Effect of semen processing methods on lumpy skin disease virus status in cryopreserved bull semen

C Henry Annandale[a*], Mario P Smuts[a], Karen Ebersohn[b], Lizette du Plessis[c], Estelle H Venter[bd], Tom AE Stout[ae]

[a]Departments of Production Animal Studies, [b]Veterinary Tropical Diseases and [c]Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[d]School of Public Health, Medical and Veterinary Sciences, Discipline: Veterinary Science, James Cook University, Townsville, Australia

[e]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht Univeristy, Utrecht, Netherlands

*__Corresponding author:__ C Henry Annandale (henry.annandale@up.ac.za)

ABSTRACT

Lumpy skin disease is an economically important disease of cattle, caused by the lumpy skin disease virus (LSDV; *Capripoxvirus*). It has a variable clinical appearance but, in severely affected animals, is associated with extensive skin damage, pneumonia and death. The LSDV can be found in the semen of infected bulls for prolonged periods of time, from where it can be transmitted by mating or artificial insemination and cause clinical disease in heifers and cows. In this study, an ejaculate was collected from a LSDV seronegative bull and confirmed free from LSDV DNA by PCR. The ejaculate was split into a control sample (C), a sample spiked with a 4 log $TCID_{50}$ dose of an LSDV isolate (HD) and a 10^3 dilution of the virus suspension (ND) and frozen routinely. Two straws from each of the different semen treatment groups (HD, ND and C) were subsequently thawed and subjected to swim-up, single layer centrifugation, Percoll® density gradient and a Percoll® density gradient with added trypsin. For one set of straws, semen quality variables were recorded, and viral DNA status determined using PCR; the other set was used for positive staining electron microscopy. Samples determined to be positive for LSDV DNA by PCR were then subjected to virus isolation (VI). Complete elimination of LSDV from semen did not occur with use of any of the processing methods. Trypsin did reduce the viral load, and eliminated LSDV from the ND sample, but severely negatively influenced semen quality. The LSDV virions, as assessed by electron microscopy, were associated with the sperm plasma membrane. Further investigation is needed to establish the efficacy of immuno-extenders for rendering semen free from LSDV.

Keywords: Lumpy skin disease virus; Bull; Semen; Sanitation methods

1. Introduction

Lumpy skin disease (LSD) is a viral disease of cattle caused by the lumpy skin disease virus (LSDV), a member of the genus C*apripoxvirus*. It is common in Africa and, more recently has been detected in the Middle East where it 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). In countries where it is present, LSD is associated with significant economic losses attributed to the extensive damage to hides, loss of production and restrictions to trade (Babiuk et al., 2008a). In recent years outbreaks have occurred in Egypt (OIE, 2006), Israel (Brenner et al., 2009) , Greece (Tasioudi et al., 2016), Azerbaijan (Zeynalova et al., 2016), Jordan (Abutarbush et al., 2015) and Iraq (Al-Salihi and Hassan, 2015). The LSDV is a direct threat to Europe and Great Britain (Tuppurainen and Oura, 2014; Beard, 2016). The origin of the outbreaks remains mostly unknown, but illegal movement of farm animals without proper health assessments and the collapse of veterinary services due to political uncertainty have been implicated (Tuppurainen et al., 2017).

Several transmission routes for LSD infection have been identified, mostly associated with mechanical transmission via blood-sucking insects (Carn and Kitching, 1995; Chihota et al., 2001) and ticks (Tuppurainen et al., 2013). Even though seminal transmission of LSDV to heifers has been demonstrated experimentally (Annandale et al., 2014), and a case of intrauterine transmission has been reported (Rouby and Aboulsoud, 2016), the potential for reproductive transmission of LSDV has not been determined.

Cattle that become infected with LSDV, experimentally and naturally, have variable clinical signs; ranging from almost none to severe generalized lumpy skin

disease. The LSDV can be shed in the semen of experimentally infected bulls for up to 159 days (Irons et al., 2005), however, the nature of this shedding is not well-documented. Semen shedding of the virus mirrors the clinical syndrome with the amount, quantity and duration of shedding being highly variable and difficult to predict (Babiuk et al., 2008b; Annandale et al., 2010).

In countries free of LSDV, 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 have no other obvious clinical signs during a herd outbreak, the likelihood of semen being contaminated with LSDV cannot be excluded. If frozen, such semen could also enter *in vitro* fertilization systems where its potential to be transmitted via resulting embryos is currently unknown. Developing and documenting methods of rendering semen and embryos free from LSDV has trade and economic advantages, and can assist in clarifying the risks of semen-based transmission of LSDV.

The objective of this study was to investigate the ability of common semen processing techniques to remove lumpy skin disease virus from cryopreserved bull semen. As a secondary objective, the way in which the virus associates with the sperm cell was also investigated.

2. Materials and methods

2.1. Study overview

Semen was collected from a 2-year old Brahman bull, confirmed to be breeding sound and sero-negative for LSDV; the semen was confirmed negative for LSDV by polymerase chain reaction (PCR) with validated primers. The semen sample was split into three aliquots, two of which were spiked with a large (LD) and small dose

(SD) of LSDV, respectively, while the third portion served as a control (C). All three aliquots were processed and frozen in 0.25 mL French mini straws. Two sets of straws from each of the different experimental groups (HD, ND and C) were subsequently thawed and subjected to swim-up, single layer centrifugation, Percoll® density gradient and a Percoll® density gradient with added trypsin. For one set of straws, semen quality variables were recorded and viral DNA status determined using PCR. Samples found to be positive for LSDV DNA by PCR were then subjected to virus isolation (VI). For the other set of straws, positive staining electron microscopy was performed, after the various semen processing methods. The study was approved by the Animal Ethics Committee of the University of Pretoria (V093/16).

2.2. Spiking of semen samples with virus

The LSDV Mireil isolate (Neethling strain) (V103/91) was obtained from the Virology Section of the Department of Veterinary Tropical Diseases and suspended in Minimum Essential Medium (MEM; Biowest, Celtic Diagnostics, Cape Town, South Africa). The ejaculate was split into three equal volume aliquots of 5 mL each and processed as follows: the LD sample was spiked with 2.5 mL of the viral suspension at a titre of 4 log $TCID_{50}$, the SD 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 triladyl® extender consisted of 20% (v/v) Triladyl® concentrate and 20% (v/v) egg yolk, 60% (v/v) distilled water and 0.5% (v/v) Equex STM paste (Minitube, Tiefenbach, Germany). Aerobic bacterial culture was perfomed on a representative aliquot of each batch of semen extender.

2.3. Cryopreservation of semen

The three semen aliquots were processed individually, making use of separate freezing equipment to prevent cross-contamination. The control sample was processed first. Semen was frozen in 0.25 mL French mini straws as per Standard Operating Procedures of the Section of Reproduction of the Faculty of Veterinary Science, University of Pretoria. Briefly, this involved extending the aliquots to a total of 15 mL with Triladyl® extender; the volume required to deliver straws with at least 20 million progressively motile sperm. Semen and extender was equilibrated at 4 °C for at least 2 h, after which the straws were filled and placed on a freezing 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.

2.4. Semen processing methods

2.4.1. Swim-up

The thawed semen sample was overlaid with 2 mL Sperm Tyrode's albumin lactate pyruvate (TALP) medium (Merck, Modderfontein, South Africa) and maintained in an incubator at 39 °C and an atmosphere of 5.5% CO_2–in-air for 60 mins. After a 1 h incubation, the top layer was aspirated and washed in 2 mL Sperm TALP medium by centrifugation at 150 *g* for 10 mins.

2.4.2. Single-layer centrifugation

Thawed semen was layered onto 2 mL of the species-specific colloid, Bovipure® (Nidacon Laboratories AB, Göthenburg, Sweden) and centrifuged for 10 mins at 150 *g*. The resulting sperm pellet was re-suspended in TALP medium.

2.4.3. Percoll gradient

A volume of 2 mL of 80% Percoll® solution (Sigma-Aldrich, Kempton Park, South Africa) was placed in a Falcon® tube and 2 mL of a 45% Percoll® solution was slowly and gently overlaid on it, to form a gradient. The semen straws were thawed in 37 °C water for 40 seconds, dried and opened using scissors disinfected in 70% ethanol, and emptied into an Eppendorf tube. Using a 1000 μl pipette, the semen was gently layered on top of the 2 Percoll® solutions and then centrifuged at 250 *g* for 25 mins. Thereafter, the supernatant was discarded, and the sperm pellet suspended in 2 mL of sperm-TALP and centrifuged at 135 *g* for 25 mins. The resultant sperm pellet was re-suspended in 2 mL of sperm-TALP and finally centrifuged at 110 *g* for 10 mins.

2.4.4. Percoll gradient with trypsin

A slight modification was made to the method described by Loskutoff (2005). Briefly, 0.25% trypsin (Sigma-Aldrich, Kempton Park, South Africa) was added to the intermediate layer of three density gradients (30%, 45% and 80%) of Percoll®. Foetal bovine serum (4%; Biowest, Celtic Diagnostics, Cape Town, South Africa) was added to the 30% and 80% Percoll® solutions to serve as a trypsin inhibitor. The samples were then processed as outlined for Percoll® gradient processing above.

2.5. Semen evaluation methods

Sperm motility was assessed immediately after thawing and processing, following the methodology outlined by Nöthling (2012). A small drop of semen was placed on a 22 mm x 22 mm glass coverslip, which was then inverted and held horizontally until it touched the glass slide, whereupon it was released and the drop allowed to spread radially between the coverslip and glass slide. No coverslips were

examined when evidence of bubbles between the coverslip and glass slide was seen. Using a phase-contrast light microscope (Zeiss AXIO Lab.A1, Zeiss South Africa) at 200 x magnification, starting at the center field of the coverslip, 10 fields along the equator of the coverslip were evaluated for progressive and aberrant motility and means were calculated. Spermatozoa not moving in a straight line were regarded as aberrantly motile.

To evaluate sperm morphology, a small drop of semen was mixed with Eosin-Nigrosin stain and a semen smear made on a glass slide. A total of 200 spermatozoa were evaluated and assessed for morphological normality using phase-contrast microscopy at 1000 x magnification according to the method described by Barth and Oko (1989). To measure spermatozoa concentration, the semen samples were diluted (1:10) in 90 µl of formaldehyde solution. A drop of the solution was placed in the chamber of a Neubauer haemocytometer. Spermatozoa in 10 of the 25 squares were counted, with each square measuring 0.04 mm^2 (volume = 0.004 mm^3, with a height of 0.1 mm). The results were converted to a concentration in sperm per mL by multiplying the number of sperm counted in five squares by 0.5×10^6.

2.6. PCR analysis

The PCR analysis was based on the methodology of Lubinga (Lubinga et al., 2013). Samples (200 µL of processed semen) were suspended in a 2X buffer (containing DTT), and Proteinase K (included in the Qiagen® kit) was added, after which the samples were incubated 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 the DNA. Real-time PCR was

performed as described by Bowden et al. (2008). Briefly, 2.5 μl of purified DNA was added to each reaction of a real-time PCR Taqman assay. Primers (Lightcycler Taqman master kit, Roche Diagnostics, Mannheim, Germany) at a concentration of 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 instrument (Roche Diagnostics, Mannhein, Germany) using the following program: A FastStart *Taq* 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 sample was regarded positive if a Ct value of less than 39 was recorded.

2.7. Virus isolation

Virus isolation was performed on LSDV PCR positive semen samples. Dermis (BD) cells of cattle were used and maintained in 5 mL of minimum essential medium (MEM) containing 4% foetal bovine serum (Biowest, Celtic Diagnostics, Cape Town, South Africa) and 1 mL gentamycin (0.1 mg/mL). Twenty-four-well plates were prepared containing 480,000 cells / mL and incubated at 37 °C for 24 h in an atmosphere of 5% CO_2. Semen samples were inoculated directly onto the cell monolayers. Cell cultures were observed daily for cytopathic effects. After 14 days, negative cultures 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 negative samples. A cell monolayer without any virus was used as negative control while a cell monolayer inoculated with 0.2-0.5 mL LSDV (strain V103/91) suspension at a titre of 4 log $TCID_{50}$ was used a positive control.

2.8. Electron microscopy

In preparation for transmission electron microscopy, 100 µL of the processed semen samples was mixed with an equal volume of 2.5% phosphate buffered glutaraldehyde and fixed overnight. Using gentle centrifugation (1096 x *g* for 10 minutes) and re-suspension, the samples were washed in Millonig's buffer, pH 7.4 before post-fixation treatment in similarly buffered 1% osmium tetroxide for 1 hour. After two subsequent washes in buffer, the samples were dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, 96%, 100%). Samples were embedded in epoxy resin, sectioned and stained with lead citrate and uranyl acetate before viewing in a Philips CM10 transmission electron microscope (Philips Electron Optical Division, Eindhoven, The Netherlands) operated at 80kV.

3. Results

*3.1. Viral status and sperm quality after processing*None of the semen processing methods tested were able to clear frozen-thawed semen of LSDV, except for the Percoll gradient with added trypsin (Table 1). The trypsin-Percoll gradient method resulted in failure to amplify LSDV DNA from frozen-thawed semen samples spiked with the smaller dose of LSDV. Virus isolation also failed to demonstrate any LSDV in samples treated this way. The sperm quality variables, however, were reduced

markedly by this treatment; progressive motility decreased to 8% to 9% and morphological defects increased significantly (Table 2).

Table 1

Viral status (PCR and VI) of control, large dose (LD) and small dose (SD) LSDV-spiked frozen-thawed semen of a bull following different semen processing methods aimed at removing virus

	Virus isolation			PCR			Ct-values		
	Control	LD	SD	Control	D	SD	Control	LD	SD
Swim-up	-	+	+	-	+	+	-	32.90	>40.00
SLC[a]	-	+	+	-	+	+	-	25.33	35.30
Percoll	-	+	+	-	+	+	-	30.81	>40.00
PT[b]	-	+	-	-	+	-	-	31.78	-

[a]SLC = single layer centrifugation

[b]PT = Percoll with trypsin

Table 2

Percentages of progressively motile and morphologically normal spermatozoa and sperm concentrations of control, Large dose (LD) and small dose (SD) LSDV-spiked frozen-thawed semen of a bull following different semen processing methods

	Progressive motility (%)			Morphologically normal (%)			Concentration (x 10^6 /mL)		
	Control	SD	LD	Control	LD	SD	Control	LD	SD
Post-thaw	40	20	28	67	62	69	110	94.2	102.1
Swim-up	53	56	34	92	91	90	0.5	0.5	0.4
SLC	53	35	36	81	82	78	35.1	61.5	42.1
Percoll	43	39	38	86	87	87	41.2	41.3	38.8
PT	8	9	9	68	70	64	31.1	29.6	28.7

PT = Percoll with trypsin

3.2. Electron microscopy

The LSDV virions, with a size of 200 to 300 nm and a double-layered envelope, could be detected in the surrounding fluid and associated with the sperm plasma membrane (Figure 1).

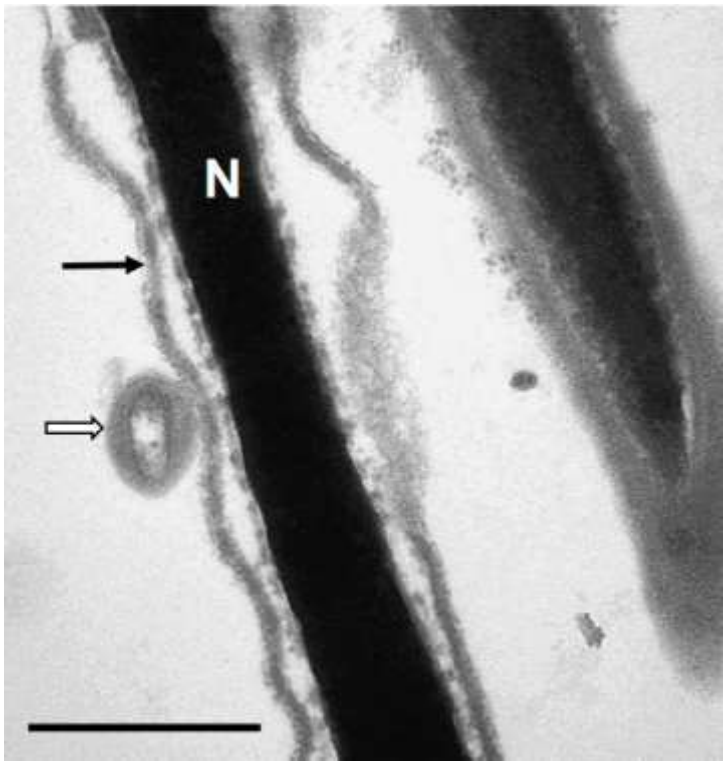

Fig. 1. Cross section of a bovine sperm demonstrating the association of an LSDV virion (block arrow) to the sperm plasma membrane (thin arrow). N = sperm nucleus. Bar = 0.5µm.

4. Discussion

This study demonstrated that common semen processing methods were not able to eliminate LSDV from cryopreserved bull semen, which is analogous to what has previously been described for bovine viral diarrhea virus (BVDV; Bielanski et al., 1992), bovine leukaemia virus (BLV; Bielanski et al., 2000), bovine immunodeficiency virus (BIV; Bielanski et al., 2001) and human papillomavirus (HPV; Niederberger, 2012) in semen. There, however, are examples in the literature of the successful removal of viruses from semen using either swim-up or Percoll®

gradient centrifugation. These viruses include equine arteritis virus (EAV; Morrell and Geraghty, 2006), porcine reproductive and respiratory syndrome virus (PRRSV; Morfeld et al., 2004), human immunodeficiency virus-1(HIV-1) and the hepatitis C (HCV) virus (Loskutoff et al., 2005).

Silva (1999) documented the removal of bovine herpesvirus-1 (BHV-1) from experimentally infected semen using 0.25% trypsin, but also demonstrated a reduction in the percentage of motile spermatozoa, and increased incidence of sperm membrane damage at a trypsin concentration of 0.3%. In the current study, adding 0.25% trypsin to the middle layer of a Percoll® gradient prior to centrifugation also succeeded in eliminating the LSDV that resulted from spiking with the smaller dose from frozen-thawed semen samples, but there were similar deleterious effects on sperm motility and membrane integrity, which precludes the use of 0.25% trypsin to sanitize semen from LSDV positive bulls. Trypsin, as a proteolytic enzyme, has a disrupting effect on BHV-1, a very labile enveloped virus with structural elements that include an icosahedral capsid, a tegument and covalently-linked components (Rechenchoski et al., 2017). In contrast, the poxvirus mature virions, responsible for viral transmission between hosts have a more complex structure including an additional outer membrane, making them very stable and resistant to decontamination processes (Tulman et al., 2001; Moss, 2006).

This study further documented the association of LSDV with the sperm plasma membrane in a processed semen sample. In a review, Moss (2016) described the attachment of poxviruses to at least four different proteins that bind to chondroitin, heparan and laminin, and ascribed viral entry and membrane fusion to a complex of 11 proteins, of which at least eight must be present. This is in contrast to entry mechanisms of other viruses which normally involve only one to three proteins. The

method by which LSDV, as a poxvirus, associates with cells may, therefore, involve a strong binding process and make dislodging and removal of the virus from cells, sperm in this case, very difficult.

It is unknown whether the concentrations of LSDV used in the current study are comparable to those found in bulls naturally infected with LSDV and shedding virus into their semen. The Ct-values for PCR amplification of LSDV DNA in this trial, however, are comparable to those described by Babiuk (2008b) for LSDV in other bodily secretions, all of which were close to the detection limit of the assay. That the semen was spiked with LSDV also raises the question of whether virus added to semen in this way behaves differently to LSDV entering semen following natural infection (Bielanski, 2007). The difficulty in sourcing LSDV-infected semen from naturally infected bulls, especially given the unpredictability of infection and viral shedding into semen, however, complicates implementation of an alternative experimental design. Furthermore, the use of spiked semen induces disease in experimentally inseminated heifers, suggesting that the infectious nature of virus added to semen experimentally is similar to that of virus entering by natural infection (Annandale et al., 2014). In the present study, there was not evaluation of the more advanced techniques of removing pathogens from the semen, which may be more successful. Such techniques include immuno-extenders such as hyperimmune yolk produced for BHV-1 (Silva et al., 2000) and acidification of the semen, which has been described for removal of foot-and-mouth disease virus (Bachrach et al., 1957). Entry of poxviruses into cells is dependent on low pH (Moss, 2016) and it, therefore, is assumed that acidification of semen will not help to free it of LSDV. The effect of alkalinisation of semen and the use of hyperimmune yolk extenders to biosanitize semen from LSDV warrant further investigation. The small number of samples

evaluated in this study precludes statistical inference, and because semen used was from a single bull a bull effect cannot be excluded. Further research, focused on the promising Percoll and trypsin method, would be indicated to exclude the possibility of an individual bull effect.

In conclusion, the present study documents that common semen processing methods are not effective in rendering semen spiked with LSDV free of the virus. It highlights the ability of trypsin to reduce the LSDV viral load in semen but, unfortunately, at the expense of significant semen quality deterioration. Furthermore, assessments in the present study indicated that there is an ultrastructural association of LSDV with the sperm plasma membrane.

Conflicts of interest

None.

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