin treatment were lower (NS) than those for embryos washed without trypsin, ie 3/10 (30%) versus 14/21 (66.7%), but the rates for trypsin and control groups transferred after freeze-thawing were the same, ie 3/11 (27.3%) versus 3/11 (27.3%). (**Otoi et al, J Vet Med Sci 1993;55:237-239**).

According to the Manual of the International Embryo Transfer Society (Stringfellow and Seidel (Eds.), 3rd Edition,1998), it is important to store trypsin as a frozen stock and to prepare working solutions immediately before their use for disinfecting embryos of specific pathogens. However, it is evident that many practitioners do not follow such guidelines and some are known to refrigerate and re-use trypsin solutions for prolonged periods. Since it is not possible to enforce or ensure that trypsin solutions are prepared properly, there is a potential risk that disease transmission will occur via embryo transfer if the embryos were processed using trypsin solutions that were unknowingly inactive. The objective of this practical study, therefore, was to test the activity of trypsin after prolonged refrigerated storage. Aliquots of a working solution of trypsin (0.25%) were prepared from a 2.5% trypsin stock (T-4549, Sigma Chemical Co, St. Louis, MO) and immediately frozen (to serve as a positive control) and the remaining volume was placed in a conventional refrigerator. Aliquots of the refrigerated and frozen (freshly thawed) working solutions were removed weekly, warmed at 38°C for 30 min, and then used to dissociate confluent Buffalo rat liver (BRL) cell monolayers cultured in Falcon 35mm 6-well plates. The time taken from the point BRL cells were initially affected (cells rounding and lifting) to the time that >90% were

dissociated and detached from the surface of the culture dishes, were recorded and compared between the refrigerated and frozen-thawed trypsin aliquots. After 5 months of the weekly exercise, the refrigerated trypsin solution would dissociate and detach the confluent BRL cell monolayers within the same timeframe from initial exposure as the freshly prepared (frozen-thawed) trypsin aliquot (P>.05, t-test), Table 1.

Table 1.Mean times for confluent BRL cell m onolayers to detach after exposure To 0.25% trypsin stored at –20°C vs. 4°C for 5 months. (Initial= first sign of cells rounding and lifting; Final = >90% cells detached).

Trypsin Storage	Initial Time (s)	Final Time (s)
–20°C	41.6 ± 4.6	238.4 ± 23.2
4°C	36.4 ± 3.3	236.2 ± 22.

In conclusion, it is evident by the results of this study that trypsin activity, as measured by the time taken for a 0.25% working solution to dissociate and detach >90% of confluent BRL cell monolayers, is not depleted after prolonged refrigerated storage of up to five months. In practice, however, it is important to stress that proper aseptic handling be maintained to avoid the risk of contaminating refrigerated trypsin solutions with psychrophilic bacteria and fungi. **(Loskutoff NM, Morfeld KA, Chrichton EG. Trypsin activity after prolonged refrigerated storage. Theriogenology 2003;59:383 abstr.)**

Porcine-origin trypsin will effectively remove bovine herpesvirus-1 (BHV-1) from *in vivo*-derived embryos. It is not known if TrypLE (Invitrogen, Carlsbad, CA, USA) could be used to remove BHV-1, but this recombinant porcine sequence trypsin-like protease would be an attractive alternative because it is highly stable at room temperature and does not pose the same threat for contamination as animal-origin trypsin. Thus, the objective of this study was to determine if TrypLE Express (1X) for 1.5 min of exposure or TrypLE Select (10X) for 10 min of exposure would be effective at removing BHV-1 from Day 7 zona pellucida-intact, *in vivo*-derived embryos after they had been exposed to the virus. Day 7 bovine *in vivo*-derived morulae and blastocysts and non-fertile degenerate (NFD) embryos were collected and shipped overnight to our facility. Upon arrival, the zona pellucida intact embryos were washed according to the International Embryo Transfer Society protocol. Developed embryos were washed separately from NFD embryos. One group of 5 or 10 NFD or developed embryos was not exposed to virus and served as the negative control. The remaining embryos and 10 NFD were exposed to 106 PFU/mL BHV-1 (Colorado strain) for 1 h. Following exposure, one group of 5 or 10 NFD or developed embryos was washed and served as the positive control. One group of 10 developed embryos was washed and treated with porcine origin trypsin. The remaining developed embryos were divided into groups of 5 or 10 and washed and treated with TrypLE Express for 1.5 min or TrypLE Select (10X) for 10 min. Following treatment, the embryos were sonicated in groups of 5 or 10 and assayed by virus isolation. The negative control embryos, porcine origin trypsin treated embryos, and TrypLE Select treated embryos were negative for virus. The positive control embryos and the TrypLE Express treated embryos were positive on virus isolation (Table 1). When it was determined that TrypLE Express was not effective at 1.5 min, TrypLE Select (10X) was used for 10 min. These preliminary results indicate that use of TrypLE Select (10X) for 10 min is effective for removal of BHV-1 associated with Day 7, zona pellucida-intact, *in vivo*-derived

embryos. In addition, TrypLE Select has the advantage of being an animal-origin-free product. However, use of TrypLE Express (1X) for 1.5 min was not effective. Because it is not practical to expose embryos to trypsin for 10 min, further research is needed to determine the ideal treatment concentration and time that will effectively remove BHV-1 without harming *in vivo*-derived bovine embryos.

Table 1. Virus isolation results

Embryo	group	Positive/total
Porcine-origin	trypsin-treated	0/6
TrypLE	Select-treated	0/2
TrypLE	Express-treated	11/11

(Marley M et al, Reproduction, Fertility and Development 2006;18:213 (abstr.))

For embryo culture, it is often desirable to use media that are devoid of animal products. One problem is that the trypsin required for treatment of embryos for export is an animal product. Therefore, we studied the efficacy of recombinant trypsin produced from the bovine gene in maize (Trypzean™, Sigma-Aldrich Corp., St Louis, MO, USA) for inactivating bovine herpes virus-1 (BHV-1), initially without ova present. We found that 525 U mL^{-1} Trypzean is equivalent in efficacy to the standard 0.25% bovine trypsin treatment. Active virus was titrated on MDBK cells using a standard plaque assay. Next, ova recovered from superovulated cows were incubated for 45 min with BHV-1-infected cells for virus adsorption to the zona pellucida. Both unfertilized ova (*n* = 26) and embryos (*n* = 22) were allotted to 4 treatments: (1) control, not exposed to virus; (2) exposed to virus and then washed 10.; (3) treatment 2 plus trypsin treatment as recommended in the IETS Manual; and (4) treatment 2 plus 525 U Trypzean mL^{-1} and 1 mM EDTA substituted for the 4th and 5th washes of 1 min each; the 6th wash of 10 washes was with soybean trypsin inhibitor at 80 µg mL^{-1} (Sigma). The chemically defined medium used for handling ova was Syngro® (AB Technology, Pullman, WA, USA), except that trypsin and inhibitor were made up in Hank's balanced salt solution with 0.2% polyvinyl alcohol, but without Ca^{2+} or Mg^{2+}. Ova were stored in 0.5 mL at -80°C until sonication and inoculation onto MDBK cells in duplicate undiluted and at 0.1. dilution. The virus was allowed to adsorb for 45 min, and then overlaid with MEM containing 0.5% agarose, 5% FBS, and antibiotics. Two days later an identical overlay was added, but containing 0.005% neutral red; plaques were counted the following day. Unfertilized ova and embryos led to similar results, which are pooled. No virus was detected in the 5 control ova not exposed to virus. As has been shown by others, BHV-1 remained adhered to zonae even after 10 washes (Table 1). In contrast, treatment with Trypzean or the IETS trypsin protocols reduced this to near zero. We thus confirm that trypsin treatment is effective in inactivating BHV-1 adhered to bovine zonae pellucidae, whether trypsin is derived from animals or genetically engineered plants. We have found that Trypzean is more efficacious than some 'trypsin-like' enzymes for this purpose. **(Seidel GE Jr., et al. Reproduction, Fertility and Development, 2007;19:236 abstr.)**

Techniques of production and transfer of embryos is safe as long as it follows the control regulations defined by the manual of the International Embryo Transfer Society (IETS) for treating oocytes with trypsin, antibiotics, and TCM-199 medium. The aim of this work was to evaluate the effectiveness of treatments, established by IETS, in bovine oocytes experimentally exposed to *Leptospira interrogans* serovar Grippotyphosa and to assist implementation of quality control standards on *in vitro* embryo production. The oocytes

were obtained through follicular puncture of ovaries derived from the slaughterhouse. They were selected and divided into 4 groups: the control group and groups exposed to 5, 10, and 30µL of an *L.interrogans* strain at 4.7×105 µL−1; and 4 additional groups exposed to the same concentrations of another *L. interrogans* strain at 6.3×105 µL−1, in which the gene for virulence is not expressed. The groups were kept in maturation medium (TCM-199 medium, 0.5µL of FSH, 50 IUmL−1 hCG, 1µLmL−1 17-βestradiol) and incubated at 38◦C, 5% CO2, and 95% humidity for 24 h. All the groups were separately subjected to the treatments with antibiotic, trypsin, and TCM-199 medium after maturation. The treatment involved 10 drops (each 200 µL), with 8 drops of TCM-199 medium and 2 drops of antibiotic (penicillin 10 000 IUmL−1 and streptomycin 10 mgmL−1) or trypsin 0.25%; exposed to trypsin and antibiotic for 120 s.

For the sequential washes, all drops contained TCM-199 medium. The analysis for presence of the pathogen by dark-field optical microscopy showed that in the groups exposed to *L. interrogans* and subjected to antibiotic washes, the effectiveness was 50% (100/200) for the group exposed to 5µL, 40% (80/200) for that exposed to 10µL, and 22.5% (45/200) for that exposed to 30 µL.We found the same results after the trypsin washes. After the washings withTCM-199 medium, the groups infected with 5 and 10µL presented 100% of effectiveness; however, for the group infected with 30µL,the washings were not effective. For the groups exposed to *L. interrogans* that did not express virulence, after the washings with antibiotics as well as with trypsin, the results showed no effectiveness in all of them (*n*=200).Yet, afterwashings withTCM-199, the group exposed to 5µL showed 28.5%(57/200) of effectiveness, whereas in those exposed to 10 and 30µL, the medium washes were not effective. Complementary studies are being madewith ultramicrotome cut and polymerase chain reaction for more reliable conclusions, to confirm the results. With such results, we conclude that the quality control regulations established by IETS for IVP could be reviewed and possibly redefined, because the effectiveness of the treatment may depend not only on the pathogen species, but also its virulence as well as its concentration and the action of the treatments on the type of pathogen.

Góes A. C., M. M. Piccolomini, D. L. Pavão, A. F. Carvalho, V. Castro, R. M. Piatti, and M. Dangelo. Assessment of trypsin and antibiotic treatment effectiveness In *in vitro*-matured oocytes experimentally exposed to *Leptospira interrogans* serovar grippotyphosa *Reprod Fertil Dev 2010,22, P 294*

Other treatments

Eight cell, ZP-I mouse embryos were exposed to the following substances or procedures reported to have germicidal effects to determine if the embryos would survive and develop under in vitro conditions: the photosensitive substances hematoporphyrin, hematoporphyrin derivative, 8-methoxypsoralen, 4,5', 8-trimethylpsoralen and thiopyronine; the enzymes lipase (0.5%), phospholipase C, (2 U/ml), chymotrypsin (0.5%) and trypsin (0.5%); pH 5.0; and helium/neon laser light (632 nm), visible light (580 nm), ultraviolet A light (400 nm) and ulraviolet C light (220-280 nm). Under the conditions used, embryos were not adversely affected by hematoporphyrin and/or helium/neon laser light; methoxypsoralen and/or ultraviolet A light; lipase; trypsin; pH 5.0 for 20 min, and visible light. Variable results were obtained from hematoporphyrin derivative with laser light. Thiopyronine, trimethylpsoralen in combination with ultraviolet A light, and ultraviolet C

light, killed embryos, while chymotrypsin and phospholipase C were harmful at 10 and 15 min exposure time, respectively (**Bielanski and Hare, J IVF and Emb Trans 1991;8:24-32**).

Bovine embryos of good (n = 27) and poor (n = 37) quality at the morula stage were exposed to continuous helium/neon laser light (250J/cm_2) and their development in vitro was compared with that of equivalent numbers (n = 30 and 36 respectively) of embryos not so exposed. The light exposure treatment had no obvious detrimental effect on numbers that reached the expanded and hatching blastocyst stages (**Bielanski and Hare, Theriogenology 1992;37:192 abstr**).

Photosensitive agents such as haematoporphyrin (HP), haematoporphyrin derivative (HPD) and thiopyronine (TP) have germicidal effects when activated by light, ie HP and HPD by helium neon laser (Ha/Ne) light, HP by white light and TP by yellow-green light. Controlled studies were done with these agents a) to inactivate BHV-1 (10_6TCID_{50}/ml) and BVDV (10_6TCID_{50}/ml) in media; b) to disinfect ZP-I in vivo derived bovine embryos exposed to the above levels of these viruses, and c) to ascertain any adverse effects of the treatments on in vitro development of these embryos. A total of 268 embryos were used in the studies. Exposure to each of the three photosensitive agents for approximately 15 minutes followed by the appropriate duration of light irradiation to give the desired energy fluences inactiviated both BHV-1 and BVDV in media. Exposure of embryos to HP or HPD followed by irradiation with He/Ne or white light was a quick, simple means of disinfecting embryos carrying BHV-1 or BVDV. Exposure to light alone or to light plus HP or HPD had no detrimental effect on their in vitro development but exposure to TP followed by light irradiation led to embryo degeneration. Exposure of embryos to photosensitive agents may be an effective method of disinfection (**Bielanski et al, Theriogenology 1992;38:663-644**).

Photosensitive agents for disinfection of bovine semen have been investigated. Haematoporphyrin, haematoporphyrin derivative and thiopyronine were effective against BHV-1, BVDV, *Mycoplasma bovigenitalium*, *Mycoplasma canadense* and *Ureaplasma diversum* in culture media. In addition, thiopyronine was effective against *Leptospira pomona* in culture medium. Similar treatments were not effective against *Leptospira hardjo*, *Mycoplasma bovis* or *Campylobacter fetus* subsp. *venerealis*. When the microorganisms were added to bovine semen, only BHV-1 was controlled by photosensitive agents used at concentrations which did not appear to be harmful to sperm cells (**Eaglesome et al. Vet Micro 1994;38:277-284**).

In preliminary studies, a cell line established from a bovine embryo was used to examine toxicities of antibiotics (penicillin G, streptomycin, amphotericin B) that have been utilized in media for handling bovine embryos. Similar treatments were then applied to day 7 bovine embryos collected from superovulated cows (a combination of the three antibiotics was also used for embryos). Bovine embryonic cells showed no detrimental effects when treated for 6 h at the recommended cell culture level or 10 times that level. Decreases in cell numbers were seen at 10 times and 100 times the concentrations of amphotericin B when exposures were for 72 h. For embryo toxicity studies, the developmental potential after treatment was assessed by comparing the time for treated and control (no antibiotic) embryos to hatch when cultured for 72 h at 37C in air and 5% CO_2. Toxicity assays using Day 7 embryos

revealed no delay in development with 1 time or 10 times the recommended concentration of penicillin G or streptomycin at either the 6 or 72 h exposure. However, 10 times the recommended concentration of amphotericin B alone or in the combination of Penicillin G/streptomycin/amphotericin B did prevent normal development when the antibiotics were in medium for 72 h of culture. The embryonic cell line was predicitive of toxicity of antibiotics to embryos. Short term exposure to even high concentrations of certain antibiotics does not result in apparent toxicity and may allow treatment to insure freedom from specific pathogems or contaminants." (**Riddell KP, et al., Theriogenology 1995;43:308 abstr**)

MISCELLANEOUS

International trade of livestock germplasm

International trading of the germplasm of farmed livestock, especially cattle, is now common. Each year, approximately 50 million doses of bovine semen with a value of about US$250 million, and approximately 80,000 bovine embryos with a value of about US$15 million, are traded internationally. The trading of germplasm instead of live animals gives distinct financial, genetic, and animal welfare benefits. There are also significant biosecurity benefits because the risks of transmitting infectious diseases via properly processed semen, and even more so embryos, are very low. **Thibier M, Wrathall AE , International Trade of Livestock Germplasm In : Encyclopedia of Biotechnology in Agriculture and Food Publication details, including instructions for authors and subscription information: http://www.tandfonline.com/doi/book/10.1081/E-EBAF.**

Review : October 1984, first formal meeting of the Association Européenne de Transfert Embryonnaire (AETE) in Lyon (France), thirty years ago…, who would have then reasonably dreamt of seeing both the European Embryo Transfer (ET) industry and the AETE up to the standard they now are 30 years later. Europe ET teams transfer a little more than 20% of the worldwide total transfers every year, hence showing its sustainability. The European evolution over the years regarding In Vivo Derived (IVD) embryos shows that it is quite parallel to that observed worldwide with perhaps a little less fluctuation from one year to another than in other continents. Referring to In Vitro Produced (IVP) embryos, although a good start was observed in Europe in the late 80's, early 90's, the numbers are still low and far from those observed in other continents.
The sustainable development of the ET industry in Europe is concomitant to the success story of AETE which has had very well attended annual meetings. AETE has been a strong support of the Industry in: communication, training and scientific information.
This sustainability of the European ET industry obviously relies on the existing market due to the benefits the farmers get from applying such a technology and hence from a good cost/benefits ratio.
In the author's opinion, there are three main technical reasons which have supported this success story. The first is that ET is the tool of choice to more efficiently implement genetic improvement programs including those deriving from the recent genomic revolution. The second is the high competence of all practitioners and people involved, together with

excellent training and high degree of innovation from the field to the bench as assessed by the high standard of scientific presentations at the AETE annual meetings and of the quality of the European pioneers in this area. The third reason is the excellent approach taken by both the veterinary community and the ET industry in elaborating an original and most efficient policy to enable ET to be recognized as the safest mean of exchanging genes from farm to farm or continent to continent. The European ET industry has participated in generating and implementing such a policy based on the definition of officially approved embryo transfer or production teams.
In conclusion, the ongoing research of innovation, the excellent expertise following well designed training of the practitioners, their sense of responsibility in taking most seriously the recommendations and rules regarding the health safety and precautions taken by the officially approved embryo transfer or production teams all explain the resulting level of excellence of this industry. Clearly, the conditions are currently met to foresee that this success story will continue as such in our European continent in the near future.
Thibier M. The european embryo transfer industry in cattle – A challenge in 1984, a success in 2014 - and well supported and reported by the AETE. in : Proc 30th Annual Meeting AETE - Dresden, Germany, 12-13 September 2014 . pp. 31 – 44.

Review : As various embryo technologies in livestock were developed and evolved to a state of usefulness over the past 40 years, scientists with a specific interest in infectious diseases sought to determine the epidemiologic consequences of movement, especially international movement, of increasing numbers of embryos. Many of the foundational studies in this area were reported in Theriogenology, beginning in the 1970s and especially throughout the 1980s and 1990s. Unquestionably, Theriogenology has been a widely used venue for dissemination of basic information on this subject, which ultimately led to the development of the now universally accepted techniques for certification of embryo health. Today it is well-recognized that movement in commerce of embryos, especially in vivo–derived embryos, is a very low risk method for exchange of animal germ plasm. This paper chronicles the evolution of strategies for health certification of embryos. An overviewis provided of the calculated efforts of practitioners, scientists, and regulators to organize, forge necessary partnerships, stimulate needed research, provide purposeful analysis of the results, and, through these processes, guarantee the universal acceptance of efficient protocols for certifying the health of embryos intended for movement in international commerce.
Gard JA, and Stringfellow DA. Shaping the norms that regulate international commerce of embryos. Theriogenology 81.1, 2014: 56-66

Review : The current world population is increasing at a fast rate. In order to feed this larger population, food production must increase by 70 percent. Recent reports show a record global production of 58.9 Carcass Equivalent Weight million metric tonnes of beef expected for 2014. It becomes clear that the worldwide agricultural community will have to integrate new technologies to assure the sustainability of global livestock and meat demands. Agriculture has benefited tremendously from the innovation of reproductive technologies such as semen artificial insemination and cryopreservation, embryo transfer and cryopreservation, and *in vitro* fertilization. Only recently have some developed countries accepted the import and export of frozen IVF embryos and more countries are currently evaluating this. Before 2003, *in vitro* embryos represented not more than 20% of all embryos

produced. After 2003, this jumped to 30 to 39% of all embryos produced, and is increasing. It is clear that South America, and more specifically Brazil, is driving this increase. However, most people in this field would agree that the trend is true for many regions active in this field. International movement of gametes or embryos must be performed in biosecure manners to make certain that pathogenic organisms are controlled and that transmission of infection to recipient animals and progeny is avoided. The embryo transfer industry has adopted appropriate procedures to manage the biosecurity risks and hence mitigate risks of pathogen transmission through international trade of bovine embryos. Techniques for biosecure production of *in vivo* bovine embryos have been well established. However, as *in vitro* embryos are relatively new to this business, there are not many papers on the subject of pathogen-interaction with this type of embryo. Certain studies demonstrate that the decontamination of *in vitro* embryos using recommended procedures is effective for specific pathogens while others have shown that this is not as evident in other conditions. All agree that more research is needed regarding washing protocols for *in vitro* embryos. It is imperative that the scientific community continues its research to validate current embryo sanitary washing procedures and recommend any modifications that would be necessary for IVF embryos. As embryos are becoming an important component of international trade of bovine genetics, such research must not only continue but augment if keyparties want to assure they meet the worldwide rising need of meat and dairy products

Blondin P. Health and safety of IVF embryos: challenges for the international ET industry. Official journal of the Brazilian College of Animal Reproduction 11.3 (2014): 270-277.

Antimicrobials/Antibiotics

In vitro susceptibilities of 16 Mycoplasma mycoides subsp. mycoides large colony type field isolates to 15 antimicrobial agents were determined using a broth microdilution method. The most effective antimicrobials were fluoroquinolones, tetracyclines and macrolides, with MIC values under 2 µg/ml. Resistance to nalidixic acid, gentamicin, streptomycin and spectinomycin was observed. **(Antunes NT et al, Veterinary Microbiology 2007;119:72-75)**

Antiviral treatments

Bovine viral diarrhea virus (BVDV) is an economically significant pathogen of cattle and a problematic contaminant in the laboratory. BVDV is often used as an in vitro model for hepatitis C virus during drug discovery efforts. Aromatic dicationic molecules have exhibited inhibitory activity against several RNA viruses. Thus, the purpose of this research was to develop and apply a method for screening the aromatic cationic compounds for in vitro cytotoxicity and activity against a noncytopathic strain of BVDV. The screening method evaluated the concentration of BVDV in medium and cell lysates after 72 h of cell culture in the presence of either a 25 or 5 µM concentration of the test compound. Five of 93 screened compounds were selected for further determination of inhibitory (90 and 50%) and cytotoxic (50 and 10%) concentration endpoints. The screening method identified compounds that exhibited inhibition of BVDV at nanomolar concentrations while exhibiting no cytotoxicity at 25 µM concentrations. The leading compounds require further investigation to determine their mechanism of action, in vivo activity, and specific activity against hepatitis C virus. **(Givens MD et al, Antimicrobial Agents and Chemotherapy 2003;47:2223-2230.)**

Bovine viral diarrhea virus (BVDV) replicates in embryo culture systems and remains associated with developing IVF bovine embryos despite washing and trypsin treatment. Previous research [Givens, et al. Theriogenology 2002; 57:572 (abstract)] determined that 2-(4-[2 -imidazolinyl] phenyl)-5-(4-methoxyphenyl)furan (DB606) can inhibit replication of BVDV in embryo culture media containing uterine tubal cells. The objective of this study was to evaluate the ability of IVF embryos to initiate and maintain pregnancy after exposure to effective antiviral concentrations of DB606 during in vitro culture. During 22 replicates, oocytes were obtained from cows via transvaginal, ultrasound-guided follicular aspiration. Presumptive zygotes that resulted from fertilization of these oocytes were cultured for 7 days in medium supplemented with 0.4 µM DB606 or medium lacking antiviral agent. All blastocysts were individually transferred by nonsurgical technique into the uterus of a synchronized recipient. Potential pregnancies were evaluated by transrectal ultrasonography 21 to 23 days after embryo transfer (gestational days 28 to 30) and subsequently every 27 to 34 days. At initial examination, pregnancy status was determined by visualization of fetal heartbeat. Rates of blastocyst development, pregnancy per cultured zygote and pregnancy per transferred embryo were compared using the Chi-square test statistic. The development of blastocysts and establishment and maintenance of pregnancies are documented in the table. Of pregnancies resulting from IVF embryos exposed to DB606, 3 of 20 expired prior to gestational day 64. Of pregnancies resulting from negative control embryos, 4 expired prior to gestational day 57 and an additional 2 expired prior to gestational day 112. Differences in rates of blastocyst development, pregnancy per cultured zygote and pregnancy per transferred embryo were not significant (p=0.05). Pending birth of normal offspring, preliminary results indicate that bovine embryo cultures might be safely supplemented with effective concentrations of antiviral agent. Addition of this antiviral agent might function to prevent viral transmission if BVDV were inadvertently introduced into the embryo culture system.

Treatment	Presumptive zygotes cultured	Blastocysts transferred	Pregnancies established	Pregnancies maintained
DB606	425	61	20	17
Negative control	424	49	24	18

(Givens MD et al, Theriogenology 2003;59:382 abstr.)

Unnoticed infections with BVDV can occur in cultured cells used for somatic cell nuclear transfer. Aromatic cationic molecules have exhibited activity against in vitro replication of BVDV. The purpose of this research was to evaluate the ability of aromatic cationic compounds to prevent or treat noncytopathic BVDV infections of fetal fibroblast cells. Aromatic compounds tested were 2-(4-[2-imidazolinyl]phenyl)furan (DB606); 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772); and 2-(1-methyl-2-benzimidazolyl)-5-[4'-(2-imidazolino)-2'-methylphenyl]furan dihydrochloride (DB824). To evaluate prevention of BVDV infections, 10 cell lines in the absence or presence of 7 dilutions of each of the three compounds were inoculated with BVDV. The concentrations of BVDV in medium and cell lysates were determined by serial dilution and virus isolation. Samples were obtained 72 hours post inoculation. Bovine viral diarrhea virus in cell culture medium and cell lysate samples was evaluated by comparison from control cultures in which no cokmpound was added (percent of control=cell culture infective doses (50%; CCID50) of BVDV in compound sample/CCID50 of BVDV in control sample lackin gcompound). The viral inhibitory

concentrations (99%) of compounds were calculated with JMP software by least squares regression techniques. Cumulatively, the 99% endpoints for inhibition of viral replication in fetal fibroblast cell lines for 3 compounds were 0.1µM, 0.007µM, and 0.028µM, respectively. To evaluate therapeutic treatment of established BVDV infections, the concentration of BVDV in medium and cell lysates of 2 fetal fibroblast cell lines were evaluated. The cell lines were previously determined to be infected with a genotype 1a strain of BVDV. Samples were obtained during 4 sequential passages in the absence or presence of 0.04µM and 4µM concentrations of DB772 or DB824. Presence of BVDV was determined by RT-nPCR assay and virus isolation. While BVDV persisted in cultures supplemented with no aromatic compound or 0.04µM, both DB772 and DB824 effectively cured BVDV infections after one passage in 4µM, and cells remained viable. Results indicate that BVDV can be effectively prevented or treated in fetal fibroblast cultures. Further research is needed to determine if exposed cells are competent for production of normal embryos via nuclear transfer. **(Givens MD, et al. Reproduction, Fertility and Development 2004;16:219 (abstr.))**

Bovine viral diarrhea virus (BVDV) has been shown to replicate in embryo culture systems and remain associated with bovine embryos developing in vitro. In this study, novel antiviral agents were evaluated for capability to inhibit replication of BVDV without affecting embryonic development. Serial concentrations of 2-[5(6)-{2-imidazolinyl}-2-benzimidazolyl]-5-(4-aminophenyl)furan (DB456) or 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) were prepared in IVC medium. Then, bovine uterine tubal epithelial cells (UTC) were placed in IVC media with varying concentrations of DB456 or DB606. Within 1h, a genotype I or II strain of BVDV was added to the cultures. Cultures were maintained for 7 days. Infectious virus was quantitated in IVC media collected on days 3 and 7 and in UTC lysates harvested on day 7. The effective antiviral concentrations of DB606 were much lower than effective antiviral concentrations of DB456. In subsequent experiments, IVF presumptive zygotes were cultured in IVC medium with or without DB456 or DB606 at multiple concentrations for 7 days to evaluate effect of the compound on conceptus development. On day 7, stage of embryonic development was observed, and blastocysts were harvested and stained using Hoechst 33342 to enumerate embryonic cells. While DB456 inhibited blastocyst development, DB606 at 20 times the effective antiviral concentration did not hinder blastocyst development or reduce the mean number of cells per blastocyst. These preliminary results indicated that bovine embryo cultures might be safely supplemented with effective concentrations of an antiviral agent. **(Givens MD, et al. Theriogenology 2005;63:1984-1894.)**

Bovine viral diarrhea virus (BVDV) replicates in embryo co-culture systems and remains associated with developing IVF bovine embryos, despite washing and trypsin treatment. Previous research demonstrated that 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) inhibits replication of BVDV in cultured cells. The objective of this study was to evaluate the capability of IVF embryos to develop into normal, weaned calves after exposure to antiviral concentrations of DB606 during IVC. Oocytes were obtained from cows via transvaginal, ultrasound-guided follicular aspiration. Presumptive zygotes (n=849) that resulted from fertilization of these oocytes were cultured for 7 d in medium supplemented with 0.4muM DB606 or medium lacking antiviral agent. All blastocysts (n=110) were transferred individually into the uterus of a synchronized recipient. The pregnancy status of recipients was determined using transrectal ultrasonography at 21-23 d after embryo transfer. Additional pregnancies as controls (n=21) were initiated by natural breeding.

Developing fetuses and resulting calves were evaluated every 27-34 d. Blastocyst development, pregnancies per transferred embryo, pregnancies maintained per pregnancies established, gestation length, gender ratio, birth weights, viability of neonates, complete blood counts, and serum chemistry profiles at 3 mo of age and adjusted 205 d weaning weights were compared for research treatments. Development to weaning after exposure to DB606 did not differ significantly from controls. In conclusion, bovine embryo cultures can be safely supplemented with antiviral concentrations of DB606; addition of DB606 agent might prevent viral transmission if BVDV were inadvertently introduced into the embryo culture system. **(Givens MD, et al. Theriogenology 2006;65:344-355.)**

Transferred embryos carry the risk of being vehicles of organisms causing diseases. Currently, the risk of *in vitro*-produced (IVP) embryos is more difficult to assess than the risk of *in vivo*-derived embryos, since less research has been published on the former. Foot and mouth disease virus (FMDV) is extremely sensitive to a low pH and is likely to be destroyed if embryos are exposed to a low pH for a short time. 2-(N-Morphalino)-ethanesulfonic acid (MES); an organic buffer with pKa 6.1; Sigma, South Africa, M2933) as been shown to destroy FMDV at a rate of 90% per minute at pH 6 and at a rate of 90% per second at pH 5 (Acharya *et al*. 1990 Vet. Microbiol. 23, 21–34; Thomson "Foot-and-mouth disease," in *Infectious Diseases of Livestock with Special Reference to Southern Africa*, ed. Coetzer JAW, Thomson GR, andTustin RC, Oxford University Press, CapeTown, 825–852). The aim of this study was to test whether exposing bovine oocytes and IVP zygotes to the organic buffer MES, buffered at pH 5.5, is detrimental to the development of bovine IVP embryos. IVM, IVF, and IVC was carried out with 1367 oocytes as described earlier [Jooste *et al.* 2003 Theriogenology 59, 443]. Oocytes were divided into three groups: 484 were used as controls (no MES exposure); 437 were in a maximal exposure group (MAX), i.e. MES treatment after washing of oocytes, after IVM and after IVF, and 446 had a minimal exposure (MIN), i.e. MES treatment after IVF only. To treat the oocytes with MES, 100 oocytes (from ten droplets) were drawn into a pipette in a maximal volume of 100µL, and placed in 3mL of MES, swirled around for 10 s, drawn up again in a maximal volume of 100µL, and placed in 3mL of culture medium. Oocytes or zygotes were then washed five times in culture medium before being processed through IVM, IVF, or IVC depending on their stage. Exposure of oocytes to MES varied from 30 to 60 s (10 s swirling and a variable time thereafter to pick up). A chi-square test was used to test for differences in cleavage and Day 7 blastocyst yield between control and treatment groups (<0.05). Cleavage (70%; 340/484) and blastocyst yield (32%; 156/484) in the control group were not different from those in MIN (68%; 304/446, and 29%; 131/446, respectively), but were significantly higher than for MAX (57%; 249/437, and 18%; 79/437, respectively). In MAX the MES had a harsh effect on the cumulus cells, making them granular and clumpy in appearance. Oocytes treated in MES solution adhered to the bottom of the dish, which made their handling difficult. Exposure time in MES was therefore variable and longer than initially planned. It is concluded that bovine IVP embryos can be exposed to MES without detrimental effect. Treatment with MAX still resulted in blastocysts but it did not yield good numbers. In future trials, treated dishes should be used to prevent oocyte and zygote adherence. Further research is needed to test whether FMDV can be removed from bovine IVP embryos with the described method. **(De Haas K, et al. Reprod Ferti Develop 2005;17:242 (abstract).)**

Bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) are the most likely viruses to be associated with abattoir-origin materials used in *in vitro* embryo production. Further, both viruses are known to associate with zona pellucida-intact embryos after

exposure and washing, and limited evidence indicates that developing, transferable embryos are able to inhibit viral replication in adjacent cells. Interferon-tau is known to have anti-BVDV and anti-BHV-1 activities, but it is not known whether interferon-tau which is secreted by developing embryos has the same effects. The objectives of this study were to evaluate the cytotoxicity and anti-viral effect of interferon-τ against a non-cytotoxic high affinity strain of BVDV (SD-1) and against a strain of BHV-1 (Colorado) in cell culture. Madin Darby bovine kidney (MDBK) cells were seeded in 96-well plates and then inoculated with serial dilutions (1:10) beginning with an initial concentration of 0.2µg of interferon- τ. Cells and interferon were incubated at 37.5NC in 5% CO2 and air for 24 h prior to addition of virus. Five concentrations of BVDV were added to the wells to give 500, 50, 25, 10 or 5 cell culture infective doses (50% endpoint) per well. Three concentrations of BHV-1—50, 10, and 5 plaque-forming units—were evaluated in separate cell cultures. Virus isolation (for BVDV) or plaque assays (for BHV-1) were utilized to determine if the addition of interferon-tau decreased the amount of infective virus. The interferon-tau produced no observable cytotoxicity in MDBK cells in any of the assays. At its three highest concentrations, the interferon- τ significantly decreased the amount of BVDV but it had no significant effect on the amount of BHV-1 in cell cultures. Thus, it is possible that interferon- τ produced ZP intact embryos (Stringfellow *et al*. 2000 Theriogenology 53, 827–839). To eliminate these sanitary risks, pre-treatment of embryos with antiviral compounds may be a promising approach (Givens *et al*. 2006 Theriogenology 65, 344–355). BPIP (5-[(4-bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine) has been reported to display antiviral activity against BVDV, with a 50% effective inhibition of BVDV-induced cytopathic effect formation at a concentration of 0.04 µM (Paeshuyse *et al*. 2006 J. Virol. 80, 149–160). However, since the short- and long-term effects of BPIP have not been described, the aim of the current study was to assess whether addition of BPIP for 2 days at a concentration of 5 µM is toxic for ZP-free cattle embryos. Oocytes were aspirated from 3–6-mm follicles of cattle ovaries, matured for 24 h, and subsequently co-incubated with 1 . 10^6 sperm cells mL^{-1} in IVF-TALP with 20 $\mu g/mL^{-1}$ heparin for 24 h at 39°C and 5% CO_2 in air. After fertilization, presumptive zygotes were put in groups of 25 into 50-µL droplets of SOF under oil in 5% CO_2, 5% O_2, and 90% N_2 for 6 days. Afterwards, morulae and blastocysts were collected, rendered ZP-free by means of pronase treatment, and divided into 4 groups: (i) ZP-free control group, (ii) ZP-free control group treated with a volume of DMSO equal to condition (iv), (iii) ZP-free group treated with 5 µM BPIP in DMSO, and (iv) ZP-free group treated with 10 µM BPIP in DMSO. Because BPIP is a fat-soluble molecule, embryos were cultured in 0.5 mL SOF without oil for 2 days. At Day 8, all embryos were fixed, TUNEL-stained, and analyzed for total cell number and percentage of apoptotic cells. Three independent replicates were performed. Results are shown in Table 1 and were analyzed by means of ANOVA. Only group iv showed a significant decrease in total cell number, indicating that at 10 µM BPIP may negatively influence embryo development. At both 5 and 10 µM, BPIP treatment resulted in an increase in percentage of apoptotic cells compared to the control group. However, a similar increase was observed using DMSO alone (group ii), indicating that the apoptotic effect may be due solely to the DMSO. In conclusion, BPIP does not appear to cause embryo toxicity at 5 µM, but an alternative, less toxic, dissolving agent may be considered. **(Mestach J, et al. Reproduction Fertility and Development, 2007;19:235-236 abstr.)**

The interferon-tau (IFN-τ) secretion levels after hatching by bovine blastocysts derived from *in vitro* maturated oocytes (Group A) and from *in vivo* (Group B) were investigated considering embryo quality. Only very homogeneous blastocysts of excellent or good quality were considered from day 7 of culture (Group A) and day 7 after artificial insemination with

frozen-thawed from the same bull used for *in vitro* fertilization (Group B). All embryos were individually cultured in a 50 µl droplet of synthetic oviduct fluid medium with 10% fetal calf serum. After 24-h culture both Group A (n=44) and B (n=40) secreted <54 pm IFN-τ. After 48-, 72-, 96-, and 120 –h culture, Group A daily secreted 143 ± 24 pM IFN-τ (n=19) vs 85 ± 12 pM IFN-τ (n=21) for Group B ($p<0.01$), 491 ± 128 IFN-τ (n=29) vs 216 ± 37 pM IFN-τ (n=23) (NS), 499 ± 135 pM IFN-τ (n=26) vs 353 ± 93 pM IFN-τ (n=21) (NS), 559 ± 136 pM IFN-τ (n=22) vs 333 ± 75 pM IFN-τ (n=20) (NS), respectively. Taken all together during 5 days, Group A produced per embryo 1690 ± 290 pM IFN-τ (n=22) vs 982 ± 182 pM IFN-τ (n=20) for Group B ($p<0.05$). For all culture time there were sizable percentages of embryos that did not produce concentrations of IFN-τ above a certain cut-off level, and as such were not used to compute the means. In respect of the embryo quality whatever the groups after days7-12 of culture, IFN-τ secretions were 1815 ± 453 pM (n=10) for the embryos of excellent quality vs 1356 ± 200 pM (n=10) for those of good quality (NS) and 360 ±188 pM (n=4) ($q<0.05$) for embryos of fair quality. A positive relationship between IFN-τ production and in vitro development of quality I embryos was observed, whatever the embryos origins and, the embryos completely produced *in vitro* secreted more IFN-τ than the embryos produced *in vivo*. **(Neira JA., et al. Reprod Dom Anim 2007;42:68-75.)**

Bovine viral diarrhea virus (BVDV) replicates in embryo culture systems and remains associated with developing IVF bovine embryos despite washing and trypsin treatment. Previous research (Givens *et al*. 2006 Theriogenology 65, 344–355) determined that 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606) can be added to an *in vitro* embryo production system to prevent replication of BVDV without inhibiting embryonic development. The objective of this research was to assess sexual development and future reproductive capacity of heifers resulting from embryos that had been exposed to DB606 while developing *in vitro*. Presumptive zygotes that resulted from *in vitro* fertilization of oocytes were cultured for 7 days in medium supplemented with 0.4 µM DB606 or medium lacking the antiviral agent. All blastocysts were individually transferred nonsurgically into the uterus of a synchronized recipient. Additional control pregnancies were established by natural breeding. At 20 to 27 months of age, 7 heifers resulting from each treatment were simultaneously exposed to a fertile bull in a single pasture during a 63-day breeding season. All but 2 heifers subjected to natural breeding were pregnant 28 days after removal of the bull. One pregnancy was aborted between 5 and 7 months of gestation from a heifer that had undergone IVF without exposure to DB606. All offspring were normal upon clinical observation at birth. One heifer resulting from IVF with exposure to DB606 would not allow its calf to nurse adequate quantities of colostrum; thus, this calf was removed from the study. Adjusted 205-day weaning weights for offspring were evaluated using ANOVA and Tukey Kramer test for pair-wise comparisons. Offspring of dams treated or untreated with DB606 during IVC or produced by natural breeding exhibited mean adjusted 205-day weaning weights of 194, 181, and 170 kg, respectively (not significantly different). Results of this research using a limited number of animals demonstrates that exposure to DB606 during the first 7 days after fertilization does not appear to cause delayed toxic effects that would impair the future reproductive capacity of heifers. **(Givens MD, et al. Reproduction, Fertility and Development, 2007:19;234 abstr.)**

Bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) are the most commonly isolated viruses from abattoir-origin materials utilized in embryo production and known to associate with zona pellucida-intact (ZP-I) embryos after exposure and washing. Some evidence indicates that developing embryos may produce substances that are able to inhibit viral replication in adjacent cells. Interferons such as recombinant human interferon-α are known to have anti-BVDV activity but no effect against BHV-1. In some preliminary studies, bovine interferon-τ has shown antiviral activities against BVDV but not against BHV-1. However, interferon-τ in other species has not been evaluated for anti-BVDV and anti-BHV-1 effects. Thus, the objective of this study was to evaluate the cytotoxicity and anti-viral effect of ovine interferon-τ against a non-cytopathic high affinity strain of BVDV (SD-1) and BHV-1 (Colorado) in cell culture. Serial dilutions (1:10) beginning with an initial concentration of 1 mg mL^{-1} of interferon-τ were made in 96-well plates and then Madin Darby bovine kidney (MDBK) cells were seeded in the wells. Cells and interferon-τ were incubated at 37.5°C in 5% CO_2 and air for 24 h prior to addition of virus. The following concentrations of BVDV were added to the wells: 6000, 3500, 1000, 625, and 350 cell culture infective doses ($CCID_{50}$) (50% endpoint) per well. In addition, four viral concentrations of BHV-1, 1000, 500, 250, and 100 $CCID_{50}$/mL were evaluated in separate cell cultures. Virus isolation was utilized to determine if the addition of interferon-τ decreased the amount of infective virus. Ovine interferon-τ produced no observable cytotoxicity in MDBK cells in any of the assays. Also, the three highest concentrations of interferon-τ significantly decreased the amount of BVDV in all of the concentrations of BVDV tested but had no apparent effect on the concentration of BHV-1 in cell cultures. Therefore ovine interferon-τ has anti-BVDV effects similar to those seen with bovine interferont-τ and neither has any apparent antiviral activity on BHV-1 in cell culture. Additionally, ovine and bovine interferon-τ might serve to limit or prevent the transmission of BVDV and curtail the négative effects of BVDV on oocyte and embryo development. However, a similar effect is not expected for BHV-1. **(Galik PK, et al. Reproduction, Fertility and Development, 2008;20:156-157 abstr.)**

Bovine viral diarrhea virus (BVDV) can associate with in vitro fertilized (IVF) bovine embryos despite washing and trypsin treatment. An antiviral compound, DB606 (2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan), inhibits the replication of BVDV in bovine uterine tubal epithelial cells, Madin Darby bovine kidney cells, and fetal fibroblast cells. As well, DB606 in in vitro culture medium does not affect embryonic development. Antiviral-treated-IVF embryos placed into recipients developed into clinically normal calves. The objective of this project was to determine if these resultant heifer calves were capable of reproducing. Seven heifers from each of the treatment groups (natural breeding, IVF embryo, and IVF embryo cultured in DB606) of the previous study were used. At 20-27 months of age, the heifers were exposed to a fertile bull in a single pasture during a 63 d breeding season. Five of the seven heifers originating from natural breeding were pregnant 35 d after removal of the bull and calved. All of the heifers resulting from transfer of untreated IVF embryos were pregnant at 35 d; however, one aborted the fetus at 5-7 months of gestation. All of the heifers derived from transfer of IVF embryos cultured in DB606 were pregnant and calved. Offspring from dams of all treatment groups were clinically normal at birth. Adjusted 205 d weaning weights were not significantly different among the offspring of the treated and untreated dams. These results indicate that culture

of bovine-IVF embryos in DB606 does not impair future reproductive capacity of resulting heifers.
Givens MD, Marley MS, Riddell KP, Galik PK, Stringfellow DA. Normal reproductive capacity of heifers that originated from in vitro fertilized embryos cultured with an antiviral compound. Anim Reprod Sci. 2009;113(1-4):283-6.

Bovine herpesvirus 1 (BoHV-1) is widely distributed among cattle populations and has been associated with cells, fluids, and tissues collected from donor animals for use in reproductive technologies. The purpose of this study was to determine if lactoferrin would inhibit BoHV-1 in cell culture and to evaluate if embryos could develop normally when cultured in vitro with lactoferrin. In Experiment 1, lactoferrin (10 mg/mL) inhibited up to 25,000 plaque forming units (PFU)/mL of BoHV-1 in Madin Darby bovine kidney (MDBK) cell culture. In Experiment 2, lactoferrin (10 mg/mL) combined with cidofovir (62.5 microg/mL) inhibited up to 100,200 PFU/mL of virus in cell culture. In Experiment 3, following fertilization, presumptive zygotes were cultured in media containing lactoferrin (10, 5, and 2.5 mg/mL). Embryonic development and quality were assessed, and embryonic viability was determined by counting the nucleated cells of developed blastocysts. While lactoferrin did not affect the nucleated cell count of the treated embryos, it did significantly decrease blastocyst development. In conclusion, lactoferrin from bovine milk can inhibit BoHV-1 in cell culture. However, supplementation of in vitro culture medium with lactoferrin inhibits blastocyst development of in vitro-produced embryos.
Marley MS, Givens MD, Galik PK, Riddell KP, Stringfellow DA. Lactoferrin from bovine milk inhibits bovine herpesvirus 1 in cell culture but suppresses development of in vitro-produced bovine embryos. Anim Reprod Sci. 2009 Jun;112(3-4):423-9.

Contaminating microorganisms

We report on microbial contamination of embryos and semen cryopreserved in sealed plastic straws and stored for 6-35 years in liquid nitrogen. There were 32 bacterial and 1 fungal species identified from randomly drawn liquid nitrogen, frozen semen, and embryos samples stored in 8 commercial and 8 research facility liquid nitrogen (LN) tanks. The identified bacteria represented commensal or environmental microorganisms and some, such as Escherichia coli, were potential or opportunistic pathogens for humans and animals. Stenotrophomonas maltophilia was the most common contaminant identified from the samples and was further shown to significantly suppress fertilization and embryonic development in vitro. Analysis of the strains by pulsed field gel electrophoresis revealed restriction patterns with no relatedness indicating that there was no apparent cross-contamination of S. maltophilia strains between the germplasm and liquid nitrogen samples. In addition, no transmission of bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) from infected semen and embryos straws to clean germplasm stored in the same LN tanks or LN was detected. **(Bielanski AF et al, Cryobiology 2003;46:146-152.)**

Human - *This paper was published in Chinese***.** OBJECTIVE: To investigate the incidence of microorganism contamination in in vitro fertilizationembryo transfer (IVFET) and to determine the sources of microorganism. METHODS: Two thousand one hundred and seventyfour cycles of in vitro fertilization from January 1999 to June 2003 were evaluated retrospectively and bacterial cultures were performed in 61 semen samples from

asymptomatic men with normal semen parameters and in 34 follicle fluid samples from infertility women through oocyte picking up procedures. RESULTS: Microorganisms were found in 11 cases. The incidence of their contamination in IVF culture system was 0.51% and the most common microorganisms were *Escherichia coli* and fungi. Microorganisms were detected in 97% of unprocessed semen, 10% in processed semen, 6% in semen mixed with media and 9% in follicle fluid. CONCLUSIONS: The incidence of microorganism contamination was 0.51% and the most common microorganisms were Escherichia coli and fungi. Semen may have the potential to contaminate IVF culture system. (**Zhu GJ et al, Zhonghau Fu Chan Ke Za Zhi 2004;39:382-384.)**

This was a retrospective study carried out in a human reproductive unit evaluating the contamination risk in open and closed vitrification devices for oocyte/embryo cryopreservation. The contaminants present (bacteria and fungi) in the thaw medium and in liquid nitrogen (LN) and storage containers. They found that no bacteria or fungi were observed in any of the devitrification media regardless of the type of device used, nor in the LN supplied by the company. No fungi were observed in any of the LN samples tested. Stenotrophomonas maltophilia and Bacillus spp.
were found in all oocyte/embryo bank LN containers. There was no relationship between the number of samples or the time that each container had been used and the presence of microbiologic contaminants in the LN. At the container's bottom, Acinetobacter lwoffii, Alcaligenes faecalis ssp. faecalis, and Sphingomonas paucimobilis were found. Bacterial cross-contamination of oocyte/embryo banking in either open or closed storage devices did not occur in this study. The bacterial cross-contamination risk was found to be no greater for open than for closed containers. However, microorganisms can survive in LN. So, cleaning of storage containers should be done periodically to prevent the risk of lost straws or small particles of contaminated material. **Molina I, Mari M, Vicente Martínez J, Novella-Maestre E, Pellicer N, Pem J. Bacterial and fungal contamination risks in human oocyte and embryo cryopreservation: open versus closed vitrification systems. *Fertility Sterility*. Vol 106(1) 2016:127-132.**

Bovine IVF systems are highly susceptible to environmental contamination, especially during oocyte collection and fertilization and due to long culture periods of at least 7 days. A persistent contamination forced us to fully characterize the identity of a contaminant. Our routine IVF procedure uses slaughterhouse derived oocytes, matured for 24 h in TCM 199 (5% CO_2). Following fertilization with Percoll separated sperm , presumptive zygotes were cultured in SOF medium, covered with oil (5% CO_2, 5% O_2 and 90% N_2). All media were supplemented with 50 µg/ml gentamicin. Two days after fertilization, a white haze was found while blastocyst formation dropped to zero (normal ±30%). Following inoculation of the SOF medium on Columbia agar with 5% sheep blood, white colonies were observed and phenotypically characterised as motile Gram-positive coccobacils. The bacteria split esculin and showed catalase and ornithine decarboxylase activity. Analysis of the 16S rDNA sequence identified the bacterium as *Microbacterium*-like. A disc diffusion test revealed gentamicin resistance (40 µg/disc). A subsequent laborious multi-step study to localize the source of the contamination revealed that the Percoll used during sperm preparation was the coccobacil reservoir. In conclusion, our results show that the addition of gentamicin to the IVF system is not a guarantee for sufficient protection, while the choice for alternatives is

very limited. **(Goovaerts IGF et al, Proceedings 10th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Ljubljana, Slovenia, 7-9 September, 2006, p. 333.)**

PURPOSE: This study was designed to examine the effect of bacterial contamination on in vitro fertilization treatment outcomes. METHOD: In a prospective clinical trial, 152 patients aged 23-38 years, mean 33.3 +/- 4.6, undergoing IVF treatment were selected for this study. During embryo transfer, separate samples were collected for microbial examination from the following sites: the fundus of the vagina, the cervix, the embryo culture medium prior and post-embryo transfer, the tip of the catheter, and the external sheet. All the samples were separately cultured to identify any bacteria or yeast present. RESULTS: Pregnancy rates in patients testing positive for Entrobacteriaceae (22.2% versus 51%) and Staphylococcus species (17.6% versus 44%) were significantly lower than those in the negative culture group ($p < 0.001$). The pregnancy rates do not seem to be affected by the other isolated microorganisms. CONCLUSION: This study shows that the presence of vaginal-cervical microbial contamination at the time of embryo transfer is associated with significantly decreased pregnancy rates. **(Selman H, et al. J Assist Reprod Genet, 2007;24(9):395-9.)**

BACKGROUND: Bacterial contamination of the transfer catheter during embryo transfer is associated with poor clinical outcomes. Antibiotics at the time of embryo transfer may improve outcomes. We evaluated the effect of co-amoxiclav on the rates of bacterial contamination of transfer catheters and clinical pregnancy. METHODS: On the day of oocyte collection, 350 patients were randomized, with sequentially numbered opaque-sealed envelopes containing treatment allocation assigned randomly by computer, to receive co-amoxiclav on the day before and the day of embryo transfer, or no antibiotics. Following transfer, the catheter tips were cultured and assessed to identify the organism(s) isolated and to quantify the level of the contamination. Couples were followed for 8 weeks to determine whether they had achieved clinical pregnancy. Outcome assessors were blinded to the treatment allocation, and the analysis was by intention to treat. RESULTS: Antibiotics significantly reduced catheter contamination rates (49.4 versus 62.3%, RR = 0.79, 95% CI: 0.64, 0.97, P = 0.03). There was no difference detected in clinical pregnancy rates between the two groups (36.0 versus 35.5%, P = 0.83) although there was a significant (P = 0.03) association between the level of bacterial contamination and clinical pregnancy rates. CONCLUSIONS: Co-amoxiclav reduces catheter contamination, but this is not translated into better clinically relevant outcomes such as clinical pregnancy rates. Our findings do not support the routine use of antibiotics at embryo transfer. **(Brook N, et al. Hum Reprod, 2006;21(11):2911-5.)**

Murine embryonic stem cells (mESCs) inoculated at passage P13 with the mycoplasma species M. hominis, M. fermentans and M. orale and cultured over 20 passages showed reduced growth rate and viability ($P < 0.0001$) compared to control mESCs. Spectral karyotypic analysis of mycoplasma-infected mESCs showed a number of non-clonal chromosomal aberrations which increased with the duration of infection. The differentiation status of the infected mESCs was most affected at passage P13+6 where the infection was strongest and 46.3% of the mESCs expressed both POU5F1 and SSEA-1 markers whereas 84.8% of control mESCs expressed both markers. The percentage of germline chimeras from mycoplasma-infected mESCs was examined after blastocyst injection and embryo transfer to

suitable recipients at different passages and, compared to the respective control group, was most affected at passage P13+5 (50% vs. 90%; P < 0.07). Further reductions were obtained at the same passage in the percentage of litters born (50% vs. 100%; P < 0.07) and in the percentage of pups born (22% vs. 45%; P < 0.001). Thirty three chimeras (39.8%) obtained from blastocyst injection with mycoplasma-infected mESCs showed reduced body weight (P < 0.0001), nasal discharge, osteoarthropathia, and cachexia. Flow cytometric analysis of plasma from chimeras produced with mycoplasma-infected mESCs revealed statistically significant differences in the proportions of T-cells and increased levels of IgG1 (P < 0.001), IgG2a (P < 0.05) and IgM (P < 0.05), anti-DNA antibodies (P < 0.05) and rheumatoid factor (P < 0.01). The present data indicate that mycoplasma contamination of mESCs affects various cell parameters, germline transmission, and postnatal development of the resulting chimeras. **Markoullis K, Bulian D, Hölzlwimmer G, Quintanilla-Martinez L, Heiliger KJ, Zitzelsberger H, Scherb H, Mysliwietz J, Uphoff CC, Drexler HG, Adler T, Busch DH, Schmidt J, Mahabir E. Mycoplasma contamination of murine embryonic stem cells affects cell parameters, germline transmission and chimeric progeny. Transgenic Res. 2009;18(1):71-87.**

A series of fused pyranopyrazole and pyranoimidazole, namely 5-(3,6-diamino-4-aryl-5-carbonitrile-pyrano(2,3-c)pyrazol-2-yl)sulphonyl-8-hydroxyquinolines (5a-e), 5-(6-amino-4-aryl-5-carbonitrile-pyrano(2,3-c)pyrazol-3-yl)sulphonamido-8-hydroxyquinolines (6a-e), 5-(2-thioxo-4-aryl-5-carbonitrile-6-amino-pyrano(2,3-d)imidazol-2-yl)sulphonyl-8-hydroxyquinolines (10a-e), and 5-(2-oxo-4-aryl-5-carbonitrile-6-amino-pyrano(2,3-d)imidazol-2-yl) sulphonyl-8-hydroxyquinolines (11a-e), have been prepared via condensation of some arylidine malononitriles with 5-sulphonamido-8-hydroxyquinoline derivatives 3, 4, 8 and 9. All the synthesized compounds were screened for their antimicrobial activities, and most of the tested compounds showed potent inhibition growth activity towards Escherichia coli, Pseudomonas aeruginosa (Gramnegative bacteria). Furthermore, six selected compounds were tested for their antiviral activity against avian paramyxovirus type1 (APMV-1) and laryngotracheitis virus (LTV), and the results showed that a concentration range of 3-4 µg per mL of compounds 2, 3, and 4 showed marked viral inhibitory activity for APMV-1 of 5000 tissue culture infected dose fifty (TCID(50)) and LTV of 500 TCID(50) in Vero cell cultures based on their cytopathic effect. Chicken embryo experiments show that compounds 2, 3, and 4 possess high antiviral activity in vitro with an inhibitory concentration fifty (IC(50)) range of 3-4 µg per egg against avian APMV-1 and LTV and their toxic concentration fifty (CC(50)) of 200-300 µg per egg. **Kassem EM, El-Sawy ER, Abd-Alla HI, Mandour AH, Abdel-Mogeed D, El-Safty MM. Synthesis, antimicrobial, and antiviral activities of some new 5-sulphonamido-8-hydroxyquinoline derivatives.Arch Pharm Res. 2012 Jun;35(6):955-64. Epub 2012 Jun 30**

Cryopreservation & Disinfection of Dry Shippers

Cryopreservation, storage, and transport of cryopreserved germplasm without the risk of disease transmission is of great concern to animal and human health authorities. Here we report on the efficacy of microbial decontamination of liquid nitrogen (LN) dry (vapor) shippers used for shortterm storage and transportation of germplasm and other biological specimens. Dry shippers containing either a hydrophobic or a nonhydrophobic LN absorbent

were experimentally contaminated with high titers of cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococus aureus*, bovine viral diarrhea virus (BVDV), and bovine herpesvirus-1 (BHV-1). Biocidals with broad-spectrum antimicrobial activity and gas vapors of formalin and ethylene oxide were used for disinfection of the shippers. All biocide solutions were diluted with milli-Q water to the concentrations recommended by the manufacturer and poured directly into the chamber of the dry shippers. The dry shipper was filled with the disinfectant for 30 min, drained, washed three times with sterile water, and drained before testing the residue for microbial contaminant. Among the biocidals used, treatment with sodium hypochlorite solution (30% of household bleach), a quaternary ammonium-based disinfectant (100% Expel), and peracetic acid (30%) were the most effective and useful for dry shippers with a hydrophobic LN absorbent. None of the bacterial or viral microorganisms were detected in samples of semen and embryos stored in dry shippers following their disinfection with these biocides. Other disinfectants (Virkon, Roccal, 242 *Reproduction, Fertility and Development* Epidemiology/Diseases 1-Stroke, Expel Odor) were not effective due to their foaming properties. Some other disinfectants (Viralex and 70% ethanol) caused irreversible damage to the permeability of the LN-absorbent hydrophobic membrane. Gas sterilization by ethylene oxide was effective for both types of dry shipper. **(Bielanski A. Repro Fertil Devel 2005;17:241(abstract).)**

Cryopreservation and storage of germplasm is an important factor in the prevention of disease transmission by embryo transfer and artificial insemination. Here we report the results of an investigation on transmission of selected bacterial and viral pathogens by the vapour phase of liquid nitrogen (VPLN) to embryos and semen in dewars designed for short-term storage and transportation of biological specimens. In this study transmission of Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, BVDV, and BHV-1 was examined from: (1) contaminated dry shippers to germplasm; (2) between contaminated and non-contaminated cryopreserved germplasm; and (3) between stock culture of pathogenic agents and germplasm. Contaminated and non-contaminated samples of embryos and semen were stored in proximity in the vapour phase LN in open containers for 7 days prior to testing for the presence of microbes. The results showed that there was no cross-contamination from either the contaminated dewars to germplasm or between contaminated and non-contaminated samples of embryos and semen during 7 days storage under LN vapours. The outcome of our investigation indicates that VPLN is a safe means for short-term storage of embryos and semen in dry shipper dewars commonly used for transportation of bovine germplasm. **(Bielanski A. Cryobiology 2005;50:206-210.)**

Cryopreservation, storage and transport of cryopreserved germplasm without the risk of disease transmission is of great concern to animal and human health authorities. Here we report on the efficacy of microbial decontamination of the liquid nitrogen (LN) dry (vapour) shippers used for short-term storage and transportation of germplasm and other biological specimens. Dry shippers containing either a hydrophobic or a non-hydrophobic LN absorbent were experimentally contaminated with high titers of cultures of Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1). Biocidals with broad spectrum antimicrobial activity and gas vapours of formalin and ethylene oxide were used for disinfection of the dewars. Among the biocidals used, treatment with sodium hypochlorite solution, the quaternary ammonium-based disinfectants and peracetic acid were the most effective and useful for dry shippers

with a hydrophobic LN absorbent. None of the bacterial or viral microorganisms were detected in samples of semen and embryos stored in dry shippers following their disinfection with these biocides. An application of some other disinfectants, due to their foaming properties or to the permeability of the absorbent hydrophobic membrane (HM) was not effective or may have caused irreversible damage to the LN absorbent. Gas sterilization by ethylene oxide in contrast to formalin was fully effective for both types of dry shippers. **(Bielanski A. Theriogenology 2005;63:1946-1957.)**

The objective was to determine the effect of cryopreservation by conventional slow controlled cooling (0.5 degrees C/min) and by vitrification on the presence of bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) infectivity associated with frozen-thawed Day 7 bovine embryos. In this study, Day 7 embryos generated by in vitro fertilization (IVF) were exposed in vitro for 1.5h to BVDV (N=393) and BHV-1 (N=242) and subsequently tested before and after cryopreservation for the presence of infectivity. Exposure of embryos to viral agents resulted in 72% of them infected prior to cryopreservation. Stepwise exposure of embryos to cryoprotectants, as well as their removal, substantially reduced the proportion of contaminated embryos (46% vs. 72%, $P<0.05$). Overall, both freezing methods reduced the percentage of infectious embryos compared with that of embryos similarly exposed to viruses but not cryopreserved (31% vs. 72%, respectively; $P<0.001$). The percentage of embryos with infectious viruses was not significantly higher after vitrification than after slow cooling (38% vs. 22%). In addition, after cryopreservation, a higher percentage ($P<0.002$) of embryos exposed to BHV-1 (42%) remained infectious than did embryos exposed to BVDV (24%). In conclusion, cryopreservation reduced the proportion of infected embryos but did not render all of them free from infectious pathogens.
Bielanski A, Lalonde A. Effect of cryopreservation by slow cooling and vitrification on viral contamination of IVF embryos experimentally exposed to bovine viral diarrhea virus and bovine herpesvirus-1. Theriogenology. 2009;72(7):919-25.

Reviews A misconception in the field of reproductive medicine is that there is a significant risk of cross-contamination during gamete or embryo cryostorage. This article is a review of the available literature on animal models and human IVF and it suggests otherwise. There is a negligible risk of cross-contamination in IVF working conditions. **Pomeroy KO, Harris S, Conaghan J, Papadakis M, Centola G, Basuray R, Battaglia D Storage of cryopreserved reproductive tissues: evidence that cross-contamination of infectious agents is a negligible risk. Fertil Steril. 2010 Sep;94(4):1181-8. Epub 2009 May 29.**

This review summarizes pertinent data and opinions regarding the potential hazard of disease transmission through cryopreserved and banked embryos in liquid nitrogen (LN). Special attention is given to the survival of pathogens in LN, new vitrification methods, sterility of LN, risks associated with the use of straws and cryovials, and LN dewars including dry shippers. It was experimentally demonstrated that cross-contamination between LN and embryos may occur, when infectious agents are present in LN and embryos are not protected by a sealed container. It is important, therefore, to prevent direct contact of embryos with LN during cryopreservation and their banking. This includes the usage of hermetically sealed, high-quality, shatter-proof freezing containers and/or the application of

a secondary enclosure such as "double bagging or straw in straw." A periodic disinfection of cryo-dewars should be considered as an additional precaution to diminish the potential for inadvertent cross-contamination. It might be advisable to use separate LN dewars to quarantine embryos derived from infected donors of valuable genotype or from unknown health status, extinction-threatened species. Nevertheless, in summary, it has been concluded that over 25 yr with no direct evidence of disease transmission by transferred cryopreserved human and animal embryos, that the present cryopreservation technology is sanitary sound, with the stipulation that biocontainment measures recommended by the International Embryo Transfer Society (IETS) and the World Organization for Animal Health - Office International des Epizooties (OIE), are strictly followed. **Bielanski A. A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. Theriogenology. 2012 Feb;77(3):467-82**

Review The introduction and widespread application of vitrification are one of the most important achievements in human assisted reproduction techniques (ART) of the past decade despite controversy and unclarified issues, mostly related to concerns about disease transmission. Guidance documents published by US Food and Drug Administration, which focused on the safety of tissue/organ donations during Zika virus spread in 2016, as well as some reports of virus, bacteria, and fungi survival to cryogenic temperatures, highlighted the need for a review of the way how potentially infectious material is handled and stored in ART-related procedures. It was experimentally demonstrated that cross-contamination between liquid nitrogen (LN2) and embryos may occur when infectious agents are present in LN2 and oocytes/embryos are not protected by a hermetically sealed device. Thus, this review summarizes pertinent data and opinions regarding the potential hazard of infectious transmission through cryopreserved and banked reproductive cells and tissues in LN2. Special attention is given to the survival of pathogens in LN2, the risk of cross-contamination, vitrification methods, sterility of LN2, and the risks associated with the use of straws, cryovials, and storage dewars. **Joaquim, D.C., Borges, E.D., Viana, I.G., Navarro, P.A. and Vireque, A.A., 2017. Risk of contamination of gametes and embryos during cryopreservation and measures to prevent cross-contamination. BioMed research international, 2017.**

Chilled conservation

Cryopreservation methods using liquid nitrogen (LN2) for gametes and embryos are prevalent in mammalian artificial reproduction. However, the pregnancy rate from frozen embryos has not improved over the past two decades because freeze–thawing causes significant damage. The strict regulation of transportation of LN2 containers by airlines also limits exchange between breeders. In this article, we introduce a medium that enabled bovine embryos to be held for up to 7 days at 4°C. Apregnancy rate of 75% (24/32) was obtained for embryos held for 7 days in this medium and transferred to primed recipients. Its constituents were medium 199, foetal bovine serum, and HEPES for buffering. This technique will enable LN2-free storage and air transportation of embryos provided transplantation to recipients can be completed within 7 days.

Ideta, A., Aoyagi, Y., Tsuchiya, K., Kamijima, T., Nishimiya, Y., &Tsuda, S. (2013). A simple medium enables bovine embryos to be held for seven days at 4° C. *Scientific reports*, *3* (1173) 1-5 (2013).

Culture systems free of animal-origin supplements

(George F, et al. Proceedings of 23rd Annual AETE meeting, Alghero, Sardinia. 2007: 168 abstr.)

Diagnostic assays on embryos

The objective was to develop a method to accurately and efficiently detect minute amounts of bovine viral diarrhea virus (BVDV) associated with a single embryo. There are two major challenges for BVDV detection in a single embryo: the test sensitivity and the efficiency of viral molecule recovery. These become even more critical when attempts are made to detect BVDV infections that occurred naturally, not through artificial exposure of the embryos to high affinity BVDV strains. We have developed a one-step sample preparation method that has increased the viral molecule recovery rate compared to the standard RNA isolation procedure by 7-100-fold. Instead of using the traditional virus exposure approach, we generated BVDV positive embryos via somatic cell nuclear transfer (SCNT) technology using BVDV positive donor cells. By combining the highly efficient sample preparation procedure with a sensitive one-step, real-time PCR system, we have developed a sensitive test that allows detection of as low as two copies of BVDV in a single embryo. This method will allow systematic risk assessment for BVDV transmission during in vitro embryo production via IVF or SCNT procedures.
Gregg K, Chen SH, Guerra T, Sadeghieh S, Xiang T, Meredith J, Polejaeva I. A sensitive and efficient detection method for bovine viral diarrhea virus (BVDV) in single preimplantation bovine embryos. Theriogenology. 2009;71(6):966-74.

This study proposes a validation strategy for an automated extraction procedure, followed by RT-qPCR analysis. To avoid false-negative results, a triplex RT-qPCR was used which detects the target viral RNA, an internal and an external control. The methods to determine the validation parameters such as linearity, efficiency, analytical sensitivity, analytical specificity and intra- and interrun variability are described in detail. Special attention is given to the analytical sensitivity, which is determined by probit analysis. The limit of detection was set at the input concentration resulting in a positive result in 95% of the repeats. The intra- and interrun variability was analysed profoundly by testing samples covering a broad range of viral loads, from strong positive to weak positive. To increase the diagnostic capacity, the extraction protocol was automated with a JANUS Automated Workstation (PerkinElmer, Waltham, MA), which can extract 186 samples in 2h and 30 min. The automation of the extraction protocol implied some additional validation parameters to be determined such as position-effect, absence of cross-contamination and comparison with the manual protocol. These parameters give essential information about the performance of the robot and are of great importance when the automated assay is used in an accreditation system.**Vandemeulebroucke E, De Clercq K, Van der Stede Y, Vandenbussche F. A proposed validation method for automated nucleic acid extraction and RT-qPCR analysis: an**

example using Bluetongue virus. J Virol Methods. 2010 Apr;165(1):76-82. Epub 2010 Jan 29.

Commercial cattle breeders produce their own herd offspring for the dairy and beef market using artificial insemination. The procedure involves sanitary risks associated with the collection and commercialization of the germplasm, and the in vitro production and transfer of the bovine embryos must be monitored by strict health surveillance. To avoid the spreading of infectious diseases, one must rely on using controlled and monitored germplasm, media, and reagents that are guaranteed free of pathogens. In this article, we investigated the use of a new mass spectrometric approach for fast and accurate identification of bacteria and fungi in bovine semen and in culture media employed in the embryo in vitro production process. The microorganisms isolated from samples obtained in a commercial bovine embryo IVP setting were identified in a few minutes by their conserved peptide/protein profile, obtained applying matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), matched against a commercial database. The successful microorganisms MS identification has been confirmed by DNA amplification and sequencing. Therefore, the MS technique seems to offer a powerful tool for rapid and accurate microorganism identification in semen and culture media samples.

Drops of semen used for IVF were collected and the MALDI-MS method was applied to detect presence of bacteria in the medium. The purpose was to assess the quality of the media. **Zampieri D, Santos VG , Braga PAC , Ferreira CR , Ballottin D, Tasic L , Basso AC , Sanches BV , Pontes JHF , Pereira da Silva B , Garboggini FF , Eberlin MN, Tata A. Microorganisms in cryopreserved semen and culture media used in the in vitro production (IVP) of bovine embryos identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).** ***Theriogenology 80 (2013) 337–345.***

Diagnosis using Multiplex testing

Potential causes of congenital infection include Toxoplasma gondii and viruses such as cytomegalovirus (CMV), enterovirus, hepatitis C virus, herpes simplex virus types 1 and 2 (HSV1 and 2), human herpesvirus types 6, 7, and 8, lymphocytic choriomeningitis virus, parvovirus, rubella virus, and varicellazoster virus. Testing for each of these agents using nucleic acid tests is time consuming and the availability of clinical samples such as amniotic fluid or neonatal blood is often limited. The aim of this study was to develop multiplex PCRs (mPCRs) for detection of DNA and RNA agents in the investigation of congenital infection and an mPCR for the viruses most commonly requested in a diagnostic virology laboratory (CMV, EpsteinBarr virus, enterovirus, HSV1, HSV2, and varicellazoster virus). The assays were assessed using known pathogenpositive tissues (cultures, placentae, plasma, and amniotic fluid) and limits of detection were determined for all the agents studied using serial dilutions of plasmid targets. Nested PCR was performed as the most sensitive assay currently available, and detection of the amplicons using hybridization to labeled probes and enzymelinked immunosorbent assay detection was incorporated into three of the four assays. This allowed detection of 10 to 10_2 copies of each agent in the samples processed. In several patients, an unexpected infection was diagnosed, including a case of encephalitis where HSV was the initial clinical suspicion but CMV was detected. In the majority of these cases the

alternative agent could be confirmed using reference culture, serology, or fluorescence methods and was of relevance to clinical care of the patient. The methods described here provide useful techniques for diagnosing congenital infections and a paradigm for assessment of new multiplex PCRs for use in the diagnostic laboratory. **(McIver CJ et al, J Clin Microbiol 2005;43:5102-5110)**

A multiplex real-time PCR was designed to detect and differentiate equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). The PCR targets the glycoprotein B gene of EHV-1 and EHV-4. Primers and probes were specific to each equine herpesvirus type and can be used in monoplex or multiplex PCRs, allowing the differentiation of these two closely related members of the Alphaherpesvirinae. The two probes were minor-groove binding probes (MGB) labelled with 6-carboxy-fluorescein (FAM) and VIC for detection of EHV-1 and EHV-4, respectively. Ten EHV-1 isolates, six EHV-1 positive clinical samples, one EHV-1 reference strain (EHV-1.438/77), three EHV-4 positive clinical samples, two EHV-4 isolates and one EHV-4 reference strain (EHV-4 405/76) were included in this study. EHV-1 isolates, clinical samples and the reference strain reacted in the EHV-1 real-time PCR but not in the EHV-4 real-time PCR and similarly EHV-4 clinical samples, isolates and the reference strain were positive in the EHV-4 real-time PCR but not in the EHV-1 real-time PCR. Other herpesviruses, such as EHV-2, EHV-3 and EHV-5 were all negative when tested using the multiplex real-time PCR. When bacterial pathogens and opportunistic pathogens were tested in the multiplex real-time PCR they did not react with either system. The multiplex PCR was shown to be sensitive and specific and is a useful tool for detection and differentiation of EHV-1 and EHV-4 in a single reaction. A comprehensive equine herpesvirus disease investigation procedure used in our laboratory is also outlined. This procedure describes the combination of alphaherpesvirus multiplex real-time PCR along with existing gel-based PCRs described by other authors. **(Diallo IS, et al. Vet Microbiol, 2007;123(1-3):93-103.)**

The objective of this study was to develop and validate a duplex quantitative polymerase chain reaction (qPCR) for simultaneous detection of bovine herpesvirus 1 (BHV-1) and bovine viral diarrhea virus (BVDV) type I and II. Follicular fluid was collected from a BHV-1 acutely infected heifer, a BVDV I persistently infected heifer, and from 10 ovaries recovered from an abattoir. Both the BHV-1 and BVDV contaminated follicular fluid were diluted 1:5 to $1:10^7$ using the pooled, abattoir-origin follicular fluid. Each dilution sample was analyzed using the duplex qPCR, virus isolation, reverse transcription nested PCR and BHV-1 qPCR. The duplex qPCR was able to simultaneously detect diluted BHV-1 (1:100) and BVDV I (1:1,000). These results corresponded with the reverse transcription nested PCR and BHV-1 qPCR. Therefore, the duplex qPCR might be used for quality assurance testing to identify these two viruses in cells, fluids and tissues collected from donor animals and used in reproductive technologies.
Marley MSD, Givens MD, Galik PK, Riddell KP, Stringfellow DA. Development of a duplex quantitative polymerase chain reaction assay for detection of bovine herpesvirus 1 and bovine viral diarrhea virus in bovine follicular fluid. Theriogenology. 2008 Jul 15;70(2):153-60.

Disinfection of fluids

Effects of 18 commercial lots of fetal calf serum (FCS) after gamma-irradiation and their non-irradiated counterparts were comparatively analyzed on CHO-K1 and MDBK MDL1 cells for genotoxicity [sister chromatid exchange (SCE), micronuclei (MNi), and single cell gel

electrophoresis (SCGE)], cytotoxicity [cell-cycle progression (CCP), proliferative replication index (PRI), mitotic index (MI), growth promotion (GP), and plating efficiency (PE)], and microbiological properties (mycoplasma and bovine viral diarrhea virus contamination). SCE and SCGE were the most informative end-points for genotoxicity since significant differences were found in 44.4% (P<0.05-0.001, Student's t-test) and 61.1% (P<0.05-0.001, chi(2) test) samples, respectively. MI was the cytotoxicity assay revealing the greatest variation, showing differences in 66.7% (P<0.05-0.001, chi(2) test) samples. Thus, these three end-points for screening bioproducts such as FCS were found most suitable for detecting potential geno-cytotoxicants in biological samples; their simultaneous use could be strongly recommended.

Pilili JP, González NV, Molinari G, Reigosa MA, Soloneski S, Larramendy ML.Testing genotoxicity and cytotoxicity strategies for the evaluation of commercial radiosterilized fetal calf sera. Biologicals. 2010 Jan;38(1):135-43. Epub 2009 Aug 28.

Endogenous retroviral sequences

Cloning may provide animals with high genetic value, but raises concerns about the risk related to mammalian endogenous retroviral sequences. No information is available on the risk related to such bovine endogenous sequences (BERV), particularly after nuclear re-programming in clones. Only three BERV sequences, present in multiple copies, have been sequenced. We first determined if they were present in controls and clones of the INRA's livestock. We then determined whether: (i) BERV are transcriptionally active, especially in clones; (ii) nuclear transfer is associated with a variation of their copy number; (iii) peripheral blood mononuclear cells (PBMC) are able to produce any type of retroviral sequences. We designed standard and Q-PCR tests to detect BERV in PBMC DNA and RNA from controls, funders and clones. We performed complementary *in vitro* co-cultures with human cells and bovine PBMC. The presence of the reverse transcriptase enzyme, associated with retroviral particles, was analysed in cell culture supernatants with the ultra-sensitive PERT assay. Our results convincingly showed that (i) the three described BERV are present in the genome of control bovine and are not transcriptionally active, (ii) transcription of BERV is not re-activated in clones derived PBMC, (iii) no variation of the BERV copy number is associated with nuclear transfer, (iv) particle-associated reverse transcriptase activity was detected neither in bovine PBMC supernatants nor in those of human cells co-cultivated with bovine cells. Therefore, our results do not suggest a 178 higher retroviral risk for clones.
(Martignant L et al, Reproduction Domestic Animals 2006;41:332(abstr.)

Exogenous DNA uptake

There is a paucity of information about sperm-mediated transmission of exogenous DNA to implanting embryos and cells of the reproductive tract. Preliminary experiments established that sperm has the capacity to actively take in exogenous DNA derived from human papilloma virus (HPV). In addition, blastocysts also take up exogenous HPV DNA, but in contrast to sperm, the process appears passive. DNA-carrying sperm migrating in an artificial glass tube or excised mouse bicornuate uteri transfected the blastocysts at the remote position using a flip-flop mechanism. There were preferential transmission of the types of

HPV DNA but this was not attributed to the gene sequence or the size of the DNA fragments. The internalized DNA became undetectable unless continuous sperm bombardment or pricking took place. Mycoplasma vectors offer a novel way to enhance the transfection of blastocyst with exogenous DNA. **(Chan PJ et al, Mol Reprod Dev 2000;56:316-318)**

Biological vectors for cell transfection are mainly viral in origin, with inherent shortcomings. Mycoplasmas are ubiquitous organisms that traverse cells easily. The objective was to determine if *Ureaplasma urealyticum* (T-mycoplasma) would vector exogenous BRCA1 DNA into blastocysts. Hatching mouse blastocysts (N = 70) were incubated in the presence of either viable or dead *Ureaplasma urealyticum* at 37 degrees C for 1 hr. The blastocysts were exposed to human BRCA1 DNA lacking homology in the mouse genome for 2 hr, followed by DNase-1 treatment and wash. Polymerase chain reaction and agarose gel electrophoresis analysis of amplified products were performed. The BRCA1 gene was detected in the blastocysts only when viable *Ureaplasma* was present. PCR analyses of control *Ureaplasma* and untreated blastocysts were negative. Viable *Ureaplasma* organisms were shown to mediate the uptake of DNA fragments into blastocysts, resulting in transgenic mouse blastocysts with a normal human BRCA1 exon 11 gene. (**Chan PJ et al, J Assist Reprod Genet 1999;16:546-550.)**

Sperm-mediated DNA transfer can be used to transfer exogenous DNA into the oocyte for the production of transgenic animals. In spite of controversy in the literature, sperm-mediated DNA transfer is a simple and quick technique that can be used in routine breeding programs (AI, embryo transfer and IVF). The main objective of this study was to determine the factors affecting the spontaneous uptake of exogenous DNA by bull spermatozoa. For this purpose, fresh and frozen spermatozoa (0.25 106), from the same ejaculate from each of four bulls were co-incubated with fluorescent-labeled green fluorescent protein (GFP) and chloremphenicol acetyltransferase (CAT) plasmids at 37 8C for 30 min. Neither bull nor plasmid significantly affected the uptake of exogenous DNA. However, transfection efficiency was higher in frozen-thawed versus fresh spermatozoa ($P < 0.001$). Regardless of whether transfected spermatozoa were alive or dead, all transfected spermatozoa were immotile. It can be concluded that a population of spermatozoa is present in bull semen which has the ability to uptake exogenous DNA spontaneously. There is tremendous scope to improve transfection efficiency of spermatozoa while maintaining motility; this needs to be achieved in order to more easily use this technique in transgenesis. However, live-transfected bull spermatozoa clearly can incorporate exogenous DNA and should be usable in intracytoplasmic sperm injection protocols. **(Anzar M, Buhr MM. Theriogenology 2006;65:683-690.)**

Fetal bovine serum

Fetal bovine serum (FBS) used in cell culture may be contaminated with viruses, among them bovine viral diarrhea virus (BVDV) affecting the production of biological reagents and the results of diagnosis. The filtration process used in the preparation of commercial FBS abrogates most viral agents that may be present in raw FBS, but BVDV may pass through the filters because of its small size and its pleomorphism. While detection of bovine herpes virus-1 and parainfluenza-3 (PI-3) is determined by observation of the cytopathic effect, and

also by hemadsorption in the case of PI-3, the most frequently isolated BVDV is non cytopathic, and infects cells without morphological alterations, inducing problems that arise after several cell generations. Batches of raw and processed FBS were analyzed. Frequencies of BVDV detection in raw serum in Argentina were similar to those published for USA. By conventional methods for BVDV detection, only 2 of 20 commercial batches of FBS had BVDV. Using cell cultures maintained with high concentrations of the serum under study for at least 2 weeks, with detection of viral antigen by indirect immunofluorescence, the percentage of BVDV detection was 80%. This method shows that most lots of commercial FBS contain BVDV. RT-PCR allows faster detection of the viral genome, but it must be validated, as it does not show viral replication. To eliminate the problem of BVDV contamination in FBS, only gamma irradiated FBS is used in our laboratory. **(Zabal O et al. Rev Argent Microbiol 2000;32:27-32)**

The recently emerging atypical bovine pestiviruses have been detected in commercial foetal bovine serum (FBS) of mainly South American origin so far. It is unclear how widely the viruses are presented in commercial FBS of different geographic origins. To further investigate the possible pestivirus contamination of commercially available FBS batches, 33 batches of FBS were obtained from ten suppliers and analysed in this study for the presence of both the recognised and the atypical bovine pestiviruses. All 33 batches of FBS were positive by real-time RT-PCR assays for at least one species of bovine pestiviruses. According to the certificate of analysis that the suppliers claimed for each batch of FBS, BVDV-1 was detected in all 11 countries and BVDV-2 was detected exclusively in the America Continent. The atypical pestiviruses were detected in 13 batches claimed to originate from five countries. Analysis of partial 5'UTR sequences showed a high similarity among these atypical bovine pestiviruses. This study has demonstrated, for the first time that commercial FBS batches of different geographic origins are contaminated not only with the recognised species BVDV-1 and BVDV-2, but also with the emerging atypical bovine pestiviruses. **Xia H, Vijayaraghavan B, Belák S, Liu L. Detection and identification of the atypical bovine pestiviruses in commercial foetal bovine serum batches. PLoS One. 2011;6(12):e28553. Epub 2011 Dec 8.**

We isolated a bovine viral diarrhea virus (BVDV) from commercial fetal bovine serum and designated it HLJ-10. The complete genome is 12,284 nucleotides (nt); the open reading frame is 11,694 nt, coding 3,898 amino acids. Phylogenetic analysis indicated that this strain belongs to BVDV group 2. **Liu H, Li Y, Gao M, Wen K, Jia Y, Liu X, Zhang W, Ma B, Wang J. Complete genome sequence of a bovine viral diarrhea virus 2 from commercial fetal bovine serum. J Virol. 2012 Sep;86(18):10233. doi: 10.1128/JVI.01581-12.**

Two European laboratories independently detected atypical bovine pestiviral nucleic acids in three commercial batches of foetal bovine serum (FBS) that was claimed by the producers to be of Australian origin. Additional batches of FBS were obtained directly from Australia to exclude possible contamination of the Australian FBS with that of South American origin during manufacturing/packaging in European countries. RT-PCR amplification of partial 5'untranslated region and the complete Npro gene yielded a specific band with expected size, which was confirmed by DNA sequencing. Bayesian analysis of sequence data demonstrated a closer phylogenetic relation of the newly detected atypical bovine pestiviruses to those of South American origin, which were related to the recognized bovine

pestivirus species, bovine viral diarrhoea virus. Taken together, the results indicated the presence of atypical bovine pestiviruses in the Australian FBS, and most likely in Australian Continent. **Xia H, Larska M, Giammarioli M, De Mia GM, Cardeti G, Zhou W, Alenius S, Belak S, Liu L. Genetic Detection and Characterization of Atypical Bovine Pestiviruses in Foetal Bovine Sera Claimed to be of Australian Origin. Transboundary and Emerging Diseases 2013;60: 284-288.**

Genetic defects

An investigation was carried out on a family of Limousin-Jersey crossbreds exhibiting low fertility in the females, to determine the impact of a previously identified X-autosome translocation (X-AT) on the reproductive performance of the carrier cows. Three of the identified translocation carriers, including a cow and two of her daughters, were maintained at our University Research Station and artificially inseminated periodically with semen from different bulls of known fertility. Attempts to breed the X-AT carriers resulted in high rates of return to estrus between days 28 and 60, abortions between days 121 and 235 after insemination, and a total of 13 live births including 4 translocation carrier calves. Results of superovulation and embryo retrieval trials on X-AT carriers revealed significantly higher proportions of unfertilized and uncleaved ova and abnormal embryos compared to those from normal cows, and no pregnancy in the recipients transferred with morphologically normal blastocysts from X-AT carriers. While the higher rates of failed fertilization and cleavage, abnormal embryos and return to estrus in X-AT carriers could be attributed to chromosome imbalance expected in their gametes, the relatively high prevalence of abortion (late in gestation) was unexpected. Our observations on the fetuses expelled by X-AT carriers after 5 months of gestation indicated that a majority (three out of four) of these fetuses were products of abnormal (3:1) segregation in meiosis I and that these chromosomally unbalanced (hyperdiploid) conceptuses were able to survive early embryogenesis and fetal life up to the end of the second trimester. We hypothesize that their relatively long in utero life and the absence of any overt birth defects may be attributable to the type of chromosomes over-represented in these fetuses and that their eventual expulsion may have been the result of selection against the clonal population of cells in which the altered X carrying a segment of chromosome 23 (Xp(+)), remained inactive. **(Basrur PK et al. Anim Repro Sci 2001;67:1-16)**

Review : Advances in biotechnology in recent decades have revolutionized our understanding of early mammalian development and promise to provide ever more finely tuned and precisely targeted techniques for genetic enhancement of domestic animal species. In demonstrating what is both technically and biologically possible, not only in mice but also in larger animal species, research has provided hope that previously intractable diseases and genetic defects can be successfully combated. Crucial to this research is the ability to culture oocytes, embryos and somatic cells in vitro and to sustain their development without inducing adverse short- or long-term consequences. There is a need to refine current culture strategies in farm animal species to avoid jeopardizing their dependent technologies. A key to resolving current limitations of culture strategies is to identify, acknowledge and then address those features of in vitro culture that compromise early regulation of mammalian development. The aim of this review is to appraise critically in vitro embryo and somatic cell production strategies in the context of their impact on

developmental competence and normality at embryonic, fetal and later stages. In addition, effects of physically manipulating embryos and cells, most notably via nuclear and gene transfer technologies, are considered with a view to identifying how detrimental consequences can be avoided. **(McEvoy TG et al. Reproduction 2001;122:507-518).**

PURPOSE: The effect of human papillomavirus (HPV) DNA from the E6-E7 region on the integrity of DNA in blastocyst stage embryonic cells was studied. The study design paralleled the event whereby HPV DNA from the infecting virus would target host cell DNA. The objectives were (a) to determine if the DNA of blastocysts were disrupted by the presence of HPV DNA and (b) to determine if the intensity of DNA damage was associated with the type of HPV. METHODS: This study involved superovulating female mice, mating, collecting one-cell embryos, and culturing to the expanded blastocyst stage. The blastocysts were infected with PCR-synthesized DNA fragments from either HPV type 16, 18, 31, or 33. The blastocyst DNA were analyzed by comet assay after 24 h of incubation. The fluorescent images were digitized and the pixel intensity of each blastocyst was measured. RESULTS: Only the DNA of HPV type 16 was associated with significant DNA fragmentation in comparison with the other HPV types. There was no relationship between HPV DNA fragment size and the intensity of DNA fragmentation. CONCLUSIONS: The data suggested that one mode of action of HPV type 16 was to initiate apoptosis of embryonic cells through DNA fragmentation. The effect of HPV 16 occurred rapidly within 24 h. The intensity of DNA damage was not linked to the specific type of HPV. However, the results do not rule out the other HPV types affecting embryos under conditions different from this study. **(Calinisan JH et al, Journal of Assisted Reproduction and Genetics 2002;19:132-136.)**

Immunoglobulins in body fluids

Immunoglobulin concentrations in bovine serum, follicular fluid and uterine and vaginal secretions were determined. The specificities of IgG, IgM and IgA for virus-neutralizing antibody against bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) viruses were also examined. High concentrations of IgG were present in both serum and follicular fluid. The IgG, IgM and IgA concentrations were low in uterine and vaginal secretions. There was more IgG in the uterus at estrus than at any other time. Virus-neutralizing antibodies against BVD and IBR in serum of cows were mainly the IgG class. There was positive correlation between serum and follicular fluid virus-neutralizing antibody titers for BVD and IBR. These antibodies may provide some protection for recently ovulated ova. **(Whitmore HL et al. Am J Vet Res 1977;38:455-457)**

Neospora caninum, infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea virus (BVDV) are important differentials for the diagnosis of infectious reproductive loss in beef herds. The objective of this study was to describe the serological status of both pregnant and non-pregnant beef cows from herds with varying levels of reproductive success. The study provided an opportunity to examine whether there were any associations between serological status for BVDV, IBR, and N. caninum and pregnancy status, as well as the subsequent risk of abortion, or stillbirth. Samples were collected from 2516 cows and heifers from 66 herds; 31 herds where the proportion pregnant was <90% and 35 randomly selected herds where the proportion pregnant was > or =90%. Of these samples 5.9% were positive for antibodies to N. caninum, 20.4% had titres >1:80 to IBR, 91.8% had titres > or =1:256 to

BVDV type 1, and 23.9% had titres > or =1:256 to BVDV type 2. N. caninum antibody concentration was associated with an increased individual animal risk of non-pregnancy (OR(logS/P), 1.9; 95% CI, 1.2-2.9) and abortion (OR(pos/neg), 2.8; 95% CI, 1.1-7.5). The proportion of animals at pregnancy testing with antibodies to BVDV type 2 above 1:3000 (OR(10%changeinprevalence), 2.3; 95% CI, 1.5-3.5) was also associated with an increased risk of abortion. No other measures of antibody status were associated with reduced reproductive performance in this group of herds. Antibodies to Mycobacterium avium spp.paratuberculosis were also measured; 0.7% of samples were positive (sample to positive (S/P) >0.25) and 3.6% were suspicious (S/P, 0.10-0.25). **(Waldner CL. Anim Reprod Sci, 2005;90(3-4):219-42.)**

Interspecies embryos

The low efficiency of somatic cell nuclear transfer may be related to the ultrastructural deviations of reconstructed embryos. The present study investigated ultrastructural differences between in vivo-produced and cloned goat embryos, including intra- and interspecies embryos. Goat ear fibroblast cells were used as donors, while the enucleated bovine and goat oocytes matured in vitro as recipients. Goat-goat (GG), goat-cattle (GC) and goat in vivo-produced embryos at the 2-cell, 4-cell, 8-cell and 16-cell stages were compared using transmission electron microscopy. These results showed that the three types of embryos had a similar tendency for mitochondrial change. Nevertheless, changes in GG embryos were more similar to changes in in vivo-produced embryos than were GC embryos, which had more extreme mitochondrial deviation. The results indicate the effects of the cytoplast on mitochondria development. The zona pellucida (ZP) in all three types of embryos became thinner and ZP pores in both GC and GG embryos showed an increased rate of development, especially for GC embryos, while in vivo-produced embryos had smooth ZP. The Golgi apparatus (Gi) and rough endoplasmic reticulum (RER) of the two reconstructed embryos became apparent at the 8-cell stage, as was found for in vivo embryos. The results showed that the excretion of reconstructed embryos was activated on time. Lipid droplets (LD) of GC and GG embryos became bigger, and congregated. In in vivo-produced embryos LD changed little in volume and dispersed gradually from the 4-cell period. The nucleolus of GC and GG embryos changed from electron dense to a fibrillo-granular meshwork at the 16-cell stage, showing that nucleus function in the reconstructed embryos was activated. The broken nuclear envelope and multiple nucleoli in one blastomere illuminated that the nucleus function of reconstructed embryos was partly changed. In addition, at a later stage in GC embryos the nuclear envelope displayed infoldings and the chromatin was concentrated, implying that the blastomeres had an obvious trend towards apoptosis. The gap junctions of the three types of embryos changed differently and GG and GC embryos had bigger perivitelline and intercellular spaces than did in vivo-produced embryos. These results are indicative of normal intercellular communication at an early stage, but this became weaker in later stages in reconstructed embryos. In conclusion, inter- and intraspecies reconstructed embryos have a similar pattern of developmental change to that of in vivo-produced embryos for ZP, rough ER, Gi and nucleolus, but differ for mitochondria, LD, vesicles, nucleus and gap junction development. In particular, the interspecies cloned embryos showed more severe destruction. These

ultrastructural deviations might contribute to the compromised developmental potential of reconstructed embryos.
Tao Y, Cheng L, Zhang M, Li B, Ding J, Zhang Y, Fang F, Zhang X, Maddox-Hyttel P. Ultrastructural changes in goat interspecies and intraspecies reconstructed early embryos. Zygote. 2008;16(2):93-110.

Miscellaneous Topics (media altern., ethylene oxide toxicity , & ZP invasion)

One- to 4-cell bovine embryos were exposed to replication defective retrovirus by microinjection of retrovirus producer cells into perivitelline space. Embryos were cultured in vitro for 3 to 4 days, then transferred to recipient cows for further development. Thirteen of 22 embryos recovered at 15 days gestation and each of four fetuses recovered at 90 days gestation were transgenic. Fetuses harbored between 2 and 12 proviruses, and within each fetus, identical patterns of integration were observed in seven tissues tested. Estimates of the number of proviruses per cell suggested that in three of the four fetuses, most, and possibly all, cells were transgenic. The authors suggested that the technique might facilitate application of transgenic technology in cattle and other agriculturally important species. (**Haskell and Bowen, Mol Reprod Develop 1995;40:386-390**)

The potential of ethylene oxide (EtO) residues in exposed plastic tissue culture dishes to adversely affect bovine oocyte maturation, fertilization, and subsequent embryonic development was monitored. In experiment 1, the effects of aeration time and aeration combined with washing of EtO-gassed culture dishes on the extent of residual toxicity were investigated. There was no cleavage in any treatment in which oocytes were matured and fertilized in dishes exposed to EtO. EtO residues caused functional degeneration of oocytes even when culture dishes were aerated for more than 12 days post EtO-exposure and repeatedly washed. In experiment 2, the residual toxicity of EtO gas on in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) were evaluated. Cleavage rate significantly decreased and post cleavage development was retarded in ova maintained in dishes treated with EtO either during IVM or IVF. EtO residues may be more detrimental to spermatozoa than to oocytes that have been the primary cause of fertilization failure during IVF. (**Holyoak et al, Toxicology 1996;108:33-38**)

The objective of the study was to produce viable bovine blastocysts by IVM/IVF/IVC in media without bovine origin products. BSA was replaced by polyvinal alcohol in wash medium, FCS was replaced by rabbit serum in IVM and IVC media, and BSA was replaced by rabbit serum albumin in IVF and IVC medium. Number of oocytes maturing to Metaphase II was similar when IVM medium was supplemented with FCS (90/96) and rabbit serum (92/96). The number of “2-pronuclei-formation” was similar after sperm separation and fertilization in Tyrode’s medium supplemented with BSA (70/97) or rabbit albumin (70/94). In a third series of experiments in which IVM/IVF/ and IVC (with cumulus) were conducted with bovine or rabbit products, the rates of cleavage and 5- to 8-cell stages at 3 d and development to blastocyst at 8 d were similar. [Bovine vs rabbit: cleavage = 81% vs 80%; 5- to 8-cell stage = 49% vs 44%; blastocysts = 37% vs 30%]. Use of rabbit origin products resulted in 0.5% delay in blastocyst detection. Also, blastocysts produced in media with rabbit products had a significantly lower

number of cells than those produced with bovine products (Rabbit vs bovine: 101+7 vs 120+6). (**Van Langendonckt et al. Theriogenology 1997;48:1387-1395**)

The report describes initial steps of a project to improve the "health and welfare" of transgenic mice at the European Molecular Biology Laboratory (EMBL), by re-deriving transgenic lines as microbiologically defined animals to be maintained in a barrier unit in a newly constructed animal facility. The pilot study showed that it was possible to transfer embryos obtained from contaminated donor mice in an old facility to specific pathogen free recipients housed in a ventilated cabinent in the new facility without transmission of disease. Offspring following embryo transfer showed no evidence of contamination with any of the pathogens present in mice in the old facility. Antibodies to various murine viruses (mouse hepatitis virus [MHV], rota virus, reo-3 virus, Theiler's encephalitis virus, adenovirus) and parasites were present in sentinel animals from the old facility, but the re-derived animals were found to be free of viral antibodies and parasites. Methods used were considered successful in terms of disease prevention and enhancement of welfare. **(Morrell JM, Lab Anim 1999;33:201-206)**

Pathogens in Materials of Animal Origin

Non-pathogenic porcine circovirus type 1 (PCV1) and pathogenic PCV2 are widespread in swine herds. In this study, the detection and characterization of PCV1 and PCV2 DNA from porcine-derived commercial pepsin are reported. The complete genomic sequences of the pepsin-derived PCV1 and PCV2 share 76% nucleotide sequence identity with each other and 95–99% identity with respective North American PCV1 and PCV2 isolates. However, the PCV-contaminated pepsin lacks infectivity in PK-15 cells. To further assess the infectivity of the contaminating pepsin in vivo, 16 5-week-old, specific-pathogen-free pigs were divided randomly into three groups: pigs in group 1 (n=5) were each inoculated intramuscularly and intranasally with 4 ml PBS buffer as negative controls, those in group 2 (n=6) were each inoculated with 400 mg contaminated pepsin dissolved in 4 ml PBS and those in group 3 (n=5) were each inoculated with 46104?3 TCID50 PCV2 as positive controls. PCV2 viraemia, seroconversion and pathological lesions were detected in group 3 pigs, but not in group 1 or 2 pigs, confirming that the contaminating PCVs were non-infectious. Nevertheless, the detection of PCV DNA in a porcine-derived commercial product raises concern for potential human infection through xenotransplantation. **(Fenaux M, et al. J Gen Virol 2004;85:3377-3382.)**

For many years users of bovine serum in the manufacture of human and veterinary biological medicinal products have relied upon USDA 9CFR to ensure the viral safety of their serum. Recently, EU regulators have formalised their position by issuing guidelines on the use of bovine serum during manufacture. Additionally, the European Pharmacopoeia has drafted a monograph on bovine serum. There is good harmonisation among the recommendations and requirements although the EU CHMP guideline calls for greater attention to be paid to the potential presence of infectious bovine polyoma virus. The EU guidelines also call for various tests to assess the effect of BVDV antibodies in the detection of BVDV. However, in response to criticisms from serum suppliers and users, the stringency of these recommendations is being relaxed. The overall viral safety of bovine serum should

be subject to a risk analysis as infectious virus will invariably be present in some batches of serum but remain undetected. Other factors such as the geographical source of the serum, the efficacy of viral inactivation/removal steps and the ability of specific viruses to grow in the production cells being used, should be taken into consideration. **(Robertson JS. Developmental Biology 2006;123:269-272.)**

During a study on Bovine Viral Diarrhoea (BVD) epidemiology in Thailand, a pestivirus was detected in serum from a calf. Comparative nucleotide sequence analysis showed that this virus was closely related to a recently described atypical pestivirus (D32/00'HoBi') that was first isolated from a batch of foetal calf serum collected in Brazil. The results from virus neutralisation tests performed on sera collected from cattle in the herd of the infected calf, showed that these cattle had markedly higher antibody titres against the atypical pestivirus 'HoBi' than against Bovine Viral Diarrhoea Virus types 1 and 2, or Border Disease Virus. The results also supported, consequently, the results from the molecular analysis, and demonstrated that a 'HoBi'-like pestivirus had been introduced to, and was now circulating in the herd. This study is the first to report a natural infection in cattle with a virus related to this atypical pestivirus, and it suggests that this group of pestiviruses may already be spread in cattle populations. The findings have implications for BVD control and for the biosafety of vaccines and other biological products produced with foetal calf serum. Consequently, these atypical pestiviruses should be included in serological assays, and any diagnostic assay aimed at detection of pestiviruses in biological products or animals should be tested for its ability to detect them. **(Ståhl K, et al. Vet Res, 2007;38(3):517-23.)**

Bovine viral diarrhea virus (BVDV) affects cattle populations causing clinical signs that range from subclinical immunosuppression to severe reproductive and respiratory problems. Detection and removal of persistently infected (PI) calves is the single most important factor for control and eradication of BVDV. Current testing strategies to detect PI calves rely heavily on immunohistochemistry (IHC) and a commercially available antigen capture ELISA (ACE) assay. These viral assays depend on 1 or 2 monoclonal antibodies which target the E(rns) glycoprotein of BVDV. The sensitivity and specificity of these two tests have been reported previously. The purpose of this research was to characterize a strain of BVDV (AU501) that was undetectable using IHC and ACE based on a single monoclonal antibody, but was consistently detected in samples from a Holstein steer using virus isolation and PCR testing. Sequencing of this AU501 viral isolate revealed a unique mutation in the portion of the genome coding for the E(rns) glycoprotein. This unique field strain of BVDV demonstrates the risk of relying on a single monoclonal antibody for detection of BVDV. Multiple testing strategies, including polyclonal or pooled monoclonal antibodies that detect more than one viral glycoprotein may be necessary to detect all PI calves and facilitate eradication of BVDV. **(Gripshover EM, et al. Veterinary Microbiology, 2007;125:11-21.)**

Both continuous UV lights and pulsed UV lasers have potentials to inactivate known and emerging viruses. Bovine viral diarrhea virus (BVDV), from the Pestivirus genus, is known to be a common viral contamination in (fetal) bovine serum (FBS). Also, BVDV has been used in the blood product industry as a surrogate for Hepatitis C virus (HCV), due to its similarity in structure and genome. Germicidal UV lamp with the wavelength of 254 nm and Nd:YAG laser (pulsed UV laser) in its third and fourth harmonic with the wavelengths of 355 and 266 nm, respectively, were used. BVDV suspended in PBS or FBS were exposed to different

intensities and doses and then reduction in BVDV titer were calculated. To complete inactivation of BVDV suspended in PBS and PBS containing 5% FBS, 1.6 (t=30 min) and 3.2 (t=60 min)J/cm(2) were used. The minimum doses for inactivation of BVDV suspended in PBS with the 355 and 266 nm of pulsed UV laser were 352 and 92.25 J/cm(2). Also, the minimum doses for inactivation of BVDV suspended in FBS with 355 and 266 nm wavelengths of pulsed UV laser were 704 and 127 J/cm(2). To evaluate the irradiated FBS quality to support cell culture growth, FBS was treated with the dose of 190.5 J/cm(2) and 266 nm pulsed UV laser and was used to grow Vero cells, in comparison with a control group. The viability of cells in two groups was identical and the statistical evaluation showed no significant difference in 12 passages.

Azar Daryany MK, Hosseini SM, Raie M, Fakharie J, Zareh A. Study on continuous (254 nm) and pulsed UV (266 and 355 nm) lights on BVD virus inactivation and its effects on biological properties of fetal bovine serum. J Photochem Photobiol B. 2009;94(2):120-4.

Risk Assessment

While thousands of *in vitro*-produced (IVP) bovine embryos have been transferred commercially with no reports of disease transmission, such risks must be considered. Due to differences in their zonae pellucidae, the disease risks with IVP embryos are known to be higher than with *in vivo*-derived embryos. Possible sources of infection include the oocytes, spermatozoa, serum, and co-culture cells. The Terrestrial Animal Health Code of the Office International des Epizooties (OIE, 2003) stipulates that disease risk management should meet standards set by theWorld Trade Organization. These standards include subjecting the IVP procedures to quantitative risk assessment to evaluate disease transmission risk. The purpose of the present work was to measure the risks of transmitting disease with IVP embryos obtained from abattoir-derived tissues. A simulation model was developed using Microsoft Excel spreadsheets with the Palisade @RISK (London, UK) software program. The model incorporates probability distributions, the shapes of which reflect the random nature of some of the data (e.g. fluid volumes in cultures and washes) and the conjectural nature of some of the scientific information (e.g. on disease agents). The model is adaptable so that, when accurate data or information become available, variability estimates and degrees of uncertainty can be replaced with fixed values. The model assumes: (1) the IVP method is as described in the IETS Manual (1998); (2) there are five possible sources of infection; donor cow, donor bull, fetal calf serum, bovine serum albumin, and co-culture cells; (3) the disease agents can survive and/or proliferate during *in vitro* maturation, fertilization and culture; (4) fluid volumes in cultures and washes follow “known” normal distributions; (5) uncertainties in current knowledge of IVP embryos and disease agents can be taken into account by use of appropriate probability distributions; (6) different methods of *in vitro* fertilization do not affect the level of risk; and (7) different methods of *in vitro* culture can affect the level of risk. The model as constructed fits comfortably into a single workbook with one worksheet allocated for the model itself and another serving to store data on diseases of interest. Data on oocytes, blastocyst numbers, etc., and on media and wash fluid volumes are held within the model while information relating to particular diseases can be selected from a drop-down list at the top of the first worksheet. The relevant data stored in the database are then retrieved and used for modelling, using Monte Carlo simulation. The model estimates the final titer of the disease agent in IVP embryos and the probability of at least one infective

transmission to a recipient, expressed as distributions. **(Perry G. Reprod Fertil Devel 2005;17:244 (abstract)).**

Bovine oocytes derived from abattoir origin are mainly used for artificial reproductive techniques in both agricultural and biomedical applications. Regulatory agencies have expressed concern for potential transmission of adventitious viruses by sourcing bovine oocytes from abattoir origin. To evaluate this concern, a viral risk assessment was conducted on batch samples collected from follicular fluid, cumulus cells, oocytes, and Day 8 embryos. These batch samples were collected from ovaries on seven randomly selected days in a 2-week period and they were frozen and stored at –80°C until tested. All samples were tested by 9 Code of Federal Regulations (9CFR) part 113.53c (animal viral testing) at a GLP compliant laboratory (American BioResearch Laboratories, Sevier, TN, USA). The 9CFR viral testing includes bovine viral diarrhea virus (BVDV), bovine parvovirus, bovine adenovirus type 3 and 5, bovine rabies virus, bovine bluetongue virus, bovine respiratory syncytial virus, bovine reovirus, viral cytopathic effect, and hemadsorption on permissive cell cultures. Batch samples were also tested for BVDV and bovine leukemia virus (BLV) by polymerase chain reaction (PCR) and follicular fluids for BVDV antibody neutralization activity at an accredited diagnostic laboratory (Animal Disease Research and Diagnostic Laboratory, SDSU, Brookings, SD, USA). The 9CFR viral testing results on all the batch samples were negative. The BVDV PCR test had a low positive (37.89 cycles) with one follicular fluid batch sample (1/7) and a low positive (37.9 cycles) on all cleaved embryos (7/7). However, BVDV virus isolation was negative for both batch samples by 9CFR testing. The BLV PCR had a positive follicular batch sample (1/7), with all other samples testing being negative. BVD serum neutralization antibody assay demonstrated that all follicular fluid had significant titers of 1:128–1:1024. Although some of the viral particles may have been detected in follicular fluid and cleaved embryos by PCR, none of the batch samples collected were positive for viral growth in this study. The BVDV PCR indicated low levels of BVDV RNA. It is speculated that the positive BVDV PCR results on cleaved embryos could possibly be attributed to the use of irradiated fetal calf serum (contaminated BVDV virus) used in culture media. Follicular fluids also have high titers to BVDV which may have neutralized the virus. These results indicate that virus-free bovine oocytes can be derived from the abattoir. Thus, with appropriately applied quality assurance testing, abattoir-origin oocytes might be used effectively in agricultural and biomedical applications. **(Pommer J, et al. Fertility and Development, 2008;20:158–159 abstr.)**

Review - In the modern biological area, the applications of pig as a laboratory model have extensive prospects, such as gene transfer, IVF, SCNT, and xenotransplantation. However, the risk of pathogen transmission by porcine embryos is always a topic to be investigated, especially the viruses related to reproductive failure, for instance, pseudorabies virus, porcine reproductive and respiratory syndrome virus, porcine parvovirus, and porcine circovirus type 2. It should be mentioned that the zona pellucida (ZP) of porcine embryos can be a barrier against the viruses, but certain pathogens may stick to or even pass through the ZP. With intact, free, and damaged ZP, porcine preimplantation embryos are susceptible to these viruses in varying degrees, which may be associated with the virus-specific receptor on embryonic cell membrane. These topics are discussed in the present review. **Zhao, H., Zhao,**

G. and Wang, W., 2016. Susceptibility of porcine preimplantation embryos to viruses associated with reproductive failure. Theriogenology, 86(7), pp.1631-1636.

Sanitary/Quality controls

The aim of this study was: 1) evaluate if bacteria and virus are present during different steps of IVF techniques, 2) determine if the contaminations could reduce the embryo production efficiency, 3) verify if slaughtering procedures could interfere with initial contamination of the ovaries. Bacteria like *Pseudomonas aeruginosa, Corynebacterium pyogenes, Streptococcus and Staphylococcus aureus* are known to cause endometritis as well as BHV1, BHV4 and BVDV. Those pathogens are supposed to be able to join at the outer COC (Cumulus-oocyte-complex) and adhere at embryo cells and ZP surface and they can cause reproductive failure or disease transmission. During 80 laboratory trials, 660 Piedmontese bovine ovaries were collected from 3 different slaughterhouse (A, B and C). The ovaries after dissection from the uterine body, were rapidly disinfected with 70% alcohol and carried to the laboratory in PBS at 26-33$_{o}$C within 3 hours from removal. Slicing method was utilized for obtaining COCs and routine IVF procedure was performed. Samples of culture media, cellular debris and rejected COCs from each trial were submitted to examination. Enrichment and insemination on selective media were executed and each plate was examined after incubation; colonies were stained with Gram method and the most typical ones for morphology, growing and staining characteristics were identified with specific biochemical method (API gallery). For virological analyses cellular debris fraction was submitted to precipitation with PEG (Polyethylene Glycol), then filtered and placed in culture on renal bovine cell monolayers. Positive samples were submitted to immunofluorescence staining method that allowed identification of the viral agent. Results are summarized in Table 1. Some identified bacteria are saphrophytic, other ones are opportunistic pathogens and further can cause alimentary infections in humans, but in any case can certainly be referred to donors pathology. With the same withdrawal ovary procedure in the slaughterhouse C we identified only one positive sample that can demonstrate more respect in hygiene during evisceration. The same tests performed on media and discard embryos at the end of the IVF procedure was also negative. These results can be determined by infectious agent dilution and by the effect of antibiotic addition. The repeated washing of oocytes and embryos requested by IETS procedures allowed a significant reduction of infection dose. The marked increased production of morulae/blastocysts in negative samples (Table 2) allows to conclude that optimal hygiene conditions enhance the production data.

Table 1. Bacteriological identification in culture media and rejected COCs (*In 3 samples an association of bacteriological agents is observed).

Bacteria	Abattoir A	Abattoir B	Abattoir C	Viruses	Abattoir A	Abattoir B	Abattoir C
Polymicrobial	1	1	0	BHV1	0	0	0
Environmental bacteria	3	0	0	BHV4	0	0	0
Staphylococcus spp.	2	1	0	BVDV	0	1	0
Escherichia hermanii	1	0	0				
Bacillus spp.	3	0	0				
Pseudomonas spp.	1	4	0				
Xanthomonas spp.	1	1	0				
Klebsiella oxytoca	1	0	0				
Coliformi	0	2	0				
Enterobacter spp.	0	3	0				
Aeromonas spp.	0	1	0				
Pasteurella multocida	0	1	0				
Moraxella lacumata	0	1	0				
Acinetobacter lwoffi	0	0	1				
Comomonas spp.	0	1	0				
Negative	17	20	20		30	32	21
Total	30	33*	21		30	33	21

Table 2. Relationship between microbiological results and IVF

Bacteria & virus	Oocytes N	1-cell %	2-cell %	4-cell %	8-cell %	M/B %	D %	NF %
Positive	822	32.12	18.98	10.83	2.8	6.56	19.83	8.88
Negative	1389	33.26	10.73	7.49	3.83	14.54	18.57	11.59

Starvaggi Cucuzza A, Nervo T, Ponzio P, Quaranta G, Vincenti L. Sanitary controls in bovine IVF embryo production. Proceedings 18e Reunion AETE, Rolduc, 06-07, September 2002, page 230.

The ability to create an optimal environment for the culture of oocytes and embryos is important to ensure that embryo viability, and therefore pregnancy outcome, is not compromised. Laboratory performance can be monitored using a quality control system. In setting up a new IVF unit, a comprehensive quality control programme was established to monitor laboratory performance and to detect any problems that potentially may have resulted in a sub-optimal service. The measures employed were designed to optimize the environment for human embryo culture by providing aseptic conditions and security for the gametes and embryos, whilst providing a safe working environment for laboratory staff. Equipment function, consumables and environmental parameters were assessed prior to the commencement of treatment in the new unit. A mouse embryo bioassay was used to assess the ability of the new laboratory and equipment to support mammalian embryo development. Prior to the start of clinical treatments a quality control program highlighted equipment that was functioning sub-optimally, which had the potential to cause problems

had it been left undetected. Once clinical treatment commenced, quality control monitoring was continued to ensure that the laboratory functioned to a consistently high standard. **(Cutting R, et al. Hum Fertil (Camb). 2004;7:119-125.)**

The implementation of suitable quality control (QC) is not only required for the accreditation of a human in vitro fertilization (IVF) laboratory, but is also fundamental to its success. Several assays have been employed to screen culture media and contact supplies. The suitability of one assay in particular, the mouse embryo assay (MEA), has been questioned over the years. Here we discuss how the conditions of such an assay, together with the stage of embryonic development used, have a profound effect on the outcome of the assay. Furthermore, by assessing embryos at multiple time points during the preimplantation period (rather than simply determining blastocyst formation), together with quantitating key parameters such as blastocyst cell number, it is possible to identify suboptimal components of a culture system. As well as identifying those components that result in outright embryonic demise, under the appropriate conditions the MEA can detect components that lead to impaired development. It is proposed that under the appropriate conditions, the MEA is a useful adjunct to quality control in human IVF, but several assays used in concert are better than a single test. **(Gardner DK, et al. Semin Reprod Med. 2005;23:319-324.)**

Objective : To assess the presence of viral RNA or DNA sequences in spent culture media used after ovum pickup (OPU) or embryo culture and in liquid nitrogen (LN) used for oocyte or embryo vitrification in patients seropositive for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) undergoing IVF cycles.
Design: Descriptive study.
Setting: Private university-affiliated IVF center.
Patient(s): Twenty-four women who underwent controlled ovarian stimulation for oocyte vitrification or IVF/ET. A total of 6, 11, and 6 patients were seropositive for HIV, HCV, and HBV, respectively, whereas 1 patient showed a coinfection with HCV and HBV. Seven patients presented positive blood viral load (HIV, n = 1; HBV, n = 1; HCV, n = 5). Sixty-three samples were analyzed: follicular fluid, n = 3; spent culture media, n = 33 (20 after OPU and 13 after embryo culture); and LN, n = 27 (14 and 10 after oocyte and embryo vitrification; and 3 LN storage tank samples).
Intervention(s): Ovum pickup, oocyte and/or embryo culture, and/or vitrification by the Cryotop open device. Reverse transcription–polymerase chain reaction analysis was performed for viral screening.
Main Outcome Measure(s): Detection of viral sequences of HIV, HCV, and HVB.
Result(s): All the samples analyzed tested negative for the detection of viral RNA or DNA sequences.
Conclusion(s): We have not detected viral sequences after culture and vitrification of oocytes/embryos from HIV-, HBV-, and HCV-seropositive patients. These findings represent good evidence of the lack of risk of cross-contamination among seropositive patients, even using an open device for vitrification.
Cobo A, Bellver J, de los Santos MJ, Remohí J. Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles. Fertil Steril. 2012 Jan;97(1):74-8. Epub 2011 Oct 26. str.)

The current world population is increasing at a fast rate. In order to feed this larger population, food production must increase by 70 percent. Recent reports show a record global production of 58.9 Carcass Equivalent Weight million metric tonnes of beef expected for 2014. It becomes clear that the worldwide agricultural community will have to integrate new technologies to assure the sustainability of global livestock and meat demands. Agriculture has benefited tremendously from the innovation of reproductive technologies such as semen artificial insemination and cryopreservation, embryo transfer and cryopreservation, and in vitrofertilization. Only recently have some developed countries accepted the import and export of frozen IVF embryos and more countries are currently evaluating this. Before 2003, in vitroembryos represented not more than 20% of all embryos produced. After 2003, this jumped to 30 to 39% of all embryos produced, and is increasing. It is clear that South America, and more specifically Brazil, is driving this increase. However, most people in this field would agree that the trend is true for many regions active in this field. International movement of gametes or embryos must be performed in biosecure manners to make certain that pathogenic organisms are controlled and that transmission of infection to recipient animals and progeny is avoided. The embryo transfer industry has adopted appropriate procedures to manage the biosecurity risks and hence mitigate risks of pathogen transmission through international trade of bovine embryos. Techniques for biosecure production of in vivo bovine embryos have been well established. However, as in vitroembryos are relatively new to this business, there are not many papers on the subject of pathogen-interaction with this type of embryo. Certain studies demonstrate that the decontamination of in vitroembryos using recommended procedures is effective for specific pathogens while others have shown that this is not as evident in other conditions. All agree that more research is needed regarding washing protocols for in vitroembryos. It is imperative that the scientific community continues its research to validate current embryo sanitary washing procedures and recommend any modifications that would be necessary for IVF embryos. As embryos are becoming an important component of international trade of bovine genetics, such research must not only continue but augment if key parties want to assure they meet the worldwide rising need of meat and dairy products.

Ponsart C., N. Pozzi. Sanitary requirements for bovine gametes and embryos in international trade Anim. Reprod., v.10, n.3, p.283-296, Jul./Sept. 2013

Viral infection effects on oocytes

Viral infection has been associated with a starvation-like state in *Drosophila melanogaster*. Because starvation and inhibiting TOR kinase activity in vivo result in blocked oocyte production, we hypothesized that viral infection would also result in compromised oogenesis. Wild-type flies were injected with flock house virus (FHV) and survival and embryo production were monitored. Infected flies had a dose-responsive loss of fecundity that corresponded to a global reduction in Akt/TOR signaling. Highly penetrant egg chamber destruction mid-way through oogenesis was noted and FHV coat protein was detected within developing egg chambers. As seen with in vivo TOR inhibition, oogenesis was partially rescued in loss of function discs large and merlin mutants. As expected, mutants in genes known to be involved in virus internalization and trafficking [Clathrin heavy chain (*chc*) and synaptotagmin] survive longer during infection. However, oogenesis was rescued only in *chc*

mutants. This suggests that viral response mechanisms that control fly survival and egg chamber survival are separable. The genetic and signaling requirements for oocyte destruction delineated here represent a novel host–virus interaction with implications for the control of both fly and virus populations. **Thomson TC, Schneemann A, Johnson J. Oocyte destruction is activated during viral infection. Genesis. 2012 Jun;50(6):453-65.**

Vitrification

After vitrification of oocytes, fertilization rates and subsequent development are unsatisfactory, possibly due in part to zona hardening. Foetal calf serum (FCS) can prevent zona hardening because of its fetuin content, but FCS composition varies among batches, and may contain viruses. In this study, we therefore compared media supplemented with different sources of macromolecules, 2% bovine serum albumin (BSA), 2% BSA + 1 mg/ml fetuin and 20% FCS, for handling oocytes for 10-30 min prior to vitrification. None of the treatments resulted in developmental rates comparable with the non-vitrified controls, but FCS inclusion in pre-vitrification handling medium resulted in higher blastocyst production per oocyte ($p < 0.05$) (10.8%) on day 9 of culture than BSA (5.3%) or BSA + fetuin (6.4%). Blastocysts developing from oocytes from all vitrification treatments were somewhat retarded relative to those developed from non-vitrified oocytes. We also tested the use of fetuin during vitrification as well as two different exposure times with cryoprotectants, 180 and 30 s. There was no significant effect of fetuin or exposure time on rates of subsequent blastocyst production. **Horvath G, Seidel GE Jr. Use of fetuin before and during vitrification of bovine oocytes. Reprod Domest Anim. 2008;43(3):333-8.**

Here we report on the outcome of the application of vitrification open pulled straw (OPS) technology on the sanitary status of embryos exposed to pathogenic agents and cryopreserved using the commercial kit Vit-SeT® (Minitube Canada, Ingersoll, Ontario, Canada). The Vit-SeT consists of 3 stainless steel chambers: the first for cooling the 0.5-mL AI straws, the second for vitrification of OPS straws, and the third for loading OPS into the AI straws. The CBC 0.5-mL straws (Cryo Bio System, Paris, France), OPS straws (Minitube Canada), and the heat sealer were used. The investigation involved: (1) pressure seal testing the CBC straws; (2) testing LN for contamination after vitrification; (3) testing the surface of the CBC straws; and (4) testing the contents of OPS straws and vitrified embryos for cross-contamination. In the first Exp., CBC straws were heat sealed at one end and the other end was connected to a compressor with a manometer. The straws were then tested for air leaks in water. In Exp. 2, OPS straws were loaded by capillary aspiration of clean or contaminated cultures ($3 \cdot 10^8$ *E. coli*, $13 \cdot 10^8$ *P. aeruginosa*, or 10^5 BVDV $TCID_{50}$ mL^{-1}, NY strain), then vitrified in LN (chamber 2) in random order, inserted into the CBC straws (chamber 3), and heat sealed at a reading of 225 on the sealer dial. In Exp. 3, IVF embryos were either exposed or not to the microbe cultures for 1 h, loaded into OPS (5 embryos per straw), and then vitrified and processed as described in Exp. 2. After vitrification, OPS straws were retrieved from CBC straws and thawed according to the original methodology (Vajta *et al*. 1997 Cryo-Lett. 18, 191–195). For bacterial isolation, standard methodology was used, and BVDV was detected by virus isolation followed by immunoperoxydase staining. In Exp. 1, no air leaks were detected up to 20 mm Hg pressure at the heat-sealed end of CBC straws ($n = 15$). In Exp. 2, two samples of LN tested positive for *E. coli* (after immersion of 10 OPS, chamber 2).

All remaining samples of LN after vitrification of OPS tested negative for pathogenic agents. Surfaces of the 0.5-mL straws all tested negative (chamber 3). The pathogenic agents were retrieved from all of the positive control OPS straws (*n* = 15). In Exp. 3, all tested samples of LN and surfaces of 0.5-mL straws were negative. The pathogenic agents retrieved from samples of control embryos previously exposed to pathogens were as follows: *E. coli*, 5/15; *P. aeroginosa*, 8/15; and BVDV, 2/15. All samples containing embryos not exposed to pathogens, surfaces of 0.5-mL straws, and LN all tested negative. On the basis of limited experimental work, it is concluded that the potential for cross-contamination of samples by application of the Vit-SeT for vitrification of embryos using OPS is negligible if: (1) LN in the chambers is frequently replaced and the chambers are disinfected between embryo donors, and (2) the protective straws (0.5-mL, AI) are applied over OPS and are properly sealed. These straws should be used without a cotton plug. However, the obtained results should be not extrapolated to other means of so-called 'sterile' methods of vitrification without specific testing. **(Bielanski A, Hanniman A. Reproduction, Fertility and Development, 2007;19:232 ab**

Xenotransplantation and allotransplantation

Allotransplantation and xenotransplantation may be associated with the transmission of pathogens from the donor to the recipient. Whereas in the case of allotransplantation the transmitted microorganisms and their pathogenic effect are well characterized, the possible influence of porcine microorganisms on humans is mostly unknown. Porcine circoviruses (PCVs) are common in pig breeds and they belong to porcine microorganisms that still have not been fully addressed in terms of evaluating the potential risk of xenotransplantation using pig cells, tissues, and organs. Two types of PCVs are known: porcine circovirus (PCV) 1 and PCV2. Whereas PCV1 is apathogenic in pigs, PCV2 may induce severe pig diseases. Although most pigs are subclinically infected, we do not know whether this infection impairs pig transplant functionality, particularly because PCV2 is immunosuppressive. In addition, vaccination against PCV2 is able to prevent diseases, but in most cases not transmission of the virus. Therefore, PCV2 has to be eliminated to obtain xenotransplants from uninfected healthy animals. Although there is evidence that PCV2 does not infect—at least immunocompetent—humans, animals should be screened using sensitive methods to ensure virus elimination by selection, Cesarean delivery, vaccination, or embryo transfer. **Denner, J. and Mankertz, A., 2017. Porcine circoviruses and xenotransplantation. Viruses, 9(4), p.83.**

Zona pellucida

Bovine ZPs from follicular oocytes obtained directly from ovaries and from embryos and degenerated ova collected on Day 7 from superovulated cows were examined by scanning electron microscopy (SEM), dimensional measurement and by total protein determination. The number of plaque-forming units (PFU) of IBR virus that were associated with the ZP-1 embryos/ova from each of the 3 sources after in vitro exposure to the virus was also determined. SEM revealed that surfaces of Day 7 embryos and degenerated ova were smoother than those of follicular oocytes. Mean diameters/thicknesses of ZPs of the 3 types were 156.7µm/12.3µm, 161.3µm/12.6µm, and 158.9µm/12.8µ respectively, and mean total

protein contents per ZP of each of the 3 types were 0.331µg, 0.349µg and 0.254µg respectively. Significantly greater quantities of IBRV were associated with follicular oocytes (mean PFU/oocyte = 68.1) than with Day 7 embryos (mean PFU/embryo = 43.0; $p < 0.05$), or with Day 7 degenerate ova (mean PFU/ovum = 31.9; $p < 0.01$).
The reliability of testing non-transferable ova/embryos for IBRV as an indicator of the presence or absence of the virus on transferable embryos from the same collection (Day 7) was supported. Although structural and biochemical differences between ZPs of the 3 types were observed, further studies are needed to see if these are accompanied by differences in their protective barrier functions against pathogens. **Riddell, KP, et al, Theriogenology 1993;40:1281-1291**.

The SDS-PAGE method was used to study the composition of isolated bovine ZPs. One dimensional SDS-PAGE revealed 3 major glycoproteins with MWts of 80-70kD, 66-63 kD, and 60kD respectively. After two dimensional SDS-PAGE the ZP electrophoretic pattern indicated a fourth glycoprotein. Analysis of ZPs from ovarian oocytes and from embryos after transit through the genital tract enabled modifications in glycoprotein composition to be described. **Bercegeay et al, Reprod Nutr Dev 1993;33:567-576**.

The lectin-binding patterns of mammalian ZPs were studied to determine differences in their carbohydrate distribution patterns. Ovaries from mouse, rat, hamster, rabbit, cat, dog and pig were fixed, sectioned and labelled with 10 different biotinylated lectins as probes and avidin-biotin-peroxidase complex as visualized. Whereas the lectin binding patterns of granulosa cells and follicular fluids were identical in all species studied, there were species-specific variations in the lectin binding patterns of the ZPs. **Skutelsky et al, J Reprod Fert 1994;100:35-41**.

"A serendipitous observation that oocytes collected from the bovine oviduct of non-bred heifers were resistent to digestion by pronase (4 mg/ml) was extended in the following experiments. Oocytes were recovered from ovaries collected at the abattoir and the cumulus cells left intact or removed by vortexing. Oocytes were also collected from the uteri of superovulated cattle bred 7 days earlier. Oocytes were washed with TL-Hepes and placed in Terasaki multiwell plates containing 4mg/ml pronase in 10µl TL-hepes. The time required (min) for complete digestion of ZP was then observed under a dissecting microscope. The zonae of follicular oocytes (n=46) incubated in pronase before or after in vitro maturation (IVM), or removal of the cumulus were digested within 2 to 6 min while those cultured for 4 hours with oviductal epithelial cells from randomly slaughtered cattle were completely dissolved within 3 min. Likewise, zonae (n=110) were digested in 2-4 min after incubation with 20%, 40%, 0r 80% of the oviductal concentration of an ovine oestrous-associated glycoprotein (88-92Kd). The zonae (n=74) of unfertilized oocytes and degenerate oocytes collected from the uteri of bred, superovulated cattle on day 7 post-insemination were digested within 5-12 min. However, cumulus intact and denuded oocytes (n=62) transferred to the oviducts of estrous ewes (oestradiol benzoate, 5 mg) for 4 h, before or after IVM, failed to digest for at least 180 min. In contrast, when transferred to the oviducts of ewes with a vaginal pessary (30 mg FGA for 6 days) the zonae were digested within 2.5 min in one ewe (n=6) and between 82 and 102 min in a second ewe (n=41). These results suggest that an oestrus-specific factor in the oviduct alters the digestibility of the zona pellucida by

pronase. It is possible that this factor may play a significant role in sperm/oocyte interaction. **Duby RT, et al, J Reprod Fert 1995;abstr series15:79**.

Structural aspects of the bovine zona pellucida (ZP) of in-vitro-matured (IVM) oocytes and in-vitro-produced (IVP) embryos were studied in 2 experiments in order to find a tentative explanation for its barrier function against viral infection. In experiment 1, the ultrastructure of the outer ZP-surface was studied. The diameter and number of the outer pores within an area of 5000 μm^2 of 10 IVM oocytes, 10 zygotes, 10 8-cell stage embryos and 10 morulae were evaluated by scanning electron microscopy (SEM). In oocytes and morulae, the ZP surface showed a rough spongy appearance with numerous pores. In zygotes, the ZP surface was found to have a smooth, melted appearance with only a few pores. In 8-cell stage embryos, both surface patterns were found. The mean number (per 5000 μm^2) and the mean diameter of the outer pores were different between the 4 stages of development ($P<0.001$): 1511 pores in oocytes, 1187 in zygotes, 1658 in 8-cell stage embryos and 3259 in morulae, with a mean diameter of 182, 223, 203 and 155 nm, respectively. In experiment 2, the continuity of the meshes towards the embryonic cells was examined by confocal laser scanning microscopy (CLSM). The passage through and the location in the ZP of fluorescent microspheres, with similar dimensions to bovine viral diarrhea virus (BVDV; 40-50 nm) and bovine herpesvirus-1 (BHV-1; 180-200 nm), were evaluated. For all stages, the smallest beads were detected half way through the thickness of the ZP, whereas the beads with a size of 200 nm were only found within the outer 25% of the ZP. It can be concluded that the intact bovine ZP of IVM oocytes and IVP embryos is constructed in such a way that BVDV and BHV-1 should not be able to traverse the ZP and reach the embryonic cells. However, the risk exists that viral particles can be trapped in the outer layers of the ZP. **Vanroose G, et al. Biol Reprod 2000;62:463-469.**

Effects of bovine plasmin and plasminogen activator recovered from bovine embryo-conditioned medium (bePA) on the polypeptide profile and solubility of bovine zonae pellucidae (ZP) were evaluated. ZP were isolated from bovine ovarian oocytes and incubated at 39 degrees C with 0, 100, or 200 microg/ml plasmin for 0, 24, or 48 hr or bePA with 0 or 100 microg/ml human plasminogen for 0 or 48 hr. ZP were evaluated either by SDS-PAGE or for changes in solubility using a zona pellucida dissolution time (ZPDT) assay. Two prominent polypeptides, molecular weight (MW) 76,000 and 65,000, and two minor polypeptides, MW 23,000 and 22,000, were resolved by SDS-PAGE. No changes occurred in the polypeptide profile for ZP incubated with 0 microg/ml plasmin for 0, 24, or 48 hr, and ZPDT did not differ ($P > 0.10$). Treatment with 100 or 200 microg/ml plasmin induced reductions in the MW 76,000, 23,000, and 22,000 polypeptides and the appearance of MW 45,000 and <10,000 polypeptides. ZPDT were less ($P < 0.05$) in 100 and 200 microg/ml compared with 0 microg/ml plasmin. Polypeptide profiles and ZPDT for ZP incubated with bePA were similar ($P > 0.10$) to ZP incubated with unconditioned medium. Addition of human plasminogen to ZP incubated with bePA reduced the MW 76,000, 23,000, and 22,000 polypeptides, caused the appearance of MW 45,000 and 20,000 polypeptides, and decreased ZPDT ($P < 0.05$). These results demonstrate that bovine plasmin is capable of proteolytically degrading the bovine ZP and that bePA can indirectly affect the ZP by converting plasminogen to plasmin. **Cannon MJ et al, Mol Reprod Dev 1998;51:330-338.**

The aim of this study was to determine the ultrastructure of cross-sectioned zonae pellucidae of in vitro-matured and ovulated pig oocytes before or after sperm penetration in vitro and in vivo, respectively. The in vitro and in vivo (ovulated) oocytes and zygotes (fertilized in vitro and in vivo) were fixed with glutaraldehyde either directly or after pretreatment with ruthenium red and saponin, processed and then examined using transmission electron microscopy. The thickness of the zona pellucida, as measured on the section of the specimens with largest diameter fixed with glutaraldehyde, differed between the in vivo (9.19 +/- 0.47 microm) and in vitro (5.95 +/- 0.51 microm) oocytes. The in vivo oocytes had a rather thick external mesh-like structure, whereas it was much thinner in the in vitro oocytes. This mesh-like external rim was less apparent in both in vivo and in vitro zygotes. Obvious differences in the density of the lattice formed by the fixed zonae pellucidae were visible between the outer and inner (ad-oolemmal) zonae. The outer area always formed a concentrically arrayed fibrillar network, whereas the inner area showed a much more compact, trabecule-like mesh. However, both areas, but particularly the outer network, were much more compacted after the zona reaction. Clear differences in the degree of fibrillar aggregation of the inner zona area were also observed between in vitro and in vivo zygotes, being much higher in the latter. This fibrillar network was more clearly visible in the zygotes pretreated with ruthenium red and saponin; the in vitro zygotes had a fibrillar, radially oriented set of parallel fibrils, whereas it was much more aggregated and trabecule-like in the in vivo zygotes. These results demonstrate that the fine structure of the zona pellucida and the zona reaction at sperm penetration differ between pig oocytes fertilized in vivo and in vitro. Moreover, the ultrastructure of the outer and inner pig zonae pellucidae has a different network organization. **Funahashi H et al. Reproduction 2001;122:443-452.**

Review : Although the transfer of embryos is much less likely to result in disease transmission than the transport of live animals, the sanitary risks associated with embryo transfer continue to be the subject of both scientific investigations and adaptations of national and international legislation. Therefore, the implications are important for veterinary practitioners and livestock breeders. In vivo-derived and in vitro-produced embryos are widely used in cattle and embryos from other species, such as sheep, goats, pigs and horses, are also currently being transferred in fairly significant numbers. Bearing in mind the wide variety of embryos of different species and the correspondingly large number of viruses that are of concern, it is expedient at this time to look again at the importance of the zona pellucida (ZP) as a barrier against viruses and at the susceptibility or otherwise of embryonic cells to viral infection if ever they are exposed. For embryos with an intact ZP, viral infection of the embryo is unlikely to occur. However, the virus may stick to the ZP and, in this case, International Embryo Transfer Society (IETS) washing procedures in combination with trypsin treatment are mandatory. A caveat is the fact that currently more and more types of embryos are becoming available for transfer and scientific data cannot be extrapolated from one species to another. These topics are discussed in the present review.
Van Soom A, Wrathall AE, Herrler A, Nauwynck HJ. Is the zona pellucida an efficient barrier to viral infection? Reprod Fertil Dev. 2010;22(1):21-31.

Review : Several studies have been performed to determine the disease transmission potential of early bovine embryos. Most of them agree that the Zona Pellucida (ZP) is a barrier that generally protects the embryo from infection but can also be a potential route for the transmission of pathogens. This review will initially focus on the characteristics of the ZP and the

potential ways in which an embryo can transmit disease and the possible alternatives to reduce this hazard. **Escobar, C.J., 2018. Embryo transfer, a potential risk in disease transmission. MOJ Anat & Physiol, 5(4), pp.259-262.**

Semen (since 2010)

Cattle

Bluetongue virus

During the recent bluetongue virus (BTV) outbreak in Germany, semen quality in bulls naturally infected with BTV-serotype 8 was evaluated. Bluetongue status was assessed by both serology and polymerase chain reaction (PCR). Six bulls became BTV–PCR positive between September and November 2007 without showing clinical signs. Between April and May 2008, all six bulls were PCR negative but remained seropositive. Semen data from non-infected test bulls recorded between 2006 and 2007 were matched for season and age and used as controls. BTV-8 infection had no effect on sperm volume and concentration, but reduced sperm motility was seen after thawing (January–August 2008: 44.1 ± 12.7% vs. 58.0 ± 7.9% in the uninfected bulls; $P < 0.001$). Malformed sperm in both in fresh and thawed semen from BTV-positive animals was above the 20% permitted maximal limit from December 2007 to February 2008. Infection with BTV-8 transiently impaired semen quality in bulls **Ute Müller a, Kirsten Kemmerling a, Denis Straet a, Ulrich Janowitz b, Helga Sauerwein. Effects of bluetongue virus infection on sperm quality in bulls: A preliminary report. The Veterinary Journal 186 (2010) 402–403**

Given that bluetongue (BT) may potentially be transmitted by semen, that the disease has significantly expanded in recent years, and that millions of doses of cattle semen are annually traded throughout the world, the transmission of bluetongue virus (BTV) by semen could have severe consequences in the cattle industry. The hypothesis that infected bulls could excrete BTV in their semen led to restrictions on international trade of ruminant semen and the establishment of measures to prevent BTV transmission by semen. However, neither the risk of BTV transmission by semen nor the effectiveness of these measures was estimated quantitatively. The objective of the study was to assess, in case of introduction of BTV into a bovine semen collection centre (SCC), both the risk of BTV transmission by bovine semen and the risk reduction achieved by some of the preventive measures, by means of a stochastic risk assessment model. The model was applied to different scenarios, depending on for example the type of diagnostic test and the interval between the controls (testing) of donor bulls, or the rate of BTV spread within the SCC. Enzyme-linked immunosorbant assay (ELISA) controls of donor bulls every 60 days seemed to be an ineffective method for reducing the risk of BTV transmission in contrast to polymerase chain reaction (PCR) tests every 28 days. An increase in the rate of spread within the SCC resulted in a reduced risk of BTV transmission by semen. The storage of semen for 30 days prior to dispatch seemed to be an efficient way of reducing the risk of transmission by semen. The sensitivity analysis identified the probability of BTV shedding in semen as a crucial parameter in the probability of BTV transmission by semen. However, there is a great degree of uncertainty associated with this parameter, with significant differences depending on the BTV serotype. **Napp S, Allepuz A, García-Bocanegra I, Alba A, Vilar MJ, Casal J. Quantitative assessment of the**

probability of bluetongue virus transmission by bovine semen and effectiveness of preventive measures. Theriogenology. 2011 Mar 15;75(5):920-32. Epub 2010 Dec 31.

Bovine herpesvirus

Bovine herpesvirus 1 (BoHV-1) and 5 (BoHV-5) are important pathogens of the respiratory and genital tract of cattle and may also affect the central nervous system and cause meningoencephalitis. Both virus types are estimated to be widely distributed in Southern Brazil. In the present study, BoHV-1 and/or BoHV-5 DNA were detected in bovine semen samples from two states of Brazil by two species-specific nested polymerase chain reactions (nPCRs). These nPCRs were used to assay 53 samples of fresh semen and 23 samples of frozen semen from breeding bulls. Viral DNA was detected in all 76 semen samples: all were positive for BoHV-5, whereas 34 of these were positive for BoHV-1 as well. Moreover, in five fresh and in 13 frozen semen samples-of a total number of 40 samples suitable for virus isolation-infectious BoHV-1 and/or BoHV-5 virus were detected. In conclusion, that both BoHV-1 and BoHV-5 were detected in bovine semen in Brazil highlighted the importance of examining bull semen in search for both agents to reduce the risk of transmitting these viruses. **Oliveira MT, Campos FS, Dias MM, Velho FA, Freneau GE, Brito WM, Rijsewijk FA, Franco AC, Roehe PM Detection of bovine herpesvirus 1 and 5 in semen from Brazilian bulls. Theriogenology. 2011 Apr 1;75(6):1139-45. Epub 2011 Jan 17.**

Infection with bovine herpesvirus-1 (BHV-1), also called infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus, is associated with a variety of respiratory, neurological and infertility health problems causing worldwide economic losses and trading restrictions to the livestock industry. Although there is a considerable amount of information about the risk of BHV-1 transmission through contaminated semen used for artificial insemination, there is no available evidence to indicate whether the resulting embryos, when used for embryo transfer (ET), can lead to the transmission of BHV-1 to recipients and offspring. For this study, cryopreserved bull semen contaminated with BHV-1 was used for artificial insemination (AI) of seronegative, superovulated heifers (N = 43). Embryos were collected post-mortem at 7 days post-insemination and were washed according to the International Embryo Transfer Society (IETS) guidelines. BHV-1 was detected in all samples of follicular fluid, oviductal epithelial cells, endometrium and corpora lutea tissues and a proportion of unwashed (52 of 120, 43%) and washed oocytes and embryos (7 of 113, 6%) collected from embryo donors. Of the 396 collected, unfertilized oocytes and embryos, only 29 (7%) were of ET quality. Most of the embryos and oocytes were degenerated (N = 224, 57%) or unfertilized (N = 143, 36%). The 13 heifers, which each received a single morula-stage washed embryo, maintained seronegative status, but only two (15%) became pregnant and delivered BHV-1-free calves. In conclusion, results suggest that embryos fertilized with BHV-1-contaminated semen may not result in disease transmission to embryo recipients or their offspring when embryos are processed according to IETS and the World Organization for Animal Health (OIE) guidelines. However, due to the transmission of BHV-1 via AI to embryo donors and the apparent adverse effect of BHV-1 on the quality of the embryos, it is unlikely that the procedure can be justified for a commercial application.

Bielanski, A., Algire, J., Lalonde, A., Garceac, A. Risk of transmission of bovine herpesvirus-1 (BHV-1) by infected semen to embryo recipients and offspring. Reproduction in Domestic Animals 2014; 49: 197-201.

Infectious pustular balanoposthitis (IPB) is one of the reproductive disorders caused by bovine herpesvirus 1 (BoHV1) that can be transmitted through artificial insemination. A herd of 63 breeding bulls at a frozen semen bank in Odisha state in India experienced a suspected outbreak of IPB, with 11 bulls showing clinical signs of the infection. Clinical signs were noticed in two bulls initially and a few days thereafter in the other nine animals. Serum samples from 53 bulls were examined for anti-BoHV1 antibodies using a virus neutralization test and a competitive enzyme-linked immunosorbent assay (cELISA); the remaining ten bulls were not included in the study because it was difficult to restrain them at that time. Paired serum samples were collected 21 days apart from ten clinically affected bulls (the eleventh clinically affected bull was not included in the study for the reason stated above). In the neutralisation test, the paired serum samples showed a two- to fourfold increase in anti-BoHV1 antibody titre; in the cELISA, the paired samples were also found positive for anti-BoHV1 antibodies. Serum samples from 43 in-contact bulls were collected about day 22 after the first observation of clinical infection in the herd. Among these serum samples, a total of 30 were found positive for anti-BoHV1 antibodies in the VNT and a total of 30 were found positive in cELISA. Ten samples were positive in one test but not the other and 25 tested positive in both tests. In all, 35 serum samples from in-contact bulls tested positive in either one or both of the two types of test. An overall agreement of 76.74% was found in detection of anti-BoHV1 antibodies in the two tests. Sensitivity was higher than specificity in detection of anti-BoHV1 antibodies in the serum samples. The glycoprotein C region of the genomic DNA of BoHV1 was amplified from semen samples by polymerase chain reaction. The findings from the outbreak indicate that continuous check-up of breeding bulls at frozen semen banks is warranted to avoid the risks associated with artificial insemination**.**
Pandey AB,. Nandi S,. Tiwari AK , Audarya SD.Sharma K,. Pradhan SK , Chauhan RS. Investigation of an outbreak of infectious pustular balanoposthitis in cattle breeding bulls at a frozen semen bank .Rev. sci. tech. Off. int. Epiz., 2014, 33 (3),

Bovine leukemia virus (BLV)

The chronic lymphoproliferative disorder, bovine leukosis, is caused by the deltaretrovirus bovine leukemia virus (BLV). Surveys indicate that 39% of the US beef cow-calf operations have at least one BLV-infected animal compared with 83% of dairy herds. Most BLV infected cattle remain asymptomatic and act as carriers of the virus while less than 5% progress to lymphosarcoma. There are limited studies on the impact of BLV in beef cattle. Understanding the impact as well as identifying important route of disease transmission in beef cattle enterprises is important to better design intervention strategies. The primary goals of the studies described in this this dissertation were to 1) further understand the risk of breeding bulls in the transmission of BLV and 2) to determine the effect of BLV on the survival of cattle in beef and dairy herds. We first determined BLV prevalence in breeding beef bulls and the presence of BLV provirus DNA in genital secretions (smegma and semen). In our study population, 44.6% of beef bulls were seropositive for BLV and 48.7% of herds had at least 1 BLV-infected bull.

Bovine leukemia virus provirus DNA was detected in smegma samples of 4/54 (7.4%) BLV-seropositive bulls and represent a potential risk for the transmission of BLV from infected bulls to uninfected cows during natural service breeding programs. To evaluate this risk, we exposed BLV negative heifers to a BLV positive bull during a defined 38-day breeding period. Although BLV provirus was found in the smegma and blood of the BLV positive bull prior to and after the breeding period, we detected no evidence of seroconversion or presence of BLV provirus DNA in the blood of naïve heifers. These results suggest that BLV infected bulls that are healthy and aleukemic may not be a significant risk of BLV transmission during a defined breeding season. We next evaluated the impact of BLV infection on beef and dairy cow' longevity within herds. The presence of BLV antibodies in blood was not associated with a change in beef cow longevity over 2 years monitoring period, but decreased survival was observed in cattle in which BLV infection had advanced clinically as indicated by a high BLV provirusload in blood. In dairy cows, we demonstrated that infected females lived significantly shorter than their negative herd mates and were at a 30% greater hazard of being culled compared with BLV negative cows. In summary, there is high prevalence of BLV in breeding beef bulls which could serve as a source of transmission both within and between herds. Based on our study results, the risk of transmission of BLV from healthy infected bulls to naïve heifers is low when bulls are housed with heifers for a defined breeding period, but this scenario should not be considered without risk. BLV does not appear to have an impact on beef cow longevity, but in contrast, is associated with decreased longevity in dairy cows. These studies provide important information for supporting and designing risk based BLV control programs.

Rojas, O.J.B., 2019. *Bovine Leukemia Virus in Beef Cattle: Sexual Transmission and Cow Survivability* (Doctoral dissertation, Michigan State University).

Highlights

- •The BLV infected bull used for natural service had a PVL of 175.90 copies/10^5 cells in the smegma and 18,405.16 copies/10^5 cells in blood.
- •Seroconversion to BLV was observed in none of the cows exposed to BLV infected bull during breeding season.
- •BLV infected bulls that are healthy and aleukemic may not be a significant risk of BLV transmission during a defined breeding season.

Abstract

Bovine leukosis is a chronic lymphoproliferative disorder that leads to significant economic losses in the beef and dairy industries. The major route of virus transmission is believed to be iatrogenic through the transfer of blood containing infected lymphocytes. In addition, BLV proviral DNA has been identified in nasal secretions, saliva, milk, colostrum, semen and smegma; however, natural transmission of BLV through these secretions has not been clearly demonstrated. The use of bulls for natural breeding has been identified as a risk factor in BLV infected dairy herds. However, the risk of BLV-infected bulls transmitting the virus is unknown. The objective of this study was to evaluate the potential for BLV transmission during natural breeding between a BLV-infected bull and uninfected heifers. Forty healthy, BLV seronegative, and proviral-negative beef heifers were randomly assigned to one of two groups: control heifers (n = 20) exposed to a BLV seronegative and proviral negative bull and challenged heifers (n = 20) exposed to a BLV seropositive and proviral-positive bull. Each group was housed with the bull for a period of 38 days in a 5-acre pasture to replicate the

housing of commercial beef cattle during the breeding season. Blood samples were collected from heifers at −60, −30 and 0 days prior to breeding and day 30, 60 and 90 after the breeding period ended. Blood samples were tested for BLV antibodies by ELISA and BLV proviral DNA by CoCoMo-qPCR. New infection was not detected by ELISA or CoCoMo-qPCR in any of the challenge or control heifers at any time point during the study. Based on these results, BLV infected bulls that are healthy and aleukemic may not be a significant risk of BLV transmission during a defined breeding season.
Benitez, O.J., Roberts, J.N., Norby, B., Bartlett, P.C., Maeroff, J.E. and Grooms, D.L., 2019. Lack of Bovine leukemia virus transmission during natural breeding of cattle. *Theriogenology*, *126*, pp.187-190.
https://doi.org/10.1016/j.theriogenology.2018.12.005

Bovine viral diarrhea virus (BVDV)

Acute or chronicle BVDV infection on bulls is associated with BVDV excretion in the semen that can be transient in case of acute infection or permanent for a persistently infected animal. Under certain circumstances BVDV acute infection on bull can lead to a permanent spermatic infection. During a 6 months follow-up, a total of 2 bulls (0.19 % , out of 1033 seropositive AI bulls) were diagnosed as permanent spermatic infected whereas the BVDV antibodies remained stable. **Pozzi N., Catinot V., Charpentier Y., Bouilloux D., Guerin B. Infection permanente spermatique chez le taureau, après infection aiguë par le virus de la diarrhée virale bovine. Epidémiol. Santé Anim. 2008; 54 :99-107.**

Bovine diarrhea virus (BVDV) causes a variety of economically important enteric and infertility problems. For that reason, several countries have eradicated the disease and some others have schemes in progress to achieve freedom from it. Although there is a considerable amount of information about the risk ofBVDVtransmission through contaminated semen used forAI, there is no available evidence to indicatewhether the resulting embryos, when used for embryo transfer (ET), can lead to the transmission of BVDV to recipients and offspring. For this experiment, semen from a bull persistently infected with BVDV (105 TCID50/mL of NY strain) was used for insemination (2 times at estrus) of BVDV-seronegative, superovulated heifers (*n*=27). All heifers seroconverted toBVDVwithin 10 days post-insemination. Embryos and unfertilized oocyteswere collected nonsurgically (*n*=92) or postmortem (*n*=52) 7 days post-insemination and were either washed according to IETS recommendations (without trypsin treatment) or left unwashed. In total, out of 144 unfertilized oocytes and embryos collected, 23 (16%) were of ET quality. Most of the embryos were degenerated or unfertilized. On 17 occasions, 1 or 2 washed embryos were transferred to BVDV-seronegative recipients. After ET, all pregnant and nonpregnant recipients remained free of BVDV and antibodies. In total, 6 heifers became pregnant and 5 calves free of BVDV and BVDV antibodies, including 2 sets of twins, have been born to date. Post-insemination, BVDV was detected in 29% (12/41) of unwashed and 10% (4/40) of washed embryos, 100% (4/4) of follicular fluid samples, oviductal epithelial cells, endometrium, and corpora lutea tissues as determined by the virus isolation test. Results herein suggest that BVDV can be transmitted by AI, resulting in the production of some proportion of contaminated embryos. However, it appears that such embryos, when washed according to the IETS guidelines, do not cause BVDV transmission to recipients or their offspring. **Bielanski A, Lalonde A, Algire J Transmission of noncytopathic bovine**

diarrhea virus by infected semen to embryo donors and by embryos to embryo transfer recipients and offspring REPRODUCTION FERTILITY AND DEVELOPMENT 2010, 22, p 251 abstract

The aim of this study was to evaluate the capacity of three semen processing techniques, Percoll gradient centrifugation, Swim-up and a combination of Swim-up and Percoll gradient centrifugation, to reduce the viral load of bovine viral diarrhea virus (BVDV) in experimentally infected semen samples. The evaluation was performed using two approaches: first, searching for the presence of virus in the processed samples (via virus titration and RT-PCR) and second, ascertaining the possible interference on in vitro embryo production. The sperm count and DNA integrity (Comet assay) of the processed samples were analyzed (Experiment 1). The amount of virus in the processed samples was determined by titration in cell culture (Experiment 2). The samples processed by Swim up/Percoll gradient centrifugation were utilized for in vitro embryo production, and the embryos produced were tested for BVDV by RT-PCR (Experiment 3). Sperm concentration, Comet assay and embryo production were analyzed by chi-squared tests ($P< 0.05$). There was a significant difference between sperm separation techniques when the sperm count and Comet assay were analyzed. The sperm count obtained from the Swim up/Percoll gradient centrifugation group was lower than that obtained in either of the two other groups (Swim up and Percoll gradient centrifugation), and the Comet assay showed that the combination of the two semen processing techniques (Swim up/Percoll gradient) produced a 1.1% prevalence of Comet level 2, which was not observed in the other groups. The BVDV titer (10(6.68) TCID(50)/ml) added to experimentally infected semen samples decreased after Percoll gradient centrifugation to 10(2.3)-10(1) TCID(50)/ml; for the Swim up group, the titer range was 10(3.3) to 10(1.87) TCID(50)/ml, and in the Swim up/Percoll gradient centrifugation group, BVDV was undetectable. The decreases in titer varied from 99.9% in the Swim up-processed group to 100% in the Swim up/Percoll gradient centrifugation group. In vitro embryo production displayed similar blastocyst development rates among all groups, and RT-PCR was negative for the produced embryos. The data showed that the combination of Swim up/Percoll gradient centrifugation promoted the elimination of BVDV from the semen samples without damaging spermatozoa cells and also allowed successful in vitro embryo production free of BVDV. Hence, the risk of BVDV contamination is negligible for the embryo recipient. **Galuppo AG, Junior NB, Arruda NS, Corbellini AO, Chiappetta CM, Pavão DL, D'Angelo M, Canal CW, Rodrigues JL Evaluation of the effectiveness of semen processing techniques to remove bovine viral diarrhea virus from experimentally contaminated semen samples. J Virol Methods. 2012 Dec 5. pii: S0166-0934(12)00421-1.**

The current report was prompted by an atypical outbreak of mucosal disease that occurred in a beef herd in the southwestern part of Buenos Aires Province, Argentina, where a total of 9/41 (21.9%) yearling bulls died. Blood samples from 73 bulls and 189 heifers were tested for evidence of persistent BVDV infection with Bovine Viral Diarrhea Virus (BVDV). Non-cytopathic BVDV was isolated from 7 (9.6%) 24- to 36-month-old bulls, and 3 (1.6%) 36-month-old heifers. Non-cytopathic BVDV was also detected in the seminal plasma of three of six persistently infected (PI) bulls. Furthermore, a 171 bp genomic fragment of BVDV was consistently detected by nested RT-PCR in one of the two samples of the commercial semen used for artificial insemination, indicating that this semen could be a possible source of

infection for the whole herd. To evaluate the possible reproductive consequences of PI heifers and bulls, ovaries and semen were obtained from PI cattle for in vitro assays. The in vitro fertilization of oocytes with semen from PI bulls was associated with decreased cleavage and embryo development rates. Additionally, non-cytopathic BVDV was isolated from the follicular fluid of PI heifers. Genetic typing revealed that all isolates BVDV from the present study had a high percentage of homology and that all of the fragments from the RT-PCR clearly fit with the BVDV 1b cluster. These findings confirm the negative impact that BVDV can have on the reproductive performance of cattle and the importance of applying the proper sanitary controls to minimize the risk of BVDV infection. **González Altamiranda EA, Kaiser GG, Weber N, Leunda MR, Pecora A, Malacari DA, Morán O, Campero CM, Odeón AC. Clinical and reproductive consequences of using BVDV-contaminated semen in artificial insemination in a beef herd in Argentina. Anim Reprod Sci. 2012 Aug;133(3-4):146-52. doi: 10.1016/j.anireprosci.2012.06.022.**

The aim of this study was to evaluate the capacity of three semen processing techniques, Percoll gradient centrifugation, Swim-up and a combination of Swim-up and Percoll gradient centrifugation, to reduce the viral load of bovine viral diarrhea virus (BVDV) in experimentally infected semen samples. The evaluation was performed using two approaches: first, searching for the presence of virus in the processed samples (via virus titration and RT-PCR) and second, ascertaining the possible interference on in vitro embryo production. The sperm count and DNA integrity (Comet assay) of the processed samples were analyzed (Experiment 1). The amount of virus in the processed samples was determined by titration in cell culture (Experiment 2). The samples processed by Swim up/Percoll gradient centrifugation were utilized for in vitro embryo production, and the embryos produced were tested for BVDV by RT-PCR (Experiment 3). Sperm concentration, Comet assay and embryo production were analyzed by chi-squared tests ($P<0.05$). There was a significant difference between sperm separation techniques when the sperm count and Comet assay were analyzed. The sperm count obtained from the Swim up/Percoll gradient centrifugation group was lower than that obtained in either of the two other groups (Swim up and Percoll gradient centrifugation), and the Comet assay showed that the combination of the two semen processing techniques (Swim up/Percoll gradient) produced a 1.1% prevalence of Comet level 2, which was not observed in the other groups. The BVDV titer (10(6.68)TCID(50)/mL) added to experimentally infected semen samples decreased after Percoll gradient centrifugation to 10(2.3)-10(1)TCID(50)/mL; for the Swim up group, the titer range was 10(3.3)-10(1.87)TCID(50)/mL, and in the Swim up/Percoll gradient centrifugation group, BVDV was undetectable. The decreases in titer varied from 99.9% in the Swim up-processed group to 100% in the Swim up/Percoll gradient centrifugation group. In vitro embryo production displayed similar blastocyst development rates among all groups, and RT-PCR was negative for the produced embryos. The data showed that the combination of Swim up/Percoll gradient centrifugation promoted the elimination of BVDV from the semen samples without damaging spermatozoa cells and also allowed successful in vitro embryo production free of BVDV. Hence, the risk of BVDV contamination is negligible for the embryo.
Galuppo AG, Junior NB, Arruda NS, Corbellini AO, Chiappetta CM, Pavão DL, D'Angelo M, Canal CW, Rodrigues JL. Evaluation of the effectiveness of semen processing techniques to

remove bovine viral diarrhea virus from experimentally contaminated semen samples. J Virol Methods. 2013 Feb;187(2):443-8

Lumpy skin disease

Lumpy skin disease is an economically important disease of cattle, caused by the lumpy skin disease virus (LSDV; *Capripoxvirus*). It has a variable clinical appearance but, in severely affected animals, is associated with extensive skin damage, pneumonia and death. The LSDV can be found in the semen of infected bulls for prolonged periods of time, from where it can be transmitted by mating or artificial insemination and cause clinical disease in heifers and cows. In this study, an ejaculate was collected from a LSDV seronegative bull and confirmed free from LSDV DNA by PCR. The ejaculate was split into a control sample (C), a sample spiked with a 4 log $TCID_{50}$ dose of an LSDV isolate (HD) and a 10^3 dilution of the virus suspension (ND) and frozen routinely. Two straws from each of the different semen treatment groups (HD, ND and C) were subsequently thawed and subjected to swim-up, single layer centrifugation, Percoll® density gradient and a Percoll® density gradient with added trypsin. For one set of straws, semen quality variables were recorded, and viral DNA status determined using PCR; the other set was used for positive staining electron microscopy. Samples determined to be positive for LSDV DNA by PCR were then subjected to virus isolation (VI). Complete elimination of LSDV from semen did not occur with use of any of the processing methods. Trypsin did reduce the viral load, and eliminated LSDV from the ND sample, but severely negatively influenced semen quality. The LSDV virions, as assessed by electron microscopy, were associated with the sperm plasma membrane. Further investigation is needed to establish the efficacy of immuno-extenders for rendering semen free from LSDV.
Annandale, C.H., Smuts, M.P., Ebersohn, K., Du Plessis, L., Venter, E.H. and Stout, T.A., 2018. Effect of semen processing methods on lumpy skin disease virus status in cryopreserved bull semen. *Animal reproduction science*, *195*, pp.24-29. https://doi.org/10.1016/j.anireprosci.2018.04.080

Semen treatments

The effect of trypsin on the fertilizing capacity of bull semen was investigated as part of the evaluation of the addition of trypsin to semen as a method for destroying or inactivating infectious agents. Parts of the ejaculates from four bulls were treated with 0.3% trypsin solution. Both the treated and untreated aliquots of semen were frozen, thawed and used for the artificial insemination of superovulated heifers. Two hundred and thirty ova and embryos were collected from 22 heifers on day 7 after oestrus (insemination). One hundred and ten out of 164 (67%) embryos and ova from 15 heifers inseminated with trypsin-treated semen were classified as of transferable quality compared to 46 out of 66 (70%) in the control group of 7 heifers (p greater than 0.05). There was no difference in the proportion of fertilized ova or degenerated embryos resulting from the control or trypsin-treated samples of frozen-thawed semen, which is consistent with results obtained previously using fresh semen. **(Bielanski A. Vet Res Communic 1989;13:251-255)**

Semen infected experimentally with infectious bovine rhinotracheitis virus (BHV-1) was treated with trypsin at concentrations of 0.30%, 0.25% and 0.15%, with or without (w or w/o) trypsin inhibitor in order to render the semen virus free. The trypsin treatments (at 0.30% and 0.25% by concentration) inactivating the virus up to $10^4 TCID_{50}$/mL, and its effect on semen quality were assessed weekly from the 1st to the 20th week after being frozen. The following parameters were determined using a computerized semen analysis system (total motility, progressive motility and linearity of sperm cells). The results showed that the total and progressive motility of sperm cells were reduced in frozen/thawed semen, principally in the semen treated with trypsin at a concentration of 0.30%. Moreover, the plasma membranes were damaged by trypsin treatments (0.30% by concentration), as determined by the hyperosmotic swelling test (HOS test). These findings suggest that trypsin treatments were effective against the virus however the effects on semen quality, and the possibility of a decrease in semen fertility were clear. Trypsin treatment could be recommended at a maximum concentration of 0.25% (w/o inhibitor) on semen with a high concentration and motility values of spermatozoa before freezing. **(Silva N et al. Anim Reprod Sci 1999;54:227-235)**

The aim of this investigation was to develop a practical method for treating bovine semen that would deter the transmission of a variety of pathogenic agents, including *Brucella abortus*. In a preliminary study, semen samples collected from 6 *Bos taurus* and 6 *Bos gaurus* bulls were processed by initial dilution (1:10) in TL Hepes solution (BioWhittaker) containing an antibiotic cocktail (tylosin, gentamycin, spectinomycin and lincomycin; Minitube) and incubated at 38^{o}C for 2 h followed by centrifugation for 10 minutes at 500xg. The sperm pellet was resuspended in 0.5 mL medium then layered on top of a Percoll density gradient: 90 % (1 mL below) and 45 % (1 mL containing 0.125 % trypsin-EDTA above) and centrifuged for 30 min at 700 xg (this trypsin-Percoll mixture effectively disassociated confluent BRL cell monolayers within 6 min of exposure before and after centrifugation with semen). The resulting sperm pellet did not differ from controls (washing using no antibiotics in the medium not trypsin in the Percoll gradient) in overall sperm motility and acrosomal integrity ($P>0.05$; chi-square).

In the next study, semen was collected by electroejaculation from 6 *Syncerus caffer* bulls, serologically negative for brucellosis, on a game farm in South Africa. Aliquots were removed as 1) negative controls, while the remaining samples were inoculated with *Brucella abortus* at 10^{3-4}/mL. (markedly higher than physiological) from an isolate originally recovered from an infected buffalo. These samples were then aliquoted into: 2) raw positive controls; then s described above 3) semen processed by dilution and incubation in TL Hepes containing no antibiotics then Percoll density centrifugation without trypsin (WASHED); and 4) dilution and incubation in medium containing the antibiotic cocktail and trypsin-EDTA in the 45 % Percoll layer (TREATED). Aliquots of the raw controls and sperm pellets after medium and Percoll treatments were plated onto bovine blood tryptose agar and cultured for up to 6 d at 37^{o}C in 7 % CO_2. As a result, all raw samples were negative, all raw brucella-inoculated samples were positive, all WASHED inoculated samples were also positive, yet all of the TREATED inoculated samples were negative for bacterial growth.

In the final study, semen was collected by electroejaculation from 14 *Syncerus caffer* bulls at Kruger National Park, and one was later flund to show strong positive serologically for brucellosis. Raw, WASHED and TREATED samples were submitted for culture; all samples were negative for brucella, indicating that the antibody titers were not necessarily diagnostic

for brucellosis or, if present, the organism would not be transmitted sexually. In conclusion, this investigation reports a novel, practical technique for treating bovine semen for specific diseases sucha s brucellosis without compromising sperm viability. **(Morfeld K et al. Theriogenology 2001;55:393 abstr.)**

REASONS FOR PERFORMING STUDY: A method of removing equine arteritis virus (EAV) from equine semen used for artificial insemination is urgently needed. Recent medical studies suggest that a double semen processing technique of density gradient centrifugation followed by a 'swim-up' can provide virus-free sperm preparations for assisted reproduction. OBJECTIVES: To investigate the use of the double semen processing technique to obtain virus-free sperm preparations from stallion semen containing EAV. METHODS: Aliquots of an ejaculate from an uninfected stallion were spiked with virus and processed by the double processing technique. The sperm preparations were tested by PCR for the presence of EAV. The procedure was repeated using an ejaculate from a known shedding stallion, testing processed and unprocessed aliquots by PCR and virus isolation. RESULTS: Virus-free sperm preparations were obtained using the double sperm processing technique. The 'swim-up' step is apparently required to ensure complete virus removal. CONCLUSIONS: The double semen processing technique is potentially a useful and simple tool for the removal of EAV from the semen of shedding stallions. POTENTIAL RELEVANCE: The inclusion of density gradient centrifugation and 'swim-up' in protocols for the processing of semen for artificial insemination could help prevent the transmission of viral diseases carried in semen, such as EAV. **(Morrell JM, et al. Equine Vet J, 2006;38(3):224-9.)**

Goats

Bluetongue virus

This study investigated the effects of the vaccination of rams with a serotype 2 bluetongue virus vaccine on the quality of their semen. One group of 23 rams was vaccinated on days 0 and 47, and 23 rams were left unvaccinated. Samples of blood, serum and semen were collected regularly in order to detect the virus genome, and to compare the quality of the semen from the vaccinated and unvaccinated rams. Segment 10 of the genome of the vaccine strain was detected in the blood of the vaccinated animals by reverse transcriptase-PCR (RT-PCR) on days 7, 13 and 19 after the first vaccination, but no virus was isolated from the RT-PCR-positive blood or from any of the semen samples from the vaccinated animals. There was a significant decrease in the concentration and motility of the spermatozoa and an increase in the proportion of abnormal and dead spermatozoa after the first vaccination ; however, after the second vaccination only smaller, non-significant changes were observed. On day 69, the quality of the semen of the vaccinated animals was not significantly different from that of the controls. **Bréard E, Pozzi N, Sailleau C, Durand B, Catinot V, Sellem E, Dumont P, Guérin B, Zientara S. Transient adverse effects of an attenuated bluetongue virus vaccine on the quality of ram semen. Vet Rec. 2007; 160 (13):431-5.**

Horses

Equine herpesvirus types 1 (EHV-1) and 4 (EHV-4)

In the horse, the risk of excretion of two major equine pathogens (equine herpesvirus types 1 (EHV-1) and 4 (EHV-4)) in semen is unknown. The objective of our study was to assess the possible risks for the horizontal transmission of equine rhinopneumonitis herpesviruses via the semen and the effect of the viruses on stallion fertility. Samples of stallion semen (n=390) were gathered from several different sources. Examination of the semen involved the detection of viral DNA using specific PCR. The mean fertility of the stallions whose sperm tested positive for viral DNA and the mean fertility of stallions whose sperm did not contain viral DNA, were compared using the Student's t-test. EHV-4 viral DNA was not detected in any of the semen samples. EHV-1 DNA was identified in 51 of the 390 samples, (13%). One hundred and eighty-two samples came from 6 studs and there was significant difference ($p<0.05$) among the proportion of stallions whose semen tested positive for viral DNA from 0 to 55% between the studs. There was a significant difference ($p<0.014$) between the fertility of stallions whose semen tested positive for viral DNA and those whose semen was free from viral DNA. The stallions that excreted the EHV-1 virus in their semen appeared to be more fertile than the non-excretors, but this difference was in fact related to the breeding technique since higher proportion of excretors were found among those whose semen was used fresh rather than preserved by cooling or freezing. In conclusion, this study suggests that the EHV-1 virus may be transmitted via the semen at mating or by artificial insemination as demonstrated with other herpes viruses in other species. **Hebia-Fellah I, Léauté A, Fiéni F, Zientara S, Imbert-Marcille BM, Besse B, Fortier G, Pronost S, Miszczak F, Ferry B, Thorin C, Pellerin JL, Bruyas JF. Evaluation of the presence of equine viral herpesvirus 1 (EHV-1) and equine viral herpesvirus 4 (EHV-4) DNA in stallion semen using polymerase chain reaction (PCR). Theriogenology. 2009; 71(9):1381-9.**

Equine arteritis virus (EAV)

A semen sample from a stallion infected during the 2010 equine arteritis virus (EAV) outbreak was received for viral isolation prior to castration of the animal. The virus was identified using a polyclonal antibody immunofluorescence test. Reverse-transcription polymerase chain reaction (RT-PCR) was used to amplify a region of the GP5 gene with primers GL105F and GL673R. The PCR products were purified and sequences of both strands were determined in a MegaBACETM 1000 with inner primers CR2 and EAV32. A phylogenetic dataset was built with the previously reported sequences of five strains isolated in Argentina, together with a group of selected sequences obtained from GenBank. The unrooted neighbour-joining tree was constructed using molecular evolutionary genetic analysis (MEGA) and bootstrap analyses were conducted using 1,000 replicate datasets. Evolutionary distances were computed using the maximum composite likelihood method. A NetNGlyc server analysis at the Technical University of Denmark (www.cbs.dtu.dk/services/NetNGlyc/) was used to predict Nglycosylation in GP5 sequences. The phylogenetic analysis revealed that the new strain (GLD-LP-ARG), together with other strains previously isolated, belongs to the European group EU-1 but in a different branch. The new strain shows 99% nucleotide identity with strain A1 and 98.1% with the Belgian

strain 08P178. Persistently infected stallions and their cryopreserved semen constitute a reservoir of EAV, which ensures its persistence in the horse population around the world. These findings reinforce the importance of careful monitoring of persistently infected stallions, as well as semen straws, by RT-PCR or test mating, in accordance with national regulations.

Metz GE, Serena MS, Panei CJ, Nosetto EO, Echeverría MG. The equine arteritis virus isolate from the 2010 Argentinian outbreak. Rev. sci. tech. Off. int. Epiz., 2014, 33 (3), ... - ...

Various pathogens

Workshop 15: Equine – Pathogen Transmission through Breeding. ***Moderator: J. Govaere (Belgium)***

Abstract 15.1

Pathogen Transmission through Breeding

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The recent outbreak of Contagious Equine Metritis (CEM) in the US demonstrates that venereal diseases are still an imminent problem for horse breeders. However, when looking at *viral* venereal diseases, numerical data on prevalences, transmission and virus shedding status of individuals, are not always available. For example, sero-prevalences for Equine Viral Arteritis (EVA) vary between 6 and 41% depending on country, season and breed. Furthermore, the European legislation prescribes a rather strict compliance with the sanitary guidelines concerning transport of animals and semen, although only little consideration is given to new purifying techniques, as density gradient centrifugation, and possible infectious risks of embryo transfer in the horse. Recently, new findings have been published on the potential risk of equine herpes virus transmission (*Hebia* et al. *2007: Theriogenology 67, 1485–1491*) and methods to purify stallion semen from viral and bacterial contaminants have become available (*Morell and Gervarthy 2006: Equine Vet J 38, 224–229*) and might feed the discussion how stringent regulations can or have to be concerning transcontinental semen and embryo transport. These data have been proven to be useful in the field and might be essential in the discussion.

Abstract 15.2

Risk of Pathogen Transmission through Breeding in Horses

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Venereal transmission: which risks? Some infectious bacterial, viral and protozoal reproductive pathogens have a well known venereal transmission. The stallion can be an asymptomatic penile carrier of pathogenic bacteria that are transmitted to the mare: Taylorella equigenitalis, Pseudomonas aeruginosa and Klebsiella pneumoniae. Streptococcus zooepidemicus may also cause significant infertility in mares but its eventual venereal transmission has never been formally proven. The ability of the Equine Arteritis Virus to be transmitted by semen is well established. Equine coital exanthema caused by Equine Herpes Virus 3 was described from many years. In some countries, the causative agent of dourine, trypanosoma equiperdum, is sometimes found. Equine herpesvirus types 1 and 4 are two major pathogens in the horse, with a large incidence of asymptomatic latent infections. In many mammalian species viruses from the Herpesviridae family are frequently found in and transmitted via semen and embryos. In the past, a few sporadic cases of the isolation of the virus in the semen have been described following the experimental or natural infection of stallions with the EHV-1 virus, but never in healthy stallions. In a recent study (Hebia-Fellah et al, 2009), samples of semen from 390 different stallions were tested using specific PCR to detect EHV-1 and EHV-4 DNA. EHV-4 viral DNA was not detected in any of the semen samples. EHV-1 DNA was identified in 51 of the 390 samples, (13%). One hundred and eighty two samples came from 6 studs and there was significant difference ($p < 0.05$) in the proportion of stallions whose semen tested positive for viral DNA from 0 to 55% among the studs. In another study (Hebia-Fellah et al, 2009) testis and epididymis tissues sampled from 60 healthy stallions during castration were tested by the same specific PCR. EHV-1 DNA was detected in 2 from 60 stallions' testis samples (3%), but was not detected in any stallions' epididymis samples. Those results demonstrate for the first time the presence of the EHV-1 in the testis and semen of naturally infected stallions and suggest that EHV-1 virus may be transmitted via semen at mating or via AI, similar to other herpesviruses in other species. Further studies are needed to determine in which testicular cells the virus resides and which consequences the genital and semen location of EHV1 has on fertility and viral transmission. Would other viruses be present in semen and could be sexually transmitted? The question has to be asked for example for Influenza virus, West-Nile virus… For a few viruses, there is a partial response: Equine Infectious Anemia (EIA) virus has been reported in semen from infected stallions, without any evidence of sexual transmission of disease (Lu & Morresey, 2007). **Transmission via embryo transfer: are there risks?** European and french sanitary regulations for the inter and intra European countries trade of equine embryos recommend embryos to be washed 10 times over in accordance with the standardized procedure described for ruminant and porcine embryos by IETS guidelines. However, the risks of any infectious transmission by embryo transfer in horses have not been really evaluated. Embryo contamination was reported once by Carvalho et al. (2000), who collected an equine embryo naturally contaminated by EHV-1 from a clinically healthy donor mare. In two studies (Hebia et al., 2007, 2008), we demonstrated that after *in vitro* contamination of D6.5, D7 and D8 equine embryos, EHV1 cannot be removed from embryos by the washing procedure (10 washes) recommended by sanitary European regulations and IETS guidelines. In the second of those experiments (Hebia et al., 2008), an enzymatic treatment (bath of 0.25% trypsin for 90 s) before the washing protocol has proven to be efficient to decontaminate equine early blastocysts (D6.5) surrounded by zona pellucida as previously reported for bovine embryos infected by BHV-1, but ineffective to do the same on older equine blastocysts (D8) surrounded only by capsule, as previously reported for porcine embryos infected by PRV. The persistence of EHV-1 after washing makes the embryo a potential means of transmitting the virus, and disease, to recipient mares. Further studies are needed to determine which different interactions exist between EHV-1 and embryos surrounded by ZP or by capsule, to evaluate the effect of virus on embryo viability and to determine whether the transfer of infected embryos could infect recipient mares. Other pathogens would be tested on equine embryos, and eventual sanitary risks of embryo transfer would be more evaluated in horses.

Abstract 15.3

Prevention of Pathogen Transmission through Breeding in Horses

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A method for removing pathogens from semen is urgently required, to protect naïve inseminated animals from viruses which may be shed in the semen before the male has sero-converted. Recently, a method was described for removing equine arteritis virus (EAV) from stallion semen to be used for artificial insemination (AI) (*Morrell and Geraghty 2006: Equine Vet J 38, 224–229*). The method, a double semen processing technique consisting of a density gradient centrifugation (DGC) followed by a 'swim-up' procedure, removed EAV from both spiked and naturally infected semen samples, using PCR and virus isolation to test for the virus (*Morrell and Geraghty 2006: Equine Vet J 38, 224–229*). Preliminary results of a similar experiment using a simplified technique, Single Layer Centrifugation (SLC) with Androcoll-P™, plus 'swim-up', considerably reduced the virus titer from boar ejaculates spiked with porcine circovirus (Morrell et al, unpublished data). Since SLC is less time-consuming than DGC and can be scaled-up to process large volumes of ejaculate, it might be feasible to use it for processing whole animal ejaculates. DGC is also reported to remove bacteria from human semen (*Nicholson* et al. *2000: Hum Reprod 15, 662–666*). Therefore, studies using SLC with animal semen are currently underway to investigate the removal of bacterial contamination occurring during semen collection. Routine use of SLC in processing animal semen for AI-doses could thus protect against accidental transmission of viruses from shedding males and reduce the amount of antibiotics used in semen doses for AI.

Abstracts from 13th Annual Conference of the European Society of Domestic Animal Reproduction, September 9-12, 2009, Ghent, Belgium. Published in Reproduction in Domestic Animals. 2009; 44(Suppl. 3): 71-72.

Humans

HIV and HCV

OBJECTIVE: To determine the effectiveness of a novel treatment designed to remove human immunodeficiency virus (HIV) -1 and hepatitis C virus (HCV) from spiked semen and to evaluate sperm motility and viability after treatment. DESIGN: A prospective clinical laboratory-based study. SETTING: The human studies were conducted in academic and national research environments. The bovine study was conducted in an accredited research facility. PATIENT(S): Healthy volunteers provided the semen samples used in the human studies; abattoir-derived material was used for the bovine embryo production study. INTERVENTIONS(S): None. MAIN OUTCOME MEASURE(S): Cytopathic, reverse transcriptase-polymerase chain reaction, and branched DNA assays were used to test the efficacy of the procedure for inactivating or removing viruses from spiked semen; standard semen evaluation criteria were used to assess the effects of the procedures on sperm motility and viability. RESULT(S): Trypsin exposure significantly reduced the infectivity of HIV-1. The triple density gradient treatment, with or without trypsin, had no detrimental affect on fresh or cryopreserved/thawed sperm 2-48 hours after treatment. The treatment of semen spiked with HIV-1 or HCV indicated that the procedure was effective for reducing viral copies to undetectable levels or below levels of clinical relevance. CONCLUSION(S): The procedure was effective for significantly inactivating or reducing HIV-1 and HCV in spiked semen without adversely affecting sperm quality. (**Loskutoff NM, et al. Fertil Steril. 2005;84:1001-1010.)**

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