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Effect of using frozen–thawed bovine semen contaminated with lumpy skin disease virus on in vitro embryo production

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Summary

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.

KEYWORDS

bovine embryo, culture media, in vitro fertilization, lumpy skin disease virus

1 | INTRODUCTION

Lumpy skin disease (LSD) is considered a transboundary animal disease of importance in cattle with significant economic impact due to the implications for food security, production losses, and restrictions in trade placed on countries where the disease is present (Tuppurainen & Oura, 2012). The causative agent of LSD, LSD virus (LSDV), is a member of the genus *Capripoxvirus* and exerts its major pathogenic

effects through granulomatous reactions in the dermis and hypodermis, following an initial vasculitis and lymphangitis (Prozesky & Barnard, 1982). LSD is associated with fever, lachrymation, lymphadenopathy, nodules on the skin and internal organs, weight loss, inappetence and, in rare cases, pneumonia and death (Coetzer, 2004). While initially restricted to Africa, LSD has recently spread further afield and is now considered a direct threat to Europe and Great Britain (Beard, 2016; Tuppurainen & Oura, 2014). The precise origin of LSD outbreaks is rarely proven, but illegal movement of farm animals without proper health checks and the collapse of veterinary services due to political uncertainty have been implicated (Tuppurainen et al., 2017).

In vitro production (IVP) of bovine embryos has grown appreciably over the last two decades, but international trade in IVP embryos is relatively uncommon, reflecting, in part, the concern of veterinary regulatory authorities with regard to the potential risks of transmitting diseases via IVP embryos (da Silva Cardoso Pinto et al., 2017). The most important viruses that have been associated with a risk of transmission via bovine IVP embryos are bovine viral diarrhea virus (BVDV), bovine herpesvirus-1 (BHV-1), enzootic bovine leucosis virus and bluetongue virus (BTV) (Wrathall, Simmons, & Van Soom, 2006). Recent reports of LSDV entering the semen of infected bulls (Irons, Tuppurainen, & Venter, 2005), transmission of the virus to heifers via contaminated embryos, infection of in vivo-derived embryos (Annandale, Holm, Ebersohn, & Venter, 2014) and intrauterine transmission to a premature calf (Rouby & Aboulsoud, 2016), have all raised concerns that LSDV might also pose a risk in IVP embryo production systems.

Cattle that become infected with LSDV, either experimentally or naturally, display variable clinical signs; ranging from almost none to severe generalized LSD. It is now known that LSDV can be shed in the semen of experimentally infected bulls for up to 159 days (Irons et al., 2005), although the nature of this shedding is not well characterized. Indeed, semen shedding of virus mirrors the general clinical syndrome i.e. that the amount, quantity and duration of shedding is variable and difficult to predict (Annandale, Irons, Bagla, Osuagwuh, & Venter, 2010; Babiuk et al., 2008).

In countries free of LSD, import restrictions apply to livestock, carcasses, hides, skins and semen (OIE, 2010). Given the potentially protracted duration of LSDV shedding into semen, even in animals that show no other obvious clinical signs during a herd outbreak, the possibility of semen from apparently healthy animals being contaminated with LSDV cannot be excluded. If frozen, such semen could also enter in vitro fertilization (IVF) systems where its potential to be transmitted via the resulting embryos is currently unknown. A recent study indicated that common semen processing methods are not sufficient to wash semen free of LSDV contamination (Annandale et al., 2018). In a previous study, LSDV DNA was detected in day 6 embryos flushed from heifers inseminated with fresh semen spiked with a high infective dose of LSDV (Annandale et al., 2014). To the authors' knowledge, no other studies have been published on the effect of LSDV on embryos, in either natural or IVP systems. Developing and documenting methods of rendering semen and embryos free from LSDV holds clear trade and economic advantages, and can assist in clarifying the risks of semen-based transmission of LSDV.

The objectives of this study were to investigate the effects of two different LSDV viral loads in cryopreserved semen on fertilization rate and yield of bovine blastocysts produced via an in vitro embryo production (IVEP) system, and to determine the viral status of embryos and culture fluids used during the process.

2 | MATERIALS AND METHODS

2.1 | Study overview

The detailed descriptions follow below, but briefly: Bovine oocytes were aspirated from ovaries collected from a local abattoir and split into three experimental groups (HD, LD and control). After maturation, the oocytes were incubated with frozen-thawed semen spiked with a high (HD) or a lower (LD) dose of LSDV, or with LSDV-free semen (control) in a conventional bovine IVF system. Normal IVEP processes were followed for all three experimental groups, with evaluation of blastocyst development on days 7 and 8. After day 8 evaluation, PCR and virus isolation were performed on all remaining structures (i.e. blastocysts and oocytes/embryos that failed to develop), taking care to separate zona-pellucida intact blastocysts from those that had already hatched. Several replicates were performed, until more than 350 oocytes per group had been used for IVF. After completion of the initial series of replicates, further replicates were performed to provide material for electron microscopy (EM) and embryo washing procedures. These samples were divided over two analyses; PCR was performed on one half, and either EM or washing was performed on the other half. The study was approved by the Animal Ethics Committee of the University of Pretoria (V093/16).

2.2 | Processing of semen samples

Processed semen samples from a previous study (Annandale et al., 2018) were used. Briefly, semen was collected from a 2-year old Brahman bull, confirmed to be breeding sound and sero-negative for LSDV antibodies; the semen was confirmed negative for LSDV by PCR using validated primers (Bowden, Babiuk, Parkyn, Copps, & Boyle, 2008). The ejaculate was split into three equal volumes of 5 ml and processed as follows: the HD sample was spiked with 2.5 ml of viral suspension (LSDV Mireil isolate (Neethling strain) (V103/91) suspended in minimum essential medium (MEM) (Biowest, Celtic Diagnostics, Cape Town, South Africa) at a titre of 4 log $TCID_{50}$, the LD sample was spiked with 2.5 ml of a 10^3 dilution of the virus suspension, and 2.5 ml of Triladyl® extender (Minitube, Tiefenbach, Germany) was added to the C sample to equalize the volumes.

The three semen aliquots were processed for cryopreservation individually, making use of separate equipment to prevent cross-contamination. The control sample was processed first. Semen was frozen in 0.25 ml French mini straws following the Standard Operating Procedures of the Section of Reproduction of the Faculty of Veterinary Science, University of Pretoria. Briefly, this involved extending the sample to a total of 15 ml with Triladyl®; the volume required to produce straws with at least 20 million progressively

motile sperm. Extended semen was equilibrated at 4°C for at least 2 hr, after which it was loaded into straws which were placed on a rack 4 cm above liquid nitrogen (LN_2) for 25 min. The straws were then plunged into LN_2, packed and stored in LN_2 flasks until used.

2.3 | Semen preparation for in vitro fertilization

Preparation of semen for IVF was as previously described (Samardzija et al., 2006). Briefly, a single straw of semen from each of the three treatment groups was thawed by plunging it into a waterbath at 37°C for 40 s, opened with sterile scissors, and emptied into an Eppendorf tube. Post-thaw motility of each sample was evaluated by placing a small drop (5 µl) of semen on a pre-warmed (37°C) coverslip on a glass slide and phase contrast microscopy under 200x magnification. Only semen samples with at least 30% post-thaw motility and ≥60% normal morphology were used. Thawed semen was then layered onto 2 ml of the species-specific colloid, Bovipure® (Nidacon Laboratories AB, Göthenburg, Sweden) and centrifuged for 10 min at 150 *g*. The resulting sperm pellet was re-suspended in Tyrode's albumin lactate pyruvate (TALP) medium (Merck, Modderfontein, South Africa). Before commencing the IVF trial, a sperm pellet from each of the experimental groups was tested for the presence of LSDV DNA by PCR; positive samples were subjected to virus isolation.

2.4 | In vitro embryo production

In vitro embryo production was performed during autumn and winter, at a time when vector numbers would be low and therefore the risk that slaughtered cows may have been exposed to virus was low. A method established for the large-scale IVP of bovine embryos was used (Zullo et al., 2016), and is briefly described below. All media and reagents were obtained from Merck (Modderfontein, South Africa), unless otherwise specified.

Ovaries were harvested from cows immediately after slaughter at local abattoirs and placed in sterile polypropylene containers containing warm (32–37°C) 0.9% sodium chloride solution supplemented with 1% kanamycin, and transported to the laboratory in a warm insulated container. Ovaries were washed thoroughly to remove blood, after which the contents were aspirated from follicles between 3 and 8 mm in diameter. Cumulus-oocyte-complexes (COCs) thus recovered in follicular fluid within 4 hours of slaughter were classified via stereomicroscopic assessment according to a standard system used in the laboratory (Gordon, 2003). Only Grade A and B oocytes were used for culture. In brief, oocytes were classified as Grade A when compact multilayered cumulus with homogenous ooplasm were present and the cytoplasm of the oocyte appeared light and transparent. Oocytes were classified as Grade B when observed to have had compact multilayered cumulus cells with homogenous ooplasm but with a coarse appearance and darker zone at the periphery of the oocyte while the cytoplasm appeared darker and less transparent. The pooled fluid containing the oocytes was confirmed to be free of BVDV using reverse transcription nested PCR, because BVDV is a known common contaminant in bovine IVF laboratories. Prior to maturation, groups of grade A and B COCs were washed at least three times in a hydroxyethylpiperazine-ethanesulfonic-buffered (HEPES) aspiration medium. Groups of 15–20 oocytes were transferred to pre-equilibrated (overnight) four-well petri dishes (NUNC™; ThermoFisher Scientific, Johannesburg, South Africa) containing 380 µl drops of TCM-maturation media under 400 µl mineral oil at 39°C. Oocytes were matured at 39°C for 24 hr in an atmosphere containing 5.5% CO_2 and saturated humidity. The temperature of media and work surfaces was maintained at a constant 38°C during the entire procedure.

The frozen-thawed semen containing different viral loads (normal, HD and LD) was used to fertilise separate groups of oocytes. Following maturation, oocytes were removed from the maturation dishes and washed at least three times in pre-equilibrated IVF (fertilisation) media under oil before they were transferred in approximately 10 µl of media to 270 µl fertilisation media drops under 400 µl of mineral oil. Each drop containing 15–20 oocytes was inseminated using semen at a final concentration of 1×10^6 motile spermatozoa per ml (calculated by using the Neubauer haemocytometer-determined sample concentration, sperm motility and a dilution factor) and cultured for 22 hr at 39°C in a humidified atmosphere containing 5.5% CO_2.

Denuding putative zygotes of all cumulus cells and non-fertilizing sperm was accomplished by vortexing for ≤1 min in 300 µl of TCM-HEPES in a 1 ml Eppendorf vial. Denuded zygotes were recovered and rinsed twice before transfer to IVC-TALP drops. Fifteen to twenty putative zygotes were transferred into 270 µl of IVC-TALP media droplets in four-well dishes (NUNC™; ThermoFisher Scientific) equilibrated overnight. Embryos were cultured in IVC-TALP in a modular chamber (Billups-Rothenberg) containing 5% CO_2, 6% O_2 and balanced nitrogen at 39°C. Blastocyst formation was determined on days 7 and 8, and grading of blastocysts performed based on the recommendations of the International Embryo Transfer Society (IETS) (Wright, 1998). Several runs were performed to account for intra-culture confounding factors and to reach the required sample size.

2.5 | Samples collected for analysis

The samples collected, and the diagnostic tests to which they were subjected, are summarized in Table 1. Samples were collected separately for each experimental group and IVEP run, and stored at −80°C until analyzed .

2.6 | PCR analysis

Samples were suspended in a 2X lysis buffer (containing DTT) to which Proteinase K (included in the Qiagen® kit) was added, before incubation at 56°C overnight. The Qiagen® QIAmp DNA mini kit (Whitehead Scientific, Cape Town, South Africa) was subsequently used according to the manufacturer's instructions to extract viral DNA.

Real-time PCR was performed as described by Bowden et al. (2008). Briefly, 2.5 µl of purified DNA was used in a real-time PCR Taqman assay. Primers (Lightcycler Taqman master kit; Roche Diagnostics, Mannheim, Germany) at a concentration of

TABLE 1 List of samples and diagnostic tests performed for detection of lumpy skin disease and bovine viral diarrhea virus at various stages of a bovine in vitro fertilization trial

Sample (pooled per IVEP run)	Diagnostic test	Infectious agent
Frozen-thawed semen, post-processing	PCR and virus isolation	LSDV
Fetal bovine serum used to supplement media	PCR and RT-nPCR	LSDV and BVDV
Follicular fluid	PCR and RT-nPCR	LSDV and BVDV
Fertilization media post denuding	PCR and virus isolation	LSDV
Unfertilized oocytes day 8	PCR and virus isolation	LSDV
Two-cell to morulae structures	PCR and virus isolation	LSDV
Blastocysts	PCR and virus isolation	LSDV
Hatched blastocysts	PCR and virus isolation	LSDV

Note. LSDV, lumpy skin disease virus; BVDV, bovine viral diarrhea virus; RT-nPCR, reverse transcription nested PCR.

20 pmol were used. These primers target an 89 bp region within the *Capripoxvirus* open reading frame 074 which encodes the intracellular mature virion protein, P32. Capripoxvirus-specific primers and probes had the following sequences: forward primer-CaPV074F1 5′-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3′, reverse primer- CaPV074R1 5′-AAA TGA AAC CAA TGG ATG GGA TA-3′ and probe CaPV074P1 50-6FAM-TGG CTC ATA GAT TTC CT-MGBNFQ-3′. The reaction was carried out using a Light Cycler® 2.0 (Roche Diagnostics, Mannhein, Germany) and the following program: A FastStart *Taq*. This involved a polymerase enzyme activation step of 10 min at 95°C. The amplification consisted of 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 60 s and extension at 72°C for 1 s (single acquisition mode). A cycle threshold (Ct) value of 40 cycles or more was deemed to be negative. The negative control consisted of water while the positive control was LSDV V103/91, extracted from cell culture material.

2.7 | Virus isolation

Virus isolation was performed on LSDV PCR positive samples. Bovine dermis (BD) cells in 25 cm^2 flasks were used and maintained in 10 ml of MEM containing 5% foetal bovine serum (Biowest, Celtic Diagnostics, Cape Town, South Africa) and 1 ml gentamycin (0.1 mg/ml) to prepare 96-well plates (due to the small sample volumes) containing 480,000 cells per ml and incubated at 37°C for 24 hr in an atmosphere of 5% CO_2 in air. Semen samples were inoculated directly onto the cell monolayers. Cell cultures were observed daily for cytopathic effects (CPE). After 14 days, cultures showing no CPE were frozen briefly at −70°C and then thawed. A second passage was performed and each culture was observed for a further 14 days. A third passage was performed for these samples. A cell monolayer without any virus was used as negative control while a cell monolayer inoculated with 0.2–0.5 ml of an LSDV (strain V103/91) suspension at a titre of 4 log $TCID_{50}$ was used as positive control.

2.8 | Electron microscopy

In preparation for transmission EM, embryos in 100 μl of media were fixed overnight with an equal volume of 2.5% phosphate buffered glutaraldehyde. The samples were washed in 0.075 mol l^{-1} phosphate buffer, pH 7.4 before post-fixation in 1% osmium tetroxide for 1 hr. After two subsequent washes, the samples were dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, 96%, 100%) and embedded in epoxy resin. Ultrathin sections of the embryos were stained with lead citrate and uranyl acetate before viewing using a Philips CM10 transmission electron microscope (Philips Electron Optical Division, Eindhoven, The Netherlands) operated at 80 kV.

2.9 | Statistical analysis

Data were analysed using STATA 14 (StataCorp, College Station, Texas). Multivariable mixed effects Poisson or negative binomial regression models were used for count data (number of antral follicles, numbers of COCs harvested and numbers of COCs and embryos reaching certain developmental stages during the IVEP procedure). Time duration of aspiration and oocyte processing, quality of oocytes (expressed as the ratio of Grade A to Grade B oocytes) and effective sperm concentration (linear motility x sperm concentration) were included as covariates in the models. Overdispersion in the Poisson model was assessed by fitting the equivalent negative binomial regression model with the overdispersion parameter α and using a likelihood ratio test to examine the null hypothesis that $\alpha = 0$ (Dohoo, Martin, & Stryhn, 2009). Statistical significance was set at 5%.

3 | RESULTS

3.1 | Embryo production parameters

The embryo production parameters for the three experimental groups are summarized in Table 2. In seven IVEP replicates, a total of 1,252 oocytes were inseminated (410 in the control group, 480 in the HD group and 362 in the LD group). A total of 346 (84.4%), 376 (78.3%) and 249 (68.8%) oocytes cleaved in the control, HD and LD groups respectively, while corresponding blastocyst production was 154 (37.6%), 107 (22.3%) and 61 (16.9%). A further three runs, producing 44 blastocysts, were performed to provide samples for PCR confirmation of the presence of viral DNA (10 embryos), embryo washing (10 embryos) and EM examination (24 embryos).

TABLE 2 Embryo production parameters for the three experimental bovine IVF groups that differed by lumpy skin disease virus load in semen used for fertilization

Group and run	Oocytes inseminated	Oocytes cleaved	Cleavage rate (%)	Day 8 blastocysts	Blastocyst rate (%)
Control 1	38	36	94.7	24	63.2
Control 2	54	46	85.2	31	57.4
Control 3	44	39	88.6	17	38.6
Control 4	36	25	67.6	7	18.9
Control 5	98	81	81.0	32	32.0
Control 6	90	79	87.8	28	31.1
Control 7	50	40	80.0	15	30.0
Control total	410	346	84.4[a]	154	37.6[a]
HD 1	37	34	89.5	9	23.7
HD 2	51	35	64.8	0	0.0
HD 3	40	32	72.7	18	40.9
HD 4	38	32	84.2	13	34.2
HD 5	109	87	79.8	20	18.3
HD 6	90	67	74.4	27	30.0
HD 7	115	89	77.4	20	17.4
HD total	480	376	78.3[a]	107	22.3[b]
LD 1	17	14	36.8	2	5.3
LD 2	54	36	66.7	16	29.6
LD 3	44	34	77.3	13	29.5
LD 4	34	25	67.6	6	16.2
LD 5	105	66	61.1	10	9.3
LD 6	60	38	63.3	9	15.0
LD 7	48	36	75.0	5	10.4
LD total	362	249	68.8[b]	61	16.9[b]

Note. IVF, in vitro fertilization; LD, lower dose; HD, high dose.
High LSD viral load.
Lower LSD viral load.
Different letters within columns indicate statistical significance ($p < 0.05$) within the negative binomial regression model.

3.2 | Viral status of media and embryos

All the frozen-thawed semen samples from the control experimental group tested negative, while samples from the HD and LD group tested consistently positive, with Ct values between 25.3–28.1 and 35.3–37.0, respectively. The viral status of media and embryos for the three experimental groups are summarized in Table 3, while the Ct values of the positive samples are represented in Table 4. All follicular fluid samples and serum used in media tested negative for LSDV DNA and BVDV RNA. No LSD viral DNA was found in the control or LD groups in any of the samples collected. In the HD group, viable LSDV and viral DNA was found in all fertilization media and in some unfertilized oocytes, zygotes, morulae, blastocysts and hatched blastocysts. A batch of 10 embryos tested positive for LSD viral DNA, while the 10 washed embryos tested negative. Viable virus could be identified by virus isolation in all samples that tested PCR-positive.

3.3 | Electron microscopy

LSDV virions, with a size of 200–300 nm and a double-layered envelope, could be seen in some hatched and unhatched day 8 blastocysts (Figure 1). The virion morphology was indicative of viral replication and evidence of cell death and embryo architecture destruction could also be seen.

3.4 | Oocyte cleavage and blastocyst production

There was significant overdispersion in the Poisson regression model, indicating that the negative binomial model better fitted the data distribution, and hence the latter was used. Oocyte cleavage success was significantly lower (count ratio [CR] = 0.77; 95% CI: 0.65–0.92; $p = 0.003$) in the LD group when compared to the control group. This lower cleavage rate was not related to any other potential predictor variables (duration of processing, quality of oocytes, progressive sperm

TABLE 3 Viral status of media and embryos after in vitro production of bovine embryos using semen spiked with lumpy skin disease virus

	Fertilization media			Unfertilized			2-cell to morula			Blastocysts			Hatched blastocysts		
Run	Ctrl	LD	HD	Ctrl	LD	HD	Ctrl	LD	HD	Ctrl	LD	HD	Ctrl	LD	HD
1	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+
5	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+
6	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+
7	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–

Lower LSD viral load.
High LSD viral load.

TABLE 4 Cycle threshold (Ct) values of samples positive for lumpy skin disease virus DNA in the HD group

Run	Fertilization media	Unfertilized	2-cell to morula	Blastocysts	Hatched blastocysts
1	33.15	–	–	–	–
2	32.55	–	–	–	–
3	34.54	–	–	–	–
4	30.87	34.17	29.67	32.79	32.08
5	31.96	31.65	31.32	30.08	31.10
6	31.72	28.26	31.72	28.69	28.27
7	34.21	–	–	–	–

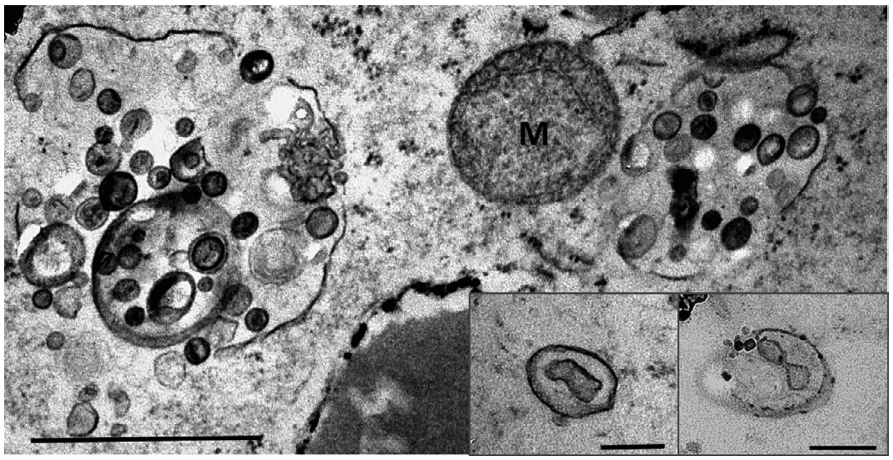

FIGURE 1 Numerous virus particles in various stages of development visible on both sides of a mitochondrion (M) within a bovine embryonic cell; bar = 1 m. Inset: Two free-lying virus particles displaying the more typical morphology of the mature virion; bars = 0.25 m

motility and sperm concentration) in the model. The success of blastocyst production was significantly lower in the HD group (CR = 0.64; 95% CI: 0.42–0.99; $p = 0.043$ and LD groups (CR = 0.55; 95% CI: 0.34–0.89; $p = 0.015$) compared to the control group. PCR positivity for LSDV was not a significant predictor of blastocyst count ($p = 0.107$). A random effect for date added to the model to account for clustering was not significant and did not influence the results.

4 | DISCUSSION

In this study, embryo yield was significantly reduced by the presence of LSDV in frozen-thawed semen used in an IVEP system, irrespective of the viral dose. In cases where semen with the lower viral dose was used, significantly reduced oocyte cleavage rates were observed. Lumpy skin disease virus could be demonstrated in fertilization media, unfertilized oocytes, degenerate embryos, blastocysts and hatched blastocysts when higher doses of LSDV were present in the frozen-thawed semen used for IVF.

Our findings of poorer embryo production parameters when using LSDV contaminated semen for IVF were similar to what has been reported for the use of BHV-1 contamined semen (Bielanski & Loewen, 1994), but different to the reported effects for BHV-5 contaminated semen (Silva-Frade, Martins, Borsanelli, & Cardoso, 2010). Failure of BLV-infected semen or oocytes to negatively affect embryo production has also been reported (Bielanski,

Maxwell, & Simard, 2000). Conflicting reports exist in the literature regarding the effect of use of semen contaminated with BVDV on embryo development. Bielanski (Bielanski & Loewen, 1994) and Guerin (Guerin, Chaffaux, Le Guienne, Allietta, & Thibier, 1992) reported a negative effect, whereas da Silva Cardoso Pinto reported no adverse effect on embryo development (da Silva Cardoso Pinto et al., 2017).

Since PCR results at the blastocyst stage were not significantly associated ($p = 0.107$) with blastocyst rate, it suggests the negative effect of LSDV on blastocyst development occurred earlier in embryo production processes and the virus did not exert its effect principally on the blastocyst at the formation stage. The finding of positive PCR and virus isolation results for the HD group only, suggests a viral dose effect of LSDV in IVEP systems. Dilution of semen in preparation for IVF likely reduced the viral dose in the LD group to levels just above the detection limit of the PCR assay whereas, subsequent to denuding, it apparently dropped below the detection limit. The Ct values of frozen-thawed semen samples within the LD group (35.3–37.0) support this statement. It is known that LSDV associates with sperm membranes (Annandale et al., 2018) and we postulate that, at low concentrations, the virus in the fertilization media may associate with the cumulus cells surrounding the putative zygotes. The degree of denuding and removing the cumulus cells, sperm and debris from the fertilization media, presumably determined the residual viral load at PCR. It should also be considered that seminal plasma constituents are known to limit the sensitivity of diagnostic tests for detecting infectious agents (Bagla, Osuagwuh, Annandale, Irons, & Venter, 2006) and could, at a low level of virus contamination, have negatively affected the sensitivity of the assays.

The PCR finding that blastocysts and hatched blastocysts were infected with LSD viral-DNA suggests that the virus associates with or enters the embryo, where it could affect early embryonic viability. Evidence for this was further supported by the EM findings. It therefore appears that LSDV does not simply associate with or adhere to the zona pellucida, as is the case for *Tritrichomonas foetus* (Bielanski, Ghazi, & Phipps-Toodd, 2004), *Neospora caninum* (Bielanski, Robinson, & Phipps-Todd, 2002) and BTV (Venter, Gerdes, Wright, & Terblanche, 2011). It is noteworthy that embryonic structures only tested positive for LSDV by PCR if the Ct value of fertilization media was <32.5 cycles; however, the experimental design did not allow statistical testing of this relationship. While it has been established that LSDV-spiked semen can trigger clinical LSD in heifers and infect in vivo derived ZP-intact embryos (Annandale et al., 2014), it is not known whether the results of experimental infections are applicable to field conditions. Clear differences between in vivo and in vitro derived embryos, especially regarding the ability of embryo washing procedures to remove viruses (Wrathall et al., 2006) have been shown, indicating that caution is required when extrapolating IVEP findings to natural conditions.

The effect of transferring LSDV infected embryos to recipient cows is not known. Bielanski et al. 2000 reported that when unwashed in vivo produced embryos positive for BHV-1 were transferred to seronegative cows, poor pregnancy rates and seroconversion resulted (Bielanski, Algire, Lalonde, & Garceac, 2013b). In another experiment, the same authors found that if BVDV-infected embryos were washed according to the IETS disinfection protocol prior to transfer, they did not trigger seroconversion in either the cows or their offspring (Bielanski, Algire, Lalonde, & Garceac, 2013a). Wrathall (Wrathall et al., 2006), quoting Bielanski (Bielanski et al., 2000), concluded that while the use of BLV-infected semen in IVEP could result in infected embryos, the likelihood of transmission of BLV to recipients was very small. In two separate experiments, one using semen infected with BTV (Thomas, Singh, & Hare, 1985) and the other infecting heifers with BTV and collecting embryos from viraemic cows (Acree et al., 1991), it was shown conclusively that the IETS washing protocol reduced the risk of transmission of BTV to negligible. Since the current experimental design resulted in low numbers of embryos available for IETS washing, its effect on embryos infected with LSDV still needs investigation.

It is noteworthy that the cleavage and blastocyst rates between individual IVF embryo runs showed a substantial range (67.6%–94.7% and 18.9%–63.2% for control; 64.8%–89.5% and 0%–40.9% for HD; and 36.8%–75.0% and 5.3%–29.6% LD experimental groups). While variation between individual replicates within IVEP systems has been described, other factors potentially impacting on embryo production parameters should also be considered. BVDV is often considered a low-level contaminant within IVEP systems, although there was no evidence of BVDV contamination in the current study. While performing the experiment in the winter months should have reduced the risk of introducing other viruses, this possibility cannot be excluded entirely.

Due to the variety of experimental models described, caution should be applied when comparing transmission of virus to embryos, and the results of embryo washing. The experimental models range from use of fresh semen from infected bulls (Thibier & Nibart, 1987), to frozen-thawed semen from naturally infected (persistently infected with BVDV) bulls (Guerin et al., 1992), artificially spiked frozen-thawed semen (Schlafer et al., 1990), incubation of oocytes with virus (Bielanski, Simard, Maxwell, & Nadin-Davis, 2001) and co-incubation of virus with morulae or embryos (Waldrop et al., 2002). Some of the experiments were carried out within an in vitro setting, while others were performed on live animals and in vivo embryo development. These experimental models therefore address different aspects of the possible mechanism of virus transmission within the reproductive system and comparison of their results may not necessarily be scientifically valid.

In conclusion, the presence of LSDV in frozen-thawed semen negatively affected embryo yield in IVEP systems. Moreover, the presence of the virus within day 8 blastocysts confirmed that embryo transfer carries a potential risk of virus transmission in cattle. Further research to quantify this potential risk is indicated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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