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SHORT COMMUNICATIONS

Association of non-cytopathogenic BVDV with bovine blastocysts: effects of washing, duration of viral exposure and degree of blastocyst expansion

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IN vitro produced bovine embryos appear to have a greater potential for carriage and transmission of bovine viral diarrhoea virus (BVDV) than their in vivo derived counterparts (Stringfellow and Wrathall 1995). This is a major concern for the future exploitation of in vitro embryo production biotechnology in cattle breeding because ovaries, from which the oocytes and co-culture cells are usually derived, are acquired from slaughterhouse animals of which approximately 1 per cent may be persistently BVDV infected (Howard and others 1986, Houe and Meyling 1991). The exact nature of the embryo-BVDV association (that is, whether the virus replicates within embryonic cells and/or simply adheres to the zona pellucida) is unknown. Furthermore, the type of association may depend on the developmental stage at which the oocyte/embryo is exposed. For example, the virus could conceivably penetrate the zona during in vitro maturation through the cumulus cell transzonal processes that provide direct communication between follicle cells and the oocyte (Allworth and Albertini 1993). Alternatively, the virus may be carried with the sperm during in vitro fertilisation, or adhere to the zona if the embryo is exposed during in vitro culture. The presence of the virus has been demonstrated in oocytes (Brownlie and others 1997) and therefore carriage of infection during in vitro maturation and/or fertilisation is a potential risk. Another possibility is that thinning and other alterations in the structure of the zona, such as those which occur during blastocyst expansion during hatching may allow virus penetration. In the mouse, a trypsin-like protease is secreted during hatching (Perona and Wassarman 1986) and this, if it occurs in the cow, could modify BVDV-embryo interactions.

In this study, a series of experiments was performed to assess the effects of exposing embryos to BVDV for varying periods of time, then washing them by methods recommended by the International Embryo Transfer Society (IETS 1990). In experiments 1 and 3, embryos were tested for BVDV following viral exposure throughout the complete in vitro production process. In experiment 2, testing was carried out after in vitro culture only, and in experiments 4 and 5 shorter periods of exposure were used (four hours and five minutes, respectively). Studies were also performed to see whether the developmental stage of the expanding blastocyst influenced these interactions.

Oocytes were aspirated from slaughterhouse ovaries and matured in TCM-199 (Sigma) medium supplemented with 4 µg/ml oestradiol (Sigma) 5 iu/ml Folligon (Intervet) and 10 per cent oestrous cow serum (BVDV-negative). After 24 hours, the oocytes were fertilised with frozen-thawed sperm (from a BVDV-negative bull) in a standard IVF-TALP medium (Parrish and others 1986) supplemented with 30 µg/ml heparin, 30µM penicillinamine, 15µM hypotaurine and 1µM epinephrine. After a further 24 hours, the zygotes were transferred to TCM-199 medium containing 10 per cent oestrous cow serum and BVDV-negative granulosa co-culture cells and were cultured for eight days.

In order for the cumulus-oocyte complexes and the embryos to be exposed to the virus either throughout the in vitro production period or only during the culture period certain minor modifications were made to the in vitro production system: cumulus-oocyte complexes were exposed to the virus (strain Pe515; titre $10^{6.2}$ $TCID_{50}$/ml) for two hours before being transferred to the in vitro maturation medium for a further 22 hours. This protocol was designed to expose the cumulus-oocyte complexes to a concentration of the virus no greater than that measured in the follicular fluid of naturally persistently infected cattle (P. J. Booth, M. C. Clarke, unpublished observations), and also to allow the cumulus cells to become infected with the virus which would consequently be carried over into the in vitro fertilisation medium. In order to infect the in vitro culture system, the granulosa co-culture cells were inoculated with BVDV four days before use.

Virus isolation was performed using calf testes cell cultures (grown on glass coverslips) derived from BVDV-free animals as described by Booth and others (1995). Each sample was cultured for seven days and passaged three times. BVDV antigen was detected in the coverslip cultures by fluorescent immuno-

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TABLE 1: Proportion of groups of 10 bovine embryos at different stages of development testing positive by virus isolation after bovine viral diarrhoea virus exposure during in vitro production (IVP), in vitro culture (IVC), and after four hours and five minutes exposure

Experiment	Duration of viral exposure	No of groups of 10 embryos virus positive/total no of groups tested: Morulae and blastocysts (%)	Unexpanded blastocysts (%)	Small expanded blastocysts (%)	Medium expanded blastocysts(%)	Total (%)
1	IVP	1/1 (100)	3/3 (100)	2/2 (100)	ND	6/6 (100)[a]
2	IVC	2/2 (100)	1/1 (100)	1/1 (100)	ND	4/4 (100)[a]
3	IVP*	ND	4/4 (100)	1/1 (100)	1/1 (100)	6/6 (100)[a]
4	4 hours	1/2 (50)	1/4 (25)	2/6 (33·3)	3/3 (100)	7/15 (47)[b]
5	5 minutes	1/3 (33·3)	0/2 (0)	0/2 (0)	ND	1/7 (14)[b]
Total		5/8 (63)	9/14 (64)	6/12 (50)	4/4 (100)	

*Bovine viral diarrhoea virus seropositive serum used during washing procedure
[a, b] Values in same column with different superscripts are different, $P<0.0001$ Data were analysed for exact P-values by Pearson's chi-square and deviance (likelihood) test in log-linear models on contingency tables
ND No data

histochemistry. The staining technique and the antibody specificity studies were as described by Booth and others (1995). Monoclonal antibodies WB162 and WB103 (specific to the viral envelope glycoprotein gp53 and the p80 non-structural protein, respectively) were tested in combination on samples. These antibodies were known to possess immunoreactivity to a range of field strains of BVDV and to the Pe515 strain.

On days 7 and 8 of in vitro culture, embryos were categorised as follows: unexpanded blastocysts; small expanding blastocysts (approximately 160 to 180 μm in diameter); and medium expanding, but unhatched blastocysts (approximately 180 to 200 μm in diameter). In cases where fewer than 10 unexpanded blastocysts were available, a fourth category, which comprised unexpanded blastocysts plus a small proportion of late morulae, was selected. Embryos were washed in groups of 10 using the washing procedure recommended by the IETS (IETS 1990). This consisted of transferring the embryos through 12 changes of medium, each wash representing a 100-fold dilution of the previous wash. Washes 6 and 7 comprised the trypsin washing steps, which were incorporated because BVDV is known to be sensitive to this enzyme (Hafez and Liess 1972). The washing medium comprised phosphate buffered saline supplemented with 0·4 per cent bovine serum albumin (for washes 1 to 5) or 2 per cent serum (for washes 8 to 12). After washing, each group of 10 embryos was sonicated before virus isolation.

In order to ensure that the only source of virus present was that introduced experimentally, virus isolation tests were performed on some of the samples before any BVDV was added. These included follicular fluids, granulosa co-culture cells, residual media in the in vitro maturation, fertilisation and culture dishes, and oocytes and degenerate embryos in addition to spare viable morulae and blastocysts (which had not been used for the virus exposure experiments). Where appropriate, the same samples were also tested in parallel control in vitro production runs (from the same pool of oocytes) in which no BVDV had been experimentally added. The oocytes, degenerate embryos and spare viable embryos were sonicated but not washed before virus isolation. BVDV was not detected in any of these samples. In the in vitro production runs that were experimentally infected with BVDV, the residual media in the in vitro maturation, fertilisation and culture dishes were collected for viral titration.

Analysis of residual media indicated mean viral titres of $10^{5.0}$, $10^{6.1}$ and $10^{6.5}$ $TCID_{50}$/ml for in vitro maturation, fertilisation and culture media, respectively, indicating exposure of oocytes and embryos to high levels of BVDV throughout the in vitro production system. The results of the five experiments are shown in Table 1. In experiments 1 and 2, all groups of embryos were infected despite being washed, therefore exposure to BVDV during in vitro culture alone did not appear to decrease the proportion of groups of embryos that were associated with the virus compared with those exposed throughout the entire in vitro production period. This could possibly suggest that the association of BVDV with oocytes/embryos was not dependent on any actual or potential viral uptake mechanisms specific to maturation and fertilisation.

Experiment 3 was conducted to ascertain whether BVDV could be inactivated by substituting BVDV-seropositive serum (that had a high titre of antibodies specific to the strain of BVDV used; 7306 U/ml) for the BVDV antibody-negative serum in the washing procedure (see Table 1). However, the presence of this serum did not reduce the proportions of infected embryos which suggests that the virus may have been located deeper than just on the zona surface.

Experiments 4 and 5 were performed to ascertain whether shorter durations of BVDV exposure would lead to lower amounts of adherent virus and increase the effectiveness of washing in removing it. The results from experiment 4 showed that exposure to BVDV for four hours significantly ($P<0.0001$) reduced the proportion of washed groups of embryos that were infected (47 per cent) compared to those exposed for the whole of the in vitro culture period (100 per cent). However, even with exposures as short as five minutes, 14 per cent of the embryo groups retained the virus after washing. Analysis of the data in experiments 4 and 5 indicated no relationship ($P>0.05$) between the degree of blastocyst expansion and the proportion of embryo groups that were infected, suggesting that the tendencies for BVDV to adhere to the zona were not altered by embryo expansion and the accompanying changes in zona structure.

The results of this study indicate that even brief exposure of in vitro produced embryos to non-cytopathogenic BVDV leads to levels of embryo-associated infectivity which cannot readily be removed by the current IETS washing procedures. They provide further evidence that 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 (Singh and others 1982, Bielanski and Hare 1988). Whether BVDV can fully penetrate the zona (as suggested by Brownlie and others 1997) or whether it merely adheres to the zona of in vitro-produced embryos (Vanroose and others 1998) is still unclear. If the latter is correct, further studies are required to establish how deeply the virus is located in the zona of in vitro produced embryos and whether zona-associated virus can be removed by alternative washing procedures. Modification of in vitro conditions might also decrease the potential for embryo-BVDV association and transmission.

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Aujeszky's disease virus infection concurrent with postweaning multisystemic wasting syndrome in pigs

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POSTWEANING multisystemic wasting syndrome (PMWS) is a recently described disease affecting late nursery and fattening pigs which has been associated with porcine circovirus (PCV) infection. Affected animals have systemic lesions, the major organs involved being lymphoid tissues, lung, liver and kidney (LeCann and others 1997, Segalés and others 1997b, Ellis and others 1998). Some evidence suggests that PMWS-affected pigs may be immunosuppressed. First, Pneumocystis carinii has been found in the lungs of approximately 5 per cent of PMWS-affected pigs in Canada (Clark 1997), and a variable degree of lymphocytic depletion in lymphoid tissues, with the presence of PCV nucleic acid in macrophage/monocyte lineage and antigen-presenting cells, is regularly found in the disease (Segalés and others 1997b, Allan and others 1998, Ellis and others 1998). Deficiency in the immune response may increase the occurrence of other infections, or even modify the clinical presentation of diseases. A relationship of concurrent viral or bacterial infections with PMWS has not yet been reported. This short communication describes two cases of concurrent PCV and Aujeszky's disease virus (ADV) infection, in two two-and-a-half-month-old pigs from a 340-pig fattening farm in Osona (Barcelona, Spain), one of which was also infected with porcine respiratory and reproductive syndrome virus (PRRSV). An unusual localised pattern of ADV antigen distribution was observed in tissues of these pigs.

TABLE 1: PCV nucleic acid distribution in tissues of pigs affected by PMWS

Tissue	PCV nucleic acid presence Pig 1	Pig 2	Pig 3
Lung	+++	–	+
Liver	+	–	–
Spleen	++	–	+
Intestine	–	–	++
Peyer's patches	+++	+	++
Tonsil	+++	+	++
Inguinal superficial LN	+++	+	++
Mesenteric LN	+++	++	+++
Mediastinal LN	+++	+	++
Submandibular LN	+++	+	++
Perirenal LN	+++	+	++
Bone marrow	++	–	NT
Stomach	–	–	–
Kidney	+	–	–

LN Lymph node, - Absence, + Low, ++ Moderate, +++ High

Approximately 10 per cent of the pigs on the fattening farm showed signs of poor body condition, mild hyperthermia (40°C), and paleness of the skin. Dyspnoea, and mild diarrhoea, with occasional bloody scours, were also seen in some of the affected animals and the mortality rate of affected pigs rose to 80 per cent. The farm had only one pig source, in which sows were vaccinated against Aujeszky's disease (live vaccine using NIA-4 strain, gE-negative, with aqueous adjuvant), erysipelas and porcine parvovirosis. The routine vaccination programme of the affected farm included only one ADV vaccine (live vaccine using Bartha K-61, gE-negative, with aqueous adjuvant) at three months of age. Affected pigs in this study were still unvaccinated. Previous necropsies made by the veterinary practitioner showed gastric ulcers in the pars oesophagica and the presence of blood in the small intestine. Proliferative ileitis was also suspected. Treatments based on oxytetracycline and tyoslin did not improve the clinical picture.

A complete necropsy was performed in three pigs. Samples from the lymph nodes (mesenteric, mediastinal, superficial inguinal, submandibular, and perirenal), lung (at least four sections for each pig), liver, kidney, spleen, ileum, pancreas, adrenal gland, tonsil, bone marrow and stomach, were fixed in 10 per cent phosphate-buffered formalin. Routine histopathological procedures were carried out. An immunohistochemical technique, based on previously described protocols (Halbur and others 1994, Segalés and others 1997a), was used to detect ADV and PRRSV antigen in tissues. An in situ hybridisation technique to detect PCV nucleic acid was also done (Segalés and others 1997b). Additional studies included: serology against PRRSV, ADV, *Mycoplasma hyopneumoniae* and swine influenza virus (SIV), haematology (complete haemogram, leucocyte differential count, and serum protein concentration of pigs 2 and 3), and hepatic enzyme profile.

Macroscopic findings consisted of a generalised lymphadenopathy, non-collapsed, grey-mottled lung, and chronic gastric ulceration of the pars oesophagica in the three examined pigs. Pigs 1 and 2 had an enlarged spleen and gallbladder wall oedema, pigs 1 and 3 had cranioventral pulmonary consolidation and pig 1 also had hepatomegaly.

Histopathological findings were characterised by a variable degree of lymphocyte depletion together with histiocytic inflammatory infiltration of lymphoid tissues (including lymph nodes, tonsils and Peyer's patches) (Fig 1), and acute to subacute interstitial pneumonia. Pigs 1 and 3 had lymphoplasmocytic periportal hepatitis, and pigs 1 and 2 had mild centrilobular hepatic necrosis. Pig 2 had slight multifocal interstitial nephritis. Multifocal necrotising lymphadeni-

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