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Caprine arthritis encephalitis: an example of risk assessment for embryo trading

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Abstract. The risk of transmission of caprine arthritis encephalitis virus (CAEV) during embryo transfer has been demonstrated *in vivo* through the detection of CAEV proviral DNA in: (1) flushing media for embryo collection; (2) cells of the cumulus oophorus surrounding the oocytes, ovarian follicle, oviduct and uterine tissues; and (3) testis, epididymis, vas deferens and vesicular glands. Experimentally infected embryos without a zona pellucida (ZP), washed 10 times with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution, were capable of transmitting CAEV. *In vitro* we demonstrated that granulosa, oviductal, epididymal and embryo cells are fully susceptible to CAEV infection and allow active replication. However, AI with *in vitro*-infected semen can result in the production, after ten washing, of CAEV-free embryos, and ten washing *in vitro*- or *in vivo*-infected embryos with an intact ZP, or ten washing oocytes with an intact ZP, resulted in the production of virus-free female gametes or embryos that can be used for IVF or embryo transfer. Therefore, we have demonstrated that: (1) that CAEV-free embryos can be produced by IVF using spermatozoa infected *in vitro* by CAEV; and (2) embryo transfer can be used under field conditions to produce CAEV-free kids from CAEV-infected biological mothers.

Additional keywords: AI, caprine arthritis encephalitis virus (CAEV), embryo transfer, IVF, oocytes, spermatozoa.

Introduction

Reproductive biotechnologies are essential to improve the gene pool in small ruminants. Although embryo transfer (ET) and AI greatly reduce the risk of pathogen transmission, few studies have been performed to quantify this risk.

Pathogenic agents can be transmitted by crossing the zona pellucida (ZP) or via infected particles adhering to the surface of that same ZP (Wrathall and Sutmöller 1998; Stringfellow 2011). Pathogens can also adhere to the surface of the spermatozoon or to non-spermatic cells in the seminal plasma (Quayle *et al.* 1997; Pudney *et al.* 1999). Successful transmission via ET or AI requires a minimum infectious dose of pathogenic agents and the presence of specific receptors in the cellular membrane of the gametes or embryos (Van Soom *et al.* 2011).

The protocols validated by the International Embryo Technology Society (IETS; Stringfellow 2011) and the OIE (Office International des Epizooties/Word Organisation for Animal Health) (OIE 2016) for bovine embryos are currently accepted and used for international exchanges of reproductive material. These protocols are designed to ensure the integrity of the ZP and the absence of any adherent epithelial cells. They involve washing the embryos with simple or associated media, rather than using trypsin and antibiotics. The efficacy of the IETS and OIE recommendations in reducing the risk of pathogen transmission via ET and AI requires verification for each pathogenic agent in each species individually (Singh 1987; Philpott 1993).

Continued improvement in the quantity and quality of embryos obtained by assisted reproductive techniques is largely reliant on ET for the exchange of genetic material between different farms, regions and countries. Embryos with an intact ZP appear to be resistant to bacterial and viral infection, and the risk of disease transmission with such embryos is minimal or non-existent (Apelo and Kanagawa 1989). The development of this technique, and the resulting dissemination of genetic material, raises the issue of introducing disease (Atwell 1987). The appearance of certain infectious diseases in countries importing embryos of high genetic value has prompted the implementation of strict sanitary measures by those countries, including certification from exporting countries that the donors are free from specific pathogens. The current lack of understanding regarding the mechanism of transmission of pathogenic agents during ET has only led to the enforcement of further restrictions by importing countries amid unfounded speculation.

As a model of risk assessment, we present research using the caprine arthritis encephalitis virus (CAEV) in goats. We describe the risk factors, safety factors and consequences for AI and ET techniques for the production of non-infected embryos using infected male or female donors.

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Caprine arthritis encephalitis

Caprine arthritis encephalitis is a very common disease in dairy goats. In adult does, after a incubation period (1–3 years), CAEV infection causes chronic arthritis, interstitial pneumonia and indurative mastitis with decreased milk production (Cheevers *et al.* 1988; de Sousa *et al.* 2014). Less frequently, CAEV causes leucoencephlomyelitis in young goats (Cork *et al.* 1974). CAEV is an enveloped, single-stranded RNA virus. It has been classified as a Lentivirus, which is a subfamily of Retroviridae (Dawson 1988). This group of cell-associated viruses is characterised by the presence of an RNA-dependent DNA polymerase, and CAEV is only functional once the viral RNA has been transformed into viral DNA and has been integrated into the host cell DNA. Thus, the viral genome becomes a part of the host's cellular DNA and replicates efficiently in non-dividing, terminally differentiated cells (Narayan *et al.* 1983). The virus has a tropism for monocytes/macrophages and causes chronic inflammatory disease (Narayan *et al.* 1993).

CAEV infection has been detected worldwide, but is most prevalent in countries with intensive dairy farming (Rowe and East 1997; Tabet *et al.* 2015). Approximately 30–80% of dairy goats in the US, Canada and Europe are infected, compared with 0–10% in Africa and South America (Crawford and Adams 1981; Adams *et al.* 1984; East *et al.* 1987; Phelps and Smith 1993; Rowe and East 1997). In dairy herds, mammary tropism has economic consequences on milk production, mammary pathology (Smith and Cutlip 1988; Greenwood 1995; Martínez-Navalón *et al.* 2013), early culling and loss of export potential (Peretz and Cimarosti 1990).

It has not been clearly demonstrated whether CAEV is transmitted between animals as a free virus or via infected cells (macrophages or epithelial cells; Blacklaws 2012), but the major route of transmission is the ingestion of virus-laden colostrum or milk from infected does (MacKenzie *et al.* 1987; East *et al.* 1993; Lerondelle *et al.* 1995). Aerosol and natural mating is also responsible for viral transmission between adults. Prevention of CAEV transmission is based on the removal of kids from their mothers at birth and feeding with heat-treated goat colostrum (Adams *et al.* 1983; Péretz *et al.* 1994). However, large herds using this pasteurisation policy have still reported an incidence of up to 10% unexplained seroconversion (Rowe *et al.* 1992; East *et al.* 1993; Leitner *et al.* 2010). Vertical transmission, *in utero*, has also been demonstrated, but the exact mechanism of this transmission has yet to be elucidated. Cases of seroconversion to CAEV have been observed in kids born to infected mothers by Caesarean section or natural delivery, and that had not consumed any colostrum from their biological mother (Adams *et al.* 1983), as well as in kids fed with pasteurised milk or milk substitute inside a control program (Rowe and East 1997).

Risk factors

Pathogenicity studies have shown that monocytes are the major target cells in CAEV infection. Only a small population of monocytes is infected. In those cells, CAEV remains latent in the form of proviral DNA in the monocytes with no infectious particles being produced; the differentiation of these cells into macrophages is a prerequisite for viral replication (Narayan and Cork 1995). The full replication cycle of CAEV continues in the differentiated macrophages, within specific target tissues (Narayan *et al.* 1983; Zanoni *et al.* 1990; Gorrell *et al.* 1992; Clements and Zink 1996). Various factors affect the expression of viruses responsible for latent infections: stress, immunodepression or a significant physiological modification (gestation) can cause viral reactivation, its expression and the onset of lesions over subsequent weeks. Tissues in which it is usual to find CAEV-infected cells are areas of inflammation in the brain, spinal cord, lung, synovium and mammary gland. However, viral transcripts have also been detected in epithelial cells from several organs of infected goats, including small intestine, thyroid and kidneys (Zink *et al.* 1990). It has also been demonstrated that epithelial cells in goat milk are susceptible to CAEV infection both *in vitro* and *in vivo* (Mselli-Lakhal *et al.* 1999).

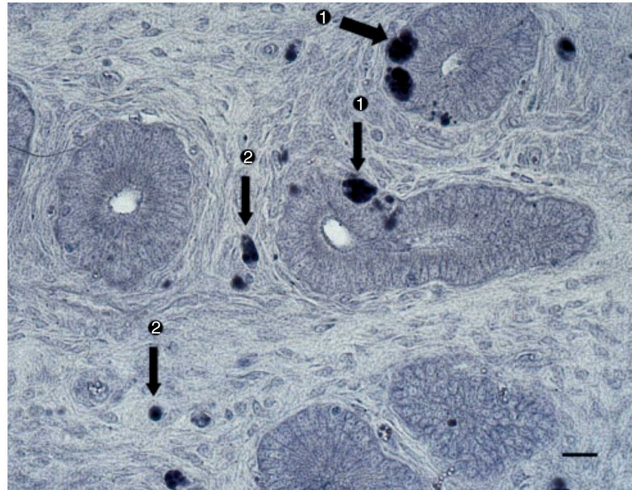

Fig. 1. *In situ* hybridisation was used to detect the presence of caprine arthritis encephalitis virus (CAEV) nucleic acids in uterine samples of naturally infected goats (Ali Al Ahmad *et al.* 2012*a*). The probe complementary to viral strand RNA was generated from the pBSCA plasmid (plasmid binding site CAEV). carrying the complete CAEV genome by polymerase chain reaction (PCR) amplification of the gag region using primers GAG EX 5′ and GAG EX 3′ (Life Technologies). CAEV RNA positive staining (brownish-purple intracytoplasmic inclusions) could be identified (arrows) in the uterine glands, ①, and in the lamina propria of the mucosa, ②, of uterine sections. Scale bar = 10 µm. Images reproduced with permission from Ali Al Ahmad *et al.* (2012*a*).

Given this viral tropism, the first step of the present risk assessment study was to detect CAEV in the tissues of the reproductive tract and/or the embryo-harvesting liquid.

Infection of the genital tract

Females

Studies using nested polymerase chain reaction (PCR) have reported the presence of CAEV proviral DNA in ovarian, oviduct and uterine tissue from naturally infected goats after superovulation (Fieni *et al.* 2003; Ali Al Ahmad *et al.* 2005). It is important to emphasise that CAEV infection of the genital tract is not correlated with the detection of antibodies or CAEV proviral DNA in peripheral blood mononuclear cells (PBMC).

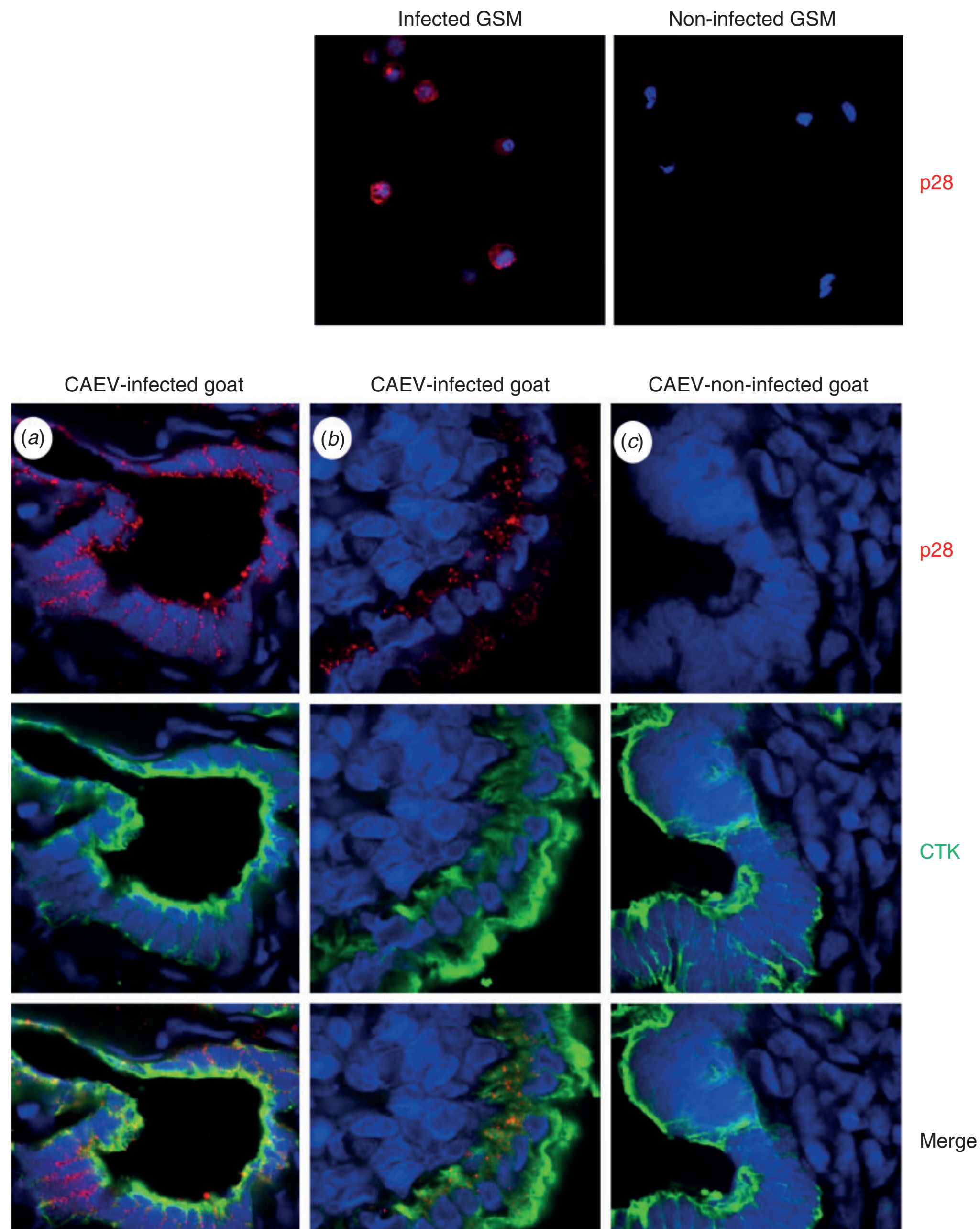

Fig. 2. Immunofluorescence results after laser scanning confocal analysis of p28 single-labelled (red) caprine arthritis encephalitis virus (CAEV) on CAEV-infected and non-infected goat synovial membrane (GSM) cells (top panels) and the same uterine sections of CAEV infected and non-infected goats double-labelled for p28 (red) and cytokeratin (CTK; green; bottom panels). (*a*) Uterine gland from a CAEV-infected goat; (*b*) external epithelium of a uterus from a CAEV-infected goat and (*c*) external epithelium of a uterus from a non-infected goat. Images reproduced with permission from Ali Al Ahmad *et al.* (2012*a*).

In vitro cell culture demonstrated that granulosa cells derived from goat ovarian and epithelial oviductal cells are fully susceptible to CAEV infection *in vitro* (Lamara *et al.* 2001; Lamara *et al.* 2002*a*). Those studies were completed using an *in situ* hybridisation probe, which was complementary to part of the CAEV group-specific antigen (*gag*) gene and confirmed the presence of CAEV nucleic acids in uterine samples from naturally infected goats (Fig. 1). The positively staining cells were concentrated in the mucosa of the lamina propria of uterine sections. Finally, laser confocal analysis of p28/cytokeratin double-immunolabelled transverse sections of CAEV-infected goat uterus demonstrated that the virus was localised in glandular and epithelial cells (Fig. 2; Ali Al Ahmad *et al.* 2012*a*).

The presence of CAEV-infected epithelial cells in genital tract tissues could have a major effect on the epidemiology of the disease. Infected epithelial cells could maintain latent infection with no accompanying inflammatory reaction; therefore, the embryo or fetus could come into contact with CAEV during pregnancy and be infected at different stages of development. This would explain the presence of amplifiable CAEV sequences in flushing media from superovulated does (Fieni *et al.* 2002; Ali Al Ahmad *et al.* 2008*a*) and in the post partum secretions of breeding goats (Rowe *et al.* 1999). The presence of the CAEV genome in uterine epithelial cells raises concerns regarding the risk of CAEV transmission via embryo transfer. After IVF, early stage embryos are cultured on a feeder layer of epithelial cells from goat oviducts (Prichard *et al.* 1992; Crozet *et al.* 1995; Martino *et al.* 1995), which are essential for early development before transfer to recipient does (Crozet *et al.* 1995; Galli and Moor 1991). These cells are derived from organs obtained at the slaughterhouse from goats of unknown CAEV status, and in many industrialised countries the incidence of CAEV infection may be as high as 60–80%.

Males

We searched for CAEV proviral DNA and viral RNA in the genital tract tissues of nine naturally infected bucks (testis, epididymis, vas deferens and vesicular gland; Ali Al Ahmad *et al.* 2008*b*). CAEV proviral-DNA was identified by PCR in the blood cells of seven of these nine bucks, in the testis in two bucks, in the epididymis in four bucks, in the vas deferens in two bucks and in the vesicular gland in two bucks. *In situ* hybridisation with a probe complementary to the viral RNA strand confirmed the presence of viral mRNA in the periphery of the epididymal epithelium (Fig. 3; Ali Al Ahmad *et al.* 2008*b*). That study clearly demonstrates the presence of viral RNA and proviral DNA in the genital tract tissues of naturally infected male goats. These results have been confirmed by others (Peterson *et al.* 2008; Turchetti *et al.* 2013) and explain the origin of CAEV proviral DNA observed in the semen of experimentally (Travassos *et al.* 1998) or naturally infected (Cruz *et al.* 2009) male goats.

CAEV has been detected in the epithelial cells that line the lumen of the epididymis from naturally infected bucks. To determine whether epididymal epithelial cells (EEC) can harbor and replicate the virus, EEC cells were isolated from CAEV-free bucks and cultured with molecularly cloned CAEV-pBSCA (plasmid binding site CAEV). Inoculated cells developed cytopathic effects (CPE), showing numerous multinucleated giant cells (MGC) in cell culture monolayers (Fig. 4) (Lamara *et al.* 2013).

CAEV proteins were detected by immunofluorescence using an anti-p28 gag-specific antibody (Fig. 5) (Lamara *et al.* 2013). Culture media of inoculated cells were shown to contain high titres ($\sim 10^6$ tissue culture infectious doses 50 per mL ($TCID_{50}$ mL^{-1})) of infectious cytopathic virus when assayed onto indicator goat synovial membrane (GSM) cells. These data demonstrate that EECs are susceptible to CAEV infection *in vitro* and able to productively replicate CAEV (Lamara *et al.* 2013).

Various male sexual organs may contribute directly to the shedding of proviral CAEV DNA in ejaculated semen. There is a suggestion of seasonality; a possible explanation for this phenomenon is an increase in sexual activity and stress coinciding with the optimal breeding period of these seasonal breeders. Increased stress is known to (re)activate viral loads and subsequent viral excretion (Cortez-Romero *et al.* 2013).

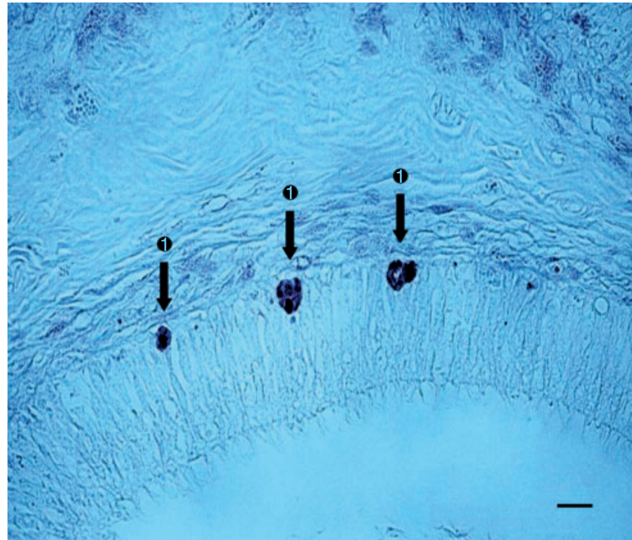

Fig. 3. *In situ* hybridisation was used to detect the presence of caprine arthritis encephalitis virus (CAEV) nucleic acids in samples of the head of the epididymis, counterstained with haematoxylin, from a naturally infected buck (Ali Al Ahmad *et al.* 2008*b*). The probe complementary to viral strand RNA was generated from the pBSCA (plasmid carrying the complete CAEV genome) by polymerase chain reaction amplification of the gag region using primers GAG EX 5′ and GAG EX 3′ (Life Technologies). CAEV RNA positive staining (brownish-purple intracytoplasmic inclusions) could be identified (arrows, ①) in the periphery of the epididymal epithelium resting on the basal membrane. The differentiated pseudostratified cylindrical cells and loose connective tissue tunic were not positive for CAEV RNA. Scale bar = 10 μm. Images reproduced with permission from Ali Al Ahmad *et al.* (2012*a*).

Presence of CAEV-infected cells in oviduct flushing media

The second step of risk assessment for ET is to look for the pathogens in the flushing media used for embryo collection. Flushing media examination provides an indication of the presence of infectious agents in the oviduct or uterine lumen of the donor.

CAEV proviral DNA (Fieni *et al.* 2002) and viral RNA (Ali-Al-Ahmad *et al.* 2008*a*) have been detected using double-nested PCR (Fig. 6) and reverse transcription–polymerase chain reaction (RT-PCR) respectively in oviduct and uterine horn washing media during embryo collection. In the first experiment (Fieni *et al.* 2002), CAEV proviral DNA was identified by double-nested PCR in 11 flushing media following the collection of oviductal-stage embryos from 20 blood CAEV-positive goats identified by PCR. Note that the mean number of embryos recovered was not significantly different between goats with PCR-positive or -negative flushing media, nor between goats with PCR-positive or -negative blood. This means that the presence of infected cells in the genital tract did not significantly affect embryo production (Fieni *et al.* 2002).

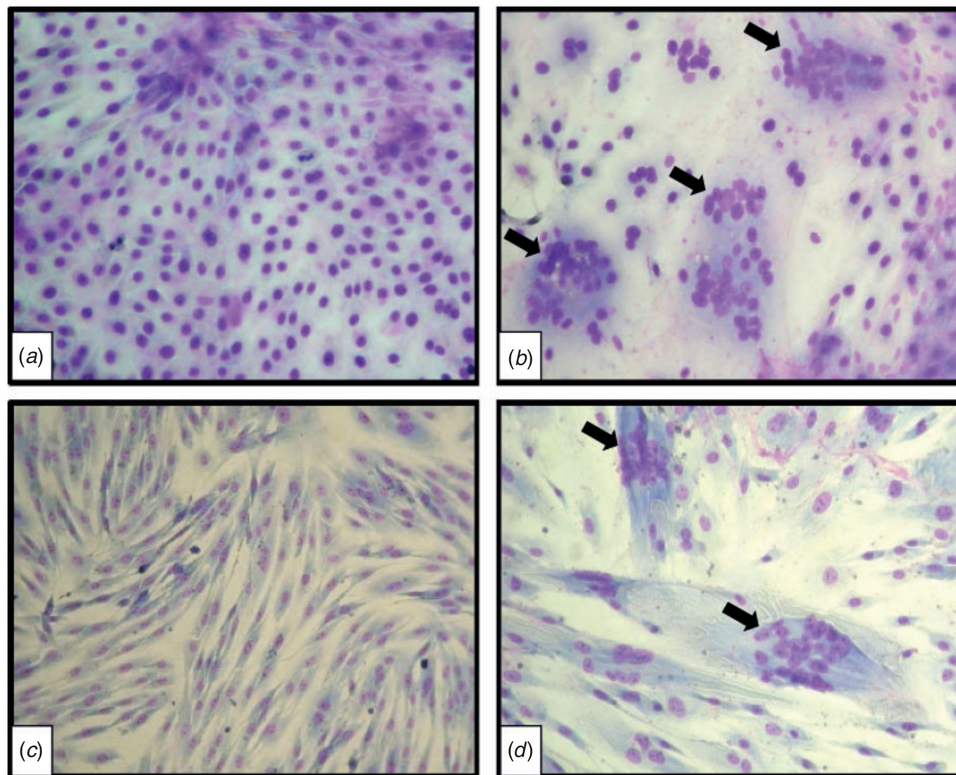

Fig. 4. Cytopathic effects of caprine arthritis encephalitis virus (CAEV) in goat epididymal epithelial cells (EEC) and goat synovial membrane (GSM) cells. To examine whether EECs are susceptible to CAEV infection, primary cultures of EECs were derived from the caput, corpus and cauda. The cell monolayers were then inoculated with CAEV-pBSCA (plasmid carrying the complete CAEV genome) at a multiplicity of infection (MOI) of 0.1 (Lamara *et al.* 2013). Monolayers of cultured goat (*a*, *b*) EEC and (*c*, *d*) GSM cells were kept uninfected (*a*, *c*) or infected with CAEV-CO at an MOI of 0.1 (*b*, *d*). At Day 6 after infection, cells were stained with May–Grünwald–Giemsa and then observed under a photonic microscope. Multinucleated giant cells are visible (arrows) in both EEC and GSM infected monolayers (*b*, *d*). (Original magnification ×200.). Images reproduced with permission from Lamara *et al.* (2013).

Embryonic cells

CAEV attachment to embryonic cells

Two studies demonstrated that CAEV attach strongly to early embryonic cells taken from goat embryos produced *in vivo* (8- to 16-cell stage; Lamara *et al.* 2002*b*; Ali-Al-Ahmad *et al.* 2006).

To determine whether goat early embryonic cells interact with CAEV, 128 8- to 16-cell *in vivo*-derived embryos whose zona pellucida (ZP) had been removed in a 1% pronase bath were studied by Lamara *et al.* (2002*b*). Seventy-eight of these ZP-free embryos were incubated for 2 h with 10^4 $TCID_{50}$ mL^{-1} CAEV-pBSCA. The remaining 50 embryos were used as controls and were incubated without CAEV. At the end of the incubation period, the embryos were washed five times with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution; each wash was at a dilution of at least 100-fold of the previous one and the pipette was changed following each wash.

The embryos were then cocultured for 72 h with a mixed monolayer containing 80% caprine oviduct epithelial cells (COEC; to support *in vitro* early embryonic development) and 20% goat synovial membrane (GSM; to indicate the presence of any virus released from the embryos). After 6 days culture, MGCs were observed in these monolayers, indicating that CAEV was transmitted from the inoculated ZP-free embryos to the indicator GSM cells. In contrast, no MGCs were observed in cell monolayers cultured with the control embryos. The embryo washing fluids were then used to inoculate monolayers of GSM cell cultures. After 6 days, the presence of infectious virus was only observed in the first two washes (Lamara *et al.* 2002*b*). In that study, the 8- to 16-cell stage was selected because it coincides with the activation of the embryonic genome (Kelk *et al.* 1994) and, consequently, with the onset of protein synthesis, which may be necessary for virus entry and replication. Early protein synthesis has been detected at the 8- to 16-cell stages in several species, including cattle (Barnes and First 1991), mice, rabbits and sheep (Crosby *et al.* 1988).

Together, these results indicate that CAEV strongly adheres to early embryonic cells and that ZP-free goat embryos are able to transmit the virus.

CAEV infection of embryonic cells

To examine the susceptibility of early goat embryo cells to caprine CAEV infection and replication *in vitro*, 41 ZP-free

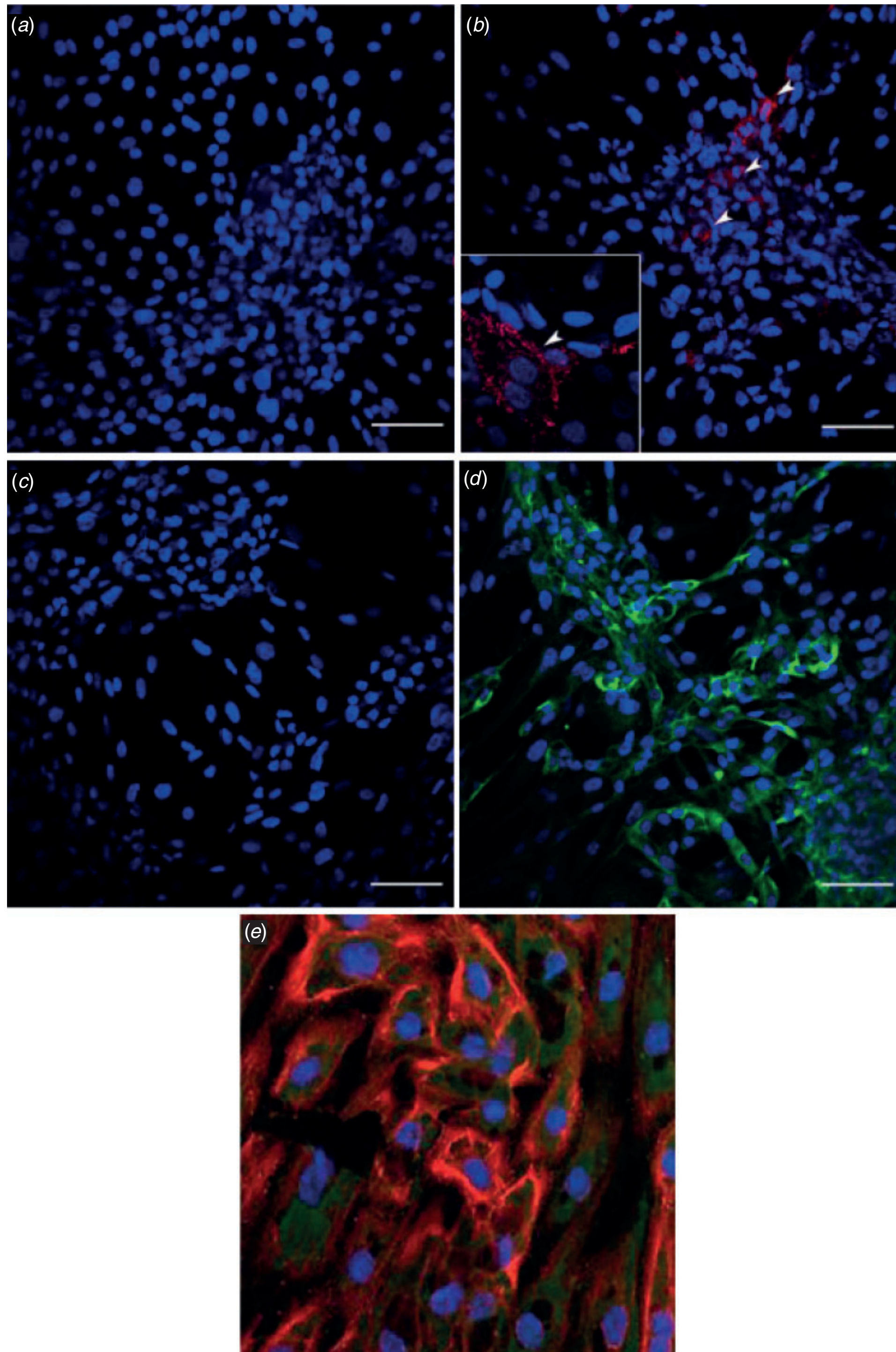

Fig. 5. Immunostaining for caprine arthritis encephalitis virus (CAEV) antigen and the endogenous cytokeratin epithelial marker in epididymal epithelial cells (EEC). Monolayers of EEC cultures were infected with CAEV at a multiplicity of infection (MOI) of 0.1, transferred to cell culture slide chambers and fixed and incubated first with mouse monoclonal antibodies directed against cytokeratin and to CAEV p28 and second with a secondary donkey-anti-mouse IgG antibody Alexa Fluor 488 or donkey-anti-mouse IgG antibody Alexa Fluor 555 for fluorescent staining of epithelial cells and CAEV respectively (Lamara *et al.* 2013). (*a*, *c*) Cells stained with TO-PRO-3 iodide (Fisher Scientific) only. (*b*) Cells stained with anti-CAEV Gag p28 monoclonal antibody (mAb) before being counterstained with TO-PRO-3 iodide. (*d*) Cells stained with anti-cytokeratin mAb before being counterstained with TO-PRO-3 iodide. (*e*) Cells were double labelled with anti-CAEV Gag-p28 and anti-cytokeratin mAbs and then counterstained with TO-PRO-3 iodide. Scale bar = 10 μm Images reproduced with permission from Lamara *et al.* (2013).

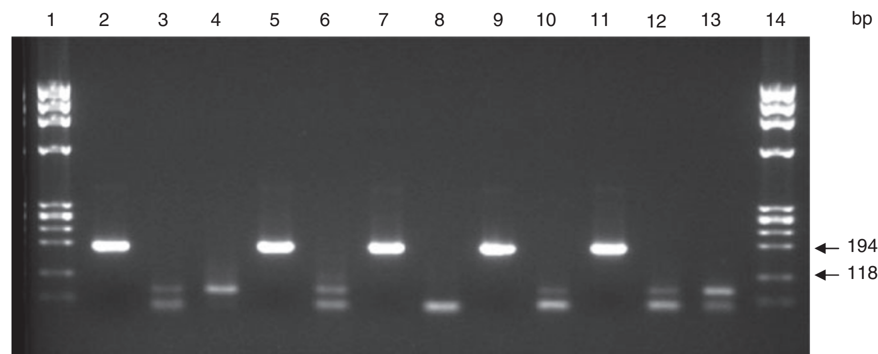

Fig. 6. Representative double-nested polymerase chain reaction products from blood, flushing media and controls. Amplification of caprine arthritis encephalitis virus (CAEV) proviral DNA using the gag primer resulted in a predicted 184-bp band between the 194- and 118-bp bands of the molecular weight standards for agarose gel electrophoresis. Lanes 1 and 14, ϕX174RFDNA *Hae*III digests used as molecular weight standards; Lanes 2 and 5, positive blood samples; Lanes 3 and 4, negative blood samples; Lanes 6, 8 and 10, negative flushing media samples; Lanes 7 and 9, positive flushing media samples; Lane 11, positive control; Lane 12, negative control; Lane 13, distilled water (Fieni *et al.* 2002).

Table 1. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of culture medium of cocultures and zona pellucida-free embryo washing fluids

Culture media of early goat embryos cocultured with non-infected or caprine arthritis encephalitis virus (CAEV)-infected caprine oviduct epithelial cells (COEC) or goat synovial membrane (GSM) cell monolayers were harvested at Day 6 after coculture and examined using RT-PCR. In parallel, harvested embryos were passed through 10 successive baths and all washing fluids were examined individually by RT-PCR (Ali Al Ahmad *et al.* 2006). Data show the number of CAEV-positive samples/total samples tested

	Cocultured medium	Washing fluid				
		First	Second	Third	Fourth	Fifth–tenth
Infected embryo	6/6	6/6	6/6	6/6	3/6	0/6
Non-infected embryo	0/6	0/6	0/6	0/6	0/6	0/6

blastocysts were cultured for 6 days in tissue culture inserts over a mixed monolayer cell culture producing 10^5 $TCID_{50}$ mL^{-1} CAEV-pBSCA (Ali Al Ahmad *et al.* 2006). These embryos were washed 10 times with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution and the washing media recovered for RT-PCR analysis. Twenty-eight of the 41 infected blastocysts were transferred for coculture in an insert, whereas the remaining 13 were directly cocultured in contact with GSM cell monolayers for 6 h. The specific CPE of CAEV in the GSM culture cells were evaluated by phase microscopy each week for 5 weeks. The 28 infected blastocysts cultured in the insert (no contact with the GSM cell monolayers) were washed 10 times with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution and cultured for 24 h in cell-free medium. The production of CAEV was determined by titration of the culture medium.

Embryos were then harvested and treated with trypsin to dissociate the cells that were cultured as monolayers over 4–8 days to look for CAEV proviral DNA by PCR, viral RNA by RT-PCR and p28 gag protein by immunocytochemistry.

Viral RNA was detected by RT-PCR from the first four wash baths of ZP-free blastocysts (Table 1; Ali Al Ahmad *et al.* 2006). After 6 h coculture in an insert (28 blastocysts) or in direct contact (13 blastocysts) with target GSM cells, CAEV RNA genome was detected by RT-PCR in the medium and CAEV-specific CPEs were observed at the end of the 4th week of cell culture. After washing and 24 h culture in cell-free medium, CAEV production by infected blastocysts ranged from $10^{3.25}$ to $10^{4.5}$ $TCID_{50}$ mL^{-1}. Finally, in the blastocyst monolayer culture, we identified viral RNA, proviral DNA and the expression of p28 gag protein (Table 2; Ali Al Ahmad *et al.* 2006).

These data clearly demonstrate that caprine early embryonic cells are susceptible to infection with CAEV, and that infection with this virus is productive (Ali Al Ahmad *et al.* 2006).

The presence of the CAEV genome in the female and male genital tract, the identification of active viral replication in the epithelial cells or macrophages of such tissues, as well as in the embryonic cells, and the detection of infected cells in the oviduct or uterine flushing media for embryo recovery present a risk of early infection of the embryo or gametes and propagation of lentivirus infection via ET or AI.

Safety concerns

ZP

The embryo is wrapped in a protective layer (ZP) until the blastocyst stage. The ZP is composed of three different glycoproteins, which create a mechanical barrier against viruses and

Table 2. Analysis of caprine arthritis encephalitis virus (CAEV) productive replication in cultured early goat embryo cell monolayers
Infected and non-infected embryos were harvested and treated with trypsin to dissociate cells that were cultured as monolayers over 4–8 days. After trypsin treatment, some cells was used for DNA and RNA isolation to detect provirus using polymerase chain reaction and viral RNA by reverse transcription–polymerase chain reaction, whereas other cells were used for immunocytochemistry to detect the expression of the major viral p28 gag protein. Positive and negative results for the six repetitions of the experimental procedure are reported in the table (Ali Al Ahmad *et al.* 2006)

	Infected embryo cells	Non-infected embryo cells
CAEV proviral DNA	6/6	0/6
CAEV ARN	6/6	0/6
p28 protein	6/6	0/6

bacteria; the porosity of the matrix is such that only the smallest of molecules can infiltrate the embryo from the external medium. However, viruses may bind to the ZP and could penetrate the outermost pores (Bolin *et al.* 1983). Pathogens may adhere or even bind to the ZP (because the ZP may express structural receptors), but because the ZP is an acellular glycoprotein coat, the virus cannot replicate in it (Van Soom *et al.* 2011).

To investigate the role of the ZP in the presence of CAEV, 99 ZP-intact 8- to 16-cell *in vivo*-derived embryos were used by Lamara *et al.* (2002*b*). Fifty-nine ZP-intact embryos were incubated for 2 h with 10^4 $TCID_{50}$ mL^{-1} CAEV-pBSCA, whereas 40 ZP-intact embryos were used as controls and incubated without CAEV. At the end of the incubation period, the ZP-intact embryos were washed five times with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution, each wash was at a dilution of at least 100-fold of the previous one, and the pipette was changed after each wash. Embryo and wash baths were tested for the presence of CAEV by coculture or culture with GSM cell indicators. No MGCs were observed in target GSM cell monolayers cocultured with the ZP-intact embryos. However, as for ZP-free embryos, the presence of infectious cytopathic CAEV was observed in the first and second washing fluids, but not in the latter three (Lamara *et al.* 2002*b*). These results clearly demonstrate the efficacy of the first two washes in removing free CAEV from ZP-free embryos. The ZP acts as a barrier to the virus *in vitro*, protecting embryo cells from infection with CAEV. Inoculated ZP-intact embryos that have been washed twice do not trap and transmit CAEV.

Embryo washing

The efficacy of washing ZP-intact embryos has been confirmed after *in vitro* (Lamara *et al.* 2002*b*) and *in vivo* (Ali Al Ahamad *et al.* 2008*a*) infection by CAEV. Following the *in vitro* infection of ZP-intact embryo with a high viral concentration ($\pm 10^4$ $TCID_{50}$ mL^{-1}), the IETS washing procedure (i.e. flushing 10 times in 100-fold dilutions of the previous one of medium) rapidly eliminates the virus. The disappearance of virus was observed from the third (Lamara *et al.* 2002*b*) or fifth (Ali Al Ahamad *et al.* 2008*a*) wash batch.

Gametes

The use of artificial reproductive techniques, such as ET or IVF followed by transfer into CAEV-free females can eliminate the risk of *in utero* contamination. However, for these techniques to effectively prevent the transmission of infection, the initial cellular material (i.e. the oocyte and the spermatozoa) must be free from contamination.

Oocytes

There have been few studies evaluating the risk of vertical CAEV transmission from mother to fetus during the oocyte phase. However, caprine lentivirus infects and replicates in granulosa cells *in vitro* (Lamara *et al.* 2001) and CAEV has been detected in granulosa cells recovered from naturally infected goats (Ali Al Ahmad *et al.* 2005).

Therefore, there is a risk of viral transmission during *in vitro* production (IVP) of embryos, because granulosa cells are commonly used for oocyte development and maturation (Staigmiller and Moor 1984; Pugh *et al.* 1991); if such cells are removed from the oocytes before maturation, the developmental capacity of oocytes is reduced (Crosby *et al.* 1981).

Therefore, CAEV has the opportunity to be in direct contact with oocytes with an intact cumulus oophorus (CO) during the oogenesis or *in vivo* maturation phase after ovulation. Thus, the sanitary safety of reproductive biotechnologies is conditioned by the quality of the oocyte–CO complex (COC).

In a study to determine whether the presence of CO cells in oocytes taken from goats naturally infected with CAEV affects the presence of proviral DNA and hence its detection using PCR, 246 ovaries were recovered from 123 goats originating from breeding herds in the south-west of France (i.e. potentially naturally infected; Ali Al Ahmad *et al.* 2005). COC were harvested and washed 10 times. The oocytes from the first 190 ovaries were divided into two batches per ovary. The first batch consisted of oocytes with their cumulus cells, whereas cumulus cells were removed from the oocytes in the second batch by incubation with hyaluronidase and then washing 10 times. The purity of the oocyte fraction was verified by searching for granulosa cell-specific mRNA using RT-PCR. In the last 56 ovaries, the cumulus cells were removed from each individual batch of oocytes and the latter grouped into one batch after 10 washes. The purity of the oocyte fraction was verified by searching for granulosa cell-specific mRNA using RT-PCR.

Examination of DNA isolated from PBMC showed that 75 of 123 (61%) goats were infected with CAEV (Ali Al Ahmad *et al.* 2005). The provirus was also identified in 116 of 246 (47.1%) of ovaries sampled. PCR analysis for CAEV proviral DNA was positive in 42 of 190 of the batches of oocytes with cumulus cells taken from the first 190 ovaries of the study, and in 22 of 56 batches of cumulus cells that had been removed by enzymatic washing from the oocytes taken from the last 56 ovaries, giving a total of 64 of 246 positive samples (26.0%; Ali Al Ahmad *et al.* 2005). However, results were negative for all batches of oocytes whose cumulus cells had been removed by enzymatic washing (Table 3).

The Ali Al Ahmad *et al.* (2005) study reports findings that support the hypothesis that oocytes in CAEV-infected goats are

Table 3. Results of double-nested polymerase chain reaction (PCR) testing for caprine arthritis encephalitis virus (CAEV) proviral DNA performed on batches of oocytes with cumulus cells (from the first 190 ovaries), on batches of cumulus cells (from the last 56 ovaries) and on batches of oocytes without cumulus cells (from all 246 ovaries sampled)

Cumulus–oocyte complexes (COC) were recovered from naturally infected goats. Cumulus cells were removed from some of these COC by enzymatic (hyaluronidase) and mechanical treatment, including 10 washings with Minimum Essential Media (MEM) and 5% Fetal Calf Serum (FCS) solution. The purity of the oocyte fraction was verified by searching for granulosa cell-specific mRNA, using reverse transcription–polymerase chain reaction (Ali Al Ahmad *et al.* 2005)

	No. batches oocytes with cumulus cells	No. batches cumulus cells	No. batches oocytes without cumulus cells
PCR positive	42	22	0
PCR negative	158	34	246
Total	190	56	246

Table 4. Results of double-nested polymerase chain reaction (PCR) tests for caprine arthritis encephalitis virus (CAEV) proviral DNA and reverse transcription–polymerase chain reaction (RT-PCR) for viral RNA tests on blood and different semen fractions recovered by electroejaculation from nine naturally infected bucks

PCR-negative sperm fractions were recovered after two centrifugations, the second being Ficoll density gradient centrifugation (Ali Al Ahmad *et al.* 2008*b*). Within columns, values with different superscript letters differ significantly ($P < 0.01$)

	Semen								
	Spermatozoa (PCR)			Non-spermatic cells (PCR)			Seminal plasma (RT-PCR)		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Blood									
PCR positive	0	7	7	3^a	4	7	4^a	3	7
PCR negative	0	2	2	0^b	2	2	1^b	1	2
Total	0	9	9	3	6	9	5	4	9

resistant to viral infection, whereas the cells of the surrounding cumulus, present during the ovarian development of the oocyte and at the beginning of its intraoviductal development, are infected. The most probable hypothesis for the resistance of the oocyte to lentivirus infection is the absence of oocyte membrane receptors, which are required for the internalisation of lentiviruses (Mselli-Lakhal *et al.* 2000). We also demonstrated that it was possible to effectively eliminate, using an enzymatic and mechanical process, all the granulosa cells surrounding the oocyte in the peri-ovulatory stage (Ali Al Ahmad *et al.* 2005). Consequently, during IVF, the complete elimination of cumulus cells from oocytes of CAEV-infected goats should make it possible to obtain CAEV-free female gametes (Ali Al Ahmad *et al.* 2005).

Spermatozoa

In a study to determine whether CAEV proviral DNA and viral mRNA could be detected in the semen fraction of naturally infected male goats, semen was obtained using electroejaculation from nine naturally infected bucks (Ali Al Ahmad *et al.* 2005). The semen was separated by centrifugation into three fractions: seminal plasma (SP), non-spermatic cells (NSC) and spermatozoa (SPZ). After a first centrifugation, the supernatant, corresponding to the SP, was removed. The pellet was diluted and centrifuged on a Ficoll density gradient. The NSC-rich fraction, located at the interface between the two phases, and the SPZ pellet were collected and washed twice in 50 mL of PBS.

CAEV RNA was identified by RT-PCR in the seminal plasma and CAEV proviral DNA was identified by PCR in blood cells of seven of nine bucks and in NSC of the SP of three of nine bucks. No CAEV proviral DNA was identified in the SPZ fraction (Table 4). Two of the three bucks with positive SP cells presented with at least one PCR-positive genital tract tissue. Proviral DNA was found in the head ($n = 3$ bucks), body ($n = 3$) and tail ($n = 2$) of the epididymis. The NSC fraction of the semen contains monocytes/macrophages, the main target cells of lentivirus replication *in vivo* (Narayan *et al.* 1982, 1983; Gendelman *et al.* 1985). Lentivirus can be present in the lumen of the spermatic tubes and epididymis in sufficient concentrations to be detected, without altering tissue function or the fertility of the semen. CAEV is thought to enter the semen from the circulation via infected macrophages (Brodie *et al.* 1995; Blacklaws *et al.* 2004). The Ali Al Ahmad *et al.* (2005) study clearly demonstrates the presence of viral mRNA and proviral DNA in naturally infected male goat semen.

Together, the results reported above indicate the possibility of caprine lentivirus transmission via semen during natural mating or AI, thus justifying the need for CAEV-free males, bred in special herds and tested regularly, for use as sperm donors for AI in genetic selection programs.

Table 5. Results of double-nested polymerase chain reaction (PCR) tests for caprine arthritis encephalitis virus (CAEV) proviral DNA on the embryos, uterine smears and flushing media of 28 donor goats inseminated under endoscopic control with thawed infected semen (AI+; *n* = 14), inseminated under endoscopic control with thawed non-infected semen (AI–; *n* = 6) or mated with a naturally infected buck (M+; *n* = 8)
Results are from Ali Al Ahmad *et al.* (2012*b*)

Method of fertilisation	Uterine smear		Flushing media		Embryos	
	PCR positive	PCR negative	PCR positive	PCR negative	PCR positive	PCR negative
AI–	0	6	0	6	0	6
AI+	8	6	9	5	0	14
M+	1	7	0	8	0	8

However, in the study of Ali Al Ahmad *et al.* (2005), CAEV proviral DNA was not found in the SPZ fraction. This *in vivo* resistance of SPZ to CAEV infection could be due to epididymal proteins, which protect the SPZ during their transit through the male genital tract. These proteins, by capping the SPZ, stabilise the plasma membrane and prevent a premature acrosomal reaction (Thibault and Levasseur 2001). The limited number of cell multiplications and the basal metabolism are factors that generally limit the incorporation of other retroviruses into the cellular genome and their replication. However, this property has not been confirmed for CAEV (Turelli *et al.* 1996; Mselli-Lakhal *et al.* 1998; Payne and Elder 2001). The most likely hypothesis to explain the resistance of SPZ to infection is the absence of SPZ plasma membrane receptors, which are required for the internalisation of the CAEV particle. The structure of these receptors remains to be elucidated for CAEV.

The inability of CAEV to infect goat oocytes, the protective nature of the ZP, the fact that CAEV does not adhere to the latter and the effectiveness of washes indicate that the IETS and OIE recommendations for bovine embryos, namely the use of ZP-intact embryos washed 10 times (Stringfellow 2011), could be used to produce CAEV-free embryos from CAEV-infected goats.

Consequences of risk assessment for AI or ET

AI

AI in small ruminants offers economic and genetic advantages to flocks specialised in milk or meat production. In order to become more widespread, the quantity and quality of the semen obtained must be improved, particularly in terms of storage. Semen represents a significant risk for the spread of infectious diseases due to the numerous pathogenic microorganisms that can be present in the semen and the high numbers of straws prepared from each individual ejaculate. This risk persists over time given that most microorganisms survive the freeze–thaw process.

In another study, Ali Al Ahmad *et al.* (2012*b*) investigated whether in *vitro*-infected cryopreserved caprine spermatozoa used for AI were able to transmit CAEV vertically to early embryo developmental stages.

Spermatozoa were 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^{-1}. 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 spermatozoa and eight CAEV-free does were mated with a naturally infected buck.

PCR was used to detect CAEV proviral DNA in the embryos at Day 7, in the embryo washing media and in uterine secretions of recipient does. At Day 7, all the harvested embryos, regardless of the fertilisation method or infectious status of the semen, were PCR-negative for CAEV proviral DNA. However, CAEV proviral DNA was detected in eight of 14 uterine smears, in nine of 14 flushing media taken from does inseminated with infected spermatozoa and in one of eight uterine smears taken from the does mated with infected bucks (Table 5; Ali Al Ahmad *et al.* 2012*b*).

The results of that study confirm that: (1) AI with infected semen or mating with infected bucks may result in the transmission of CAEV to the genital tract of does 7 days after insemination; and (2) regardless of the medical status of the semen or the recipient doe, it is possible to obtain CAEV-free early embryos usable for embryo transfer (Ali Al Ahmad *et al.* 2012*b*).

IVP embryos

ET can also be performed using embryos that have been produced *in vitro* using IVF oocytes. *In vitro* embryo production has become a routine technique for fundamental research, such as gene transfer or cloning, and for commercial exchanges. Oocytes may be collected at the slaughterhouse from goats of unknown CAEV status, and sperm bucks are generally selected for fertility rather than disease status. Fieni *et al.* (2012) investigated whether the use of spermatozoa that had been experimentally infected with CAEV at the capacitation stage resulted in infected embryos after IVF.

For each of the five fertilisation trials in that experiment, frozen semen was prepared for *in vitro* capacitation at a concentration of 1×10^7 spermatozoa mL^{-1} and divided into three groups. One group was used as a control, whereas the other two groups were inoculated with 100 μL mL^{-1} 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^{-1} (infected group). In all, 789 oocytes were used for IVF. For each

Table 6. Results of reverse transcription–polymerase chain reaction (RT-PCR) analyses for caprine arthritis encephalitis virus (CAEV) RNA of sperm fertilisation media, washing media (Washes 1–12) and *in vitro*-derived embryos in infected and non-infected (control and placebo) groups for the five trials

Fertilisation media of oocytes fertilised with non-infected or CAEV-infected spermatozoa were collected and examined using RT-PCR (Fieni *et al.* 2012). In parallel, harvested *in vitro*-derived embryos were passed through 12 successive baths. Embryos and all washing media were examined individually using RT-PCR. Data show the number of positive samples/total number of samples (Fieni *et al.* 2012). Within rows, values with different superscript letters differ significantly ($P < 0.05$, Chi-squared test)

Group	Fertilisation media	Embryo washing media				Embryos
		1st	2nd	3rd	4th–12th	
Infected	5/5	5/5^{a}	4/5	3/5	0/5^{b}	0/5
Non-infected (placebo and control)	0/5	0/5	0/5	0/5	0/5	0/5

of the five trials, a group of oocytes was used as a non-infected control and found to be CAEV free. The other oocytes were divided into two equal batches. Oocytes in the first batch were subjected to IVF with CAEV-infected spermatozoa (infected group), whereas the second batch was fertilised with CAEV-free spermatozoa (placebo and control groups).

After IVF, the zygotes in 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 batches of washing media from the infected group were consistently found to be positive for the CAEV genome (five of five), whereas subsequent washing media were CAEV free ($P < 0.05$; Fieni *et al.* 2012). After 7 days culture, embryos obtained using all semen groups tested negative for both the provirus and genome of CAEV (Table 6) (Fieni *et al.* 2012). Considering the methods and results of that study, and assuming a 5% risk, the probability for CAEV infection of cleaved oocytes and embryos following IVF with infected semen is less than 1/4000 and 1/2000 respectively.

These results clearly show that the first four washes were sufficient to remove viral particles from CAEV-infected fertilisation media and that CAEV-free embryos can be produced by IVF using spermatozoa infected *in vitro* with CAEV (Fieni *et al.* 2012).

ET under field conditions

In goats, few studies have been undertaken under field conditions to examine the consequences of ET on the vertical transmission of CAEV. Wolfe *et al.* (1987) did not report any cases of seroconversion in 4-month-old kids issuing from ET from seropositive donor goats that had been inseminated by seropositive bucks. Later, a Brazilian study confirmed the absence of seroconversion at 6 months of age in kids born after ET and issuing from seropositive goats with clinical disease (Cavalcante *et al.* 1998). These are the only two studies to report serological findings in kids obtained by ET. However, delayed seroconversion may be observed following natural or experimental infection, up to 8 months of age (Rimstad *et al.* 1993). Molecular biology now enables earlier and more sensitive diagnosis, as well as the detection of animals with latent infection that are seronegative despite the presence of proviral DNA in the cells of their genital apparatus (Fieni *et al.* 2003).

Ali Al Ahmad *et al.* (2008*a*) investigated whether, *in vivo* and under in field conditions, it is possible to produce CAEV-free kids with a significant number of ETs from donor goats with infected genital tracts. Thirty goats that had repeatedly tested seropositive for CAEV using ELISA and that had been confirmed as positive using PCR of leucocytes and vaginal secretions, underwent superovulation for use as embryo donors. Embryos with an intact ZP were selected and washed 10 times before being frozen and subsequently used for transfer into CAEV-free recipient goats. Nineteen of the 49 recipient goats gave birth, producing a total of 23 kids. Three blood samples were taken from each recipient goat, 10 days before, during and 10 days after parturition; these samples were tested for CAEV antibodies using ELISA and for CAEV proviral DNA using PCR. The mothers were then killed. Tissue samples were taken from the lungs, udder and retromammary and prescapular lymph nodes.

The kids were separated from their mothers at birth; seven died. At 4 months of age, 16 kids were subjected to drug-induced immunosuppression, as described by Guiguen *et al.* (1990). Blood samples were taken monthly from birth to 4 months of age; then, on Days 15, 21 and 28 after the start of immunosuppressive treatment kids were killed and tissue samples were taken from the carpal synovial membrane, lung tissue, prescapular lymph nodes, inguinal and retromammary lymph nodes and uterus.

The CAEV provirus genome was identified in DNA isolated from cells harvested from the embryo collection fluid and uterine smear samples from 28 of 30 donor goats (93.3%; Ali Al Ahmad *et al.* 2008*a*). Only good embryos from these infected donors were used for transfer. RT-PCR analysis using RNA harvested from ultracentrifuged products from the embryo washing fluids demonstrated that although CAEV RNA was detected in the first three baths, the seven subsequent baths were free of CAEV (Ali Al Ahmad *et al.* 2008*a*). However, all samples from the 19 recipient goats (blood, lochial discharge, tissues; Table 7) and 23 kids (blood, tissues; Table 8) before and after immunosuppression were negative for CAEV antibodies and/or CAEV proviral DNA.

To determine the risk of CAEV transmission through embryo transfer and using an *in vivo*/*in vivo* approach, embryos were transferred from CAEV-seropositive goats, whose blood and

Table 7. Results of reverse transcription–polymerase chain reaction (RT-PCR) analyses for caprine arthritis encephalitis virus (CAEV) RNA viral diagnostic tests performed on female recipients 10 days before, during and 10 days after parturition

These goats had been implanted with embryos that had been treated in accordance with International Embryo Transfer Society guidelines for bovine embryos (i.e. zona pellucida-intact embryos washed with 10 successive 100-fold dilutions) (Stringfellow 2011) and recovered from CAEV-seropositive goats, whose blood, uterine tissues and uterine flushing media were infected with CAEV. Data are from Ali Al Ahmad *et al.* (2008*a*)

Tissue sample	10 days before parturition	At parturition	10 days after parturition
Blood	Negative	Negative	Negative
Vaginal smear	Negative	Negative	Negative
Udder			Negative
Lung			Negative
Prescapular lymph nodes			Negative
Retromammary lymph nodes			Negative

Table 8. Results of monthly double-nested polymerase chain reaction (PCR) tests for caprine arthritis encephalitis virus (CAEV) proviral DNA in neonates during the first 4 months of life and then 15, 21 and 28 days after the start of immunosuppressive treatment

Those neonates issued from embryos that had been treated in accordance with International Embryo Transfer Society guidelines for bovine embryos (i.e. zona pellucida-intact embryos washed with 10 successive 100-fold dilutions) (Stringfellow 2011) and recovered from CAEV-seropositive donor goats, whose blood, uterine tissues and uterine flushing media were infected with CAEV. Data are from Ali Al Hamad *et al.* (2008*a*)

Neonate tissue sample	Time after birth (days)				Time (days) after starting immunosuppressive treatment (Day 0 = 4 months after birth)		
	30	60	90	120	15	21	28
Blood	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Synovial membrane							Negative
Lung							Negative
Superficial cervical lymph nodes							Negative
Pelvic lymph node							Negative
Retromammary lymph node							Negative
Uterus[A]							Negative

[A]In females.

uterine tissues were infected with CAEV, into CAEV-free recipient females (CAEV; Ali Al Ahmad *et al.* 2008*a*), in accordance with the guidelines of the International Embryo Transfer Society (Stringfellow 2011) for bovine embryos.

That study, performed under field conditions, clearly demonstrated that ET can be used to produce CAEV-free kids from CAEV-infected biological mothers. Indeed, none of the 16 kids collected from infected mothers at the embryonic stage, transferred to CAEV-free recipient goats and subjected to immunosuppressive treatment at 4 months of age was found to be positive for CAEV using any of the diagnostic methods in all target tissues analysed. Similarly, none of the 20 recipient goats seroconverted and none of the sampled tissues tested positive for CAEV proviral DNA (Ali Al Ahmad *et al.* 2008*a*).

In addition, that study (Ali Al Ahmad *et al.* 2008*a*), performed on a significant number of donors, has the originality of using females with confirmed uterine infection, and thus a maximal risk of viral transmission. The second particularity of that study is the panel of techniques used to detect viral infection in the recipient goats and kids. In the study of Wolfe *et al.* (1987), viral infection was studied by testing the recipients and kids (up to 4 months of age) for seroconversion and performing viral cultures on colostrum, placenta and tissue samples from still-born kids and those that died as neonates. Cavalcante *et al.* (1998) looked for viral infection by testing the kids for seroconversion up to 6 months of age. However, the lack of detection of seroconversion in the kids could be explained by delayed seroconversion, as reported previously (Adams *et al.* 1983; Rimstad *et al.* 1993; Knowles 1997), and false negatives may be observed in kids over 6 months of age (Rimstad *et al.* 1993). Thus, animals that are seronegative on ELISA may still be healthy carriers of the virus (Vitu *et al.* 1988).

Together, these results emphasise the importance of the IETS recommendations to use ZP-intact embryos and successive washings, with the additional restriction of the strict elimination of any non-ZP-intact embryos. Such precautions will minimise the risk of the emergence of endogenous CAEV genomes in animals produced by ET from lentivirus-positive females. This is further supported by the absence of any substantiated report, anywhere in the world, of disease transmission to an uninfected recipient following the commercial transfer of *in vivo*-derived embryos (Wrathall and Sutmöller 1998; Stringfellow and

Givens 2000), despite the high numbers of embryos that are transferred each year.

Conclusion

The studies described herein demonstrate that CAEV is present in the male and female genital track. So, the risk of horizontal or vertical transmission by ET and AI is present and sometime demonstrated. In addition, the studies clearly demonstrate that oocytes, spermatozoa and ZP-intact embryos after washing are free of CAEV while they are harvested from infected males and females.

Selection via the male line, using AI, is the most powerful route for genetic progress. Despite infection of the SP and NSC, SPZ seem to resist CAEV infection. Blacklaws *et al.* (2004) indicated that the use of semen for AI represents a minor risk. Although it is preferable to use CAEV-free bucks as semen donors, human immunodeficiency virus (HIV)-free spermatozoa have been produced from HIV-positive humans using centrifugation in a Ficoll density gradient followed by the swim-up method (Kato *et al.* 2006).

Experiments using *in vitro*/*in vitro* and *in vivo*/*in vivo* approaches have demonstrated that ET from infected donors to healthy females, performed in accordance with the IETS protocols (ZP integrity and 10 washes), can be safely used to produce CAEV-free neonates from infected CAEV donors. Therefore, this technique of artificial reproduction can play a useful role in a prophylactic health program against CAEV in the goat. In France, ET is an integral part of the genetic scheme to produce CAEV-free young bucks from CAEV-infected goats of high genetic value.

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