Seminal Transmission of Lumpy Skin Disease Virus in Heifers

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Summary

It is known that lumpy skin disease virus (LSDV) can be shed in bull semen following infection and also that artificial insemination (AI) poses a biosecurity risk. However, it is not known whether the use of LSDV infected semen in AI poses a biosecurity risk. The aim of this study was to investigate whether LSDV, transmitted through semen, can infect cows and their embryos. Two controlled trials were performed simultaneously. Eleven young beef heifers, naive to LSDV, were synchronized using an OvSynch protocol and inseminated on Day 0 with fresh semen spiked with a field strain of LSDV on day 0. Six of the heifers were superovulated on Day 1 using pregnant mare serum gonadotropin, and embryos were flushed from these heifers on Day 6. Blood and serum samples were collected from Day 4 until Day 27 to determine the presence of LSDV by PCR and virus isolation, and the presence of antibodies against LSDV by SNT. The first clinical signs of LSD were noticed on Day 10, followed by severe generalized LSD in three heifers and mild LSD in two more heifers. Two heifers were humanely euthanized due to severe unresponsive stranguria. LSDV was detected by PCR, virus isolation or electron microscopy in blood, embryos and organs of experimentally infected animals; and eight heifers had seroconverted by Day 27. Two control animals were not affected. This is the first report of experimental seminal transmission of LSDV in cattle.

Introduction

Lumpy skin disease virus (LSDV), a member of the genus Capripoxivirus within the family Poxviridae, causes an imperceptible to acute disease in cattle, characterized by firm, circumscribed skin nodules, necrotic plaques in the mouth and nares, fever and generalized lymphadenopathy (Coetzer, 2004). Due to pneumonia, infertility reduced feed intake and a harmful effect on the quality of hides, economic losses associated with the disease can be substantial (Woods, 1988). Although endemic to sub-Saharan Africa, lumpy skin disease (LSD) has the potential to spread to other parts of Africa, the Middle East and Europe (Tuppurainen and Oura, 2012).

A number of possible routes of transmission for LSDV have been investigated and identified. These include arthropods, biting insects and mosquitoes (Kitching and Taylor, 1985; Kitching and Mellor, 1986; Carn and Kitching, 1995; Chihota et al., 2001, 2003). The role of ticks in transmission has been suspected (Aiel, 2009) with observations of the presence of ticks made on affected animals in some outbreaks (especially Amblyomma ticks) (Ali and Obeid, 1977). A recent study indicated that transstadial and transovarial transmission of LSDV by Rhipicephalus (Boophilus) decoloratus ticks and mechanical or intrastadial transmission by Rhipicephalus appendiculatus and Amblyomma hebraeum ticks were possible (Tuppurainen et al., 2010). The mechanical transmission of LSDV by R. appendiculatus male ticks has also now been confirmed (publication submitted).

Transmission through artificial insemination (AI) has been observed in different animal species e.g., for equine arteritis virus (Guthrie et al., 2003), porcine reproductive and respiratory syndrome virus (Christopher-Hennings et al., 1995), classical swine fever virus (Smit et al., 1999) and feline immunodeficiency virus (Jordan et al., 1996). In bovines, foot-and-mouth disease virus and bovine herpes virus 1 can be transmitted to susceptible heifers by
insemination with infected semen (Afshar and Eaglesome, 1990). Intra-uterine inoculation with ephemeral fever virus did not result in seroconversion or production of the disease (Kahrs et al., 1980), and bovine leukaemia virus (BLV) could not be transmitted via leucocyte-free semen from BLV-infected bulls (Kaja and Olson, 1982).

Recent research has indicated the presence of LSDV in semen and the possible risk of transmission of the disease via this route. In a study by Irons et al. (2005), virus particles were detected in semen of experimentally infected animals for up to five months, when clinical signs were no longer evident. Following an initial drastic deterioration in semen quality during the acute phase of the infection, the quality of the semen recovered before the end of the period of virus excretion (Irons et al., 2005).

It was also shown that LSDV is not limited to specific fractions of the ejaculate and the tests, and that the epididymides are most profoundly affected (Annandale et al., 2010). The virus was not thought to be sperm or blood associated, and it was concluded that the ejaculate was likely to have been contaminated with virus that was being shed during emission from necrotic lesions in the genital tract (Annandale et al., 2010). The role posed to in vitro fertilization (IVF) procedures were also demonstrated. It was established that the virus was sperm-associated in 9% of spermatozoa, and that the early blastocyst contained viral DNA (Irons, 2008). The contradictory finding of sperm association of virus in these two studies needs further investigation to establish the significance thereof.

There is a high demand by other countries for breeding material such as embryos and semen from South African cattle (Kahrs et al., 1980; de la Rey, 2003). In particular, AI is an extremely effective means of disseminating genetic material, and at present semen exports exceed embryo exports. Lumpy skin disease virus is one of the viruses identified in semen risk analysis as a potential hazard in the international movement of bovine semen (Hentzen, 2000). There is currently no published information on the ability of LSDV-infected semen to establish clinical disease in cows naturally or artificially by insemination i.e., trans-uterine infection. No further data are available on the effect of the use of infected semen for in vitro fertilization of oocytes, or on the potential for the spread of the disease to recipients of embryos produced in this way.

The objectives of the current study were therefore to assess the possibility of transmission of LSDV through semen and subsequent LSDV infection in cows and embryos.

**Materials and Methods**

**Animals**

Thirteen healthy, thin-skinned European breed heifers from a diverse genetic pool were sourced from herds where LSD had not been seen for three years, and where no vaccination was practiced. Only animals that tested negative for antibodies to LSDV by using the serum neutralization test (SNT) were selected and kept at the insect-free stables of the Biological Research Centre of the University of Pretoria (UPBRC), Faculty of Veterinary Science.

The experiment was divided into two trials – each with one control animal. The two trials ran simultaneously and were only denoted as two trials for simplification of the experimental design.

Trial I investigated the possible effect of LSDV on embryo parameters in vivo. Heifers in this trial were called ‘Embryo heifers’. Trial II investigated the response of heifers to insemination with semen spiked with an infective dose of LSDV. Heifers in this trial were called ‘AI heifers’.

The heifers were divided into groups of six (Embryo Group) and seven animals (AI Group) each. Oestrus was synchronized in all heifers using the OvSynch protocol. Briefly, this involved intramuscular injections of 100 µg buserelin (Fertagyl®) on Day -10, a luteolytic dose of prostaglandin F2α (Lutalyse®) on Day -3, another injection of buserelin on Day –1 and insemination 17–24 h after the second buserelin injection. The day of insemination was denoted Day 0. Embryo heifers were superovulated using a single injection of pregnant mare serum gonadotropin (PMSG) on the day of the second buserelin injection (Pursley and Bello, 2007).

**LSDV preparation**

A South African field isolate (V248/93) was used to spike semen as challenge material for experimental animals. The infectivity of the field isolate had previously been established (Tuppurainen et al., 2005). Bovine dermis cells prepared from a foetal calf’s ear were cultured in 75 cm² tissue culture flasks in Minimum Essential Medium (MEM) supplemented with l-glutamine (1 ml/l), 0,2% sodium bicarbonate (Highveld Biological, Lyndhurst, Johannesburg, Gauteng), 5% foetal calf serum (Adcock Ingram, Midrand, Gauteng) and 1 ml/l gentamycin (50 mg/ml Genta 50 Phenix). When 70–80% of the cell monolayer was confluent, a volume of 0.5 ml of virus isolate was inoculated into a 75 cm² flask. Virus was harvested when 90–100% of the cell monolayer was infected and showed typical cytopathic changes. Culture flasks were frozen briefly at –70°C, then thawed and the flasks shaken gently to release the cell-associated virus. The cell cultures were centrifuged at 448 g for 3 min. The supernatant was removed and aliquoted in volumes of 1.8 and 3.5 ml in cryo tubes (Nunc, Amersham, Johannesburg, South Africa) and stored as reference stock virus at –70°C until used. The reference stock virus was
titrated on bovine dermis cells using 96-well microtitre plates, including the cell control. Cells were observed daily for CPE, and the TCID\textsubscript{50} of the virus was calculated using the method of Reed and Miuench (1938).

**Experimental challenge with spiked semen**

On Day 0, 11 animals were inseminated with 1 ml of fresh semen spiked with 1 ml of LSDV virus suspension. The titre of the virus used was 5.5 log TCID\textsubscript{50}/ml. A control in each group consisted of an animal inseminated with an equivalent dose of unspiked semen.

**Sample Collection and Observation**

All animals were observed daily for habitus and development of clinical signs. Rectal temperature was taken twice daily. Blood samples were collected in EDTA and heparin from all animals on 2, 6, 9, 12, 16, 20, 23 and 27 days post-infection (dpi), and subjected to PCR and virus isolation. Serum samples were collected on 9, 12, 16, 20, 23 and 27 dpi and subjected to SNT.

On Day 6, the five treatment heifers and one control heifer (EM heifers) received epidural anaesthesia, an embryo flushing catheter was passed into the uterine horns alternately and the embryos were flushed from the horns with embryo flushing media. Before use, the flushing media was confirmed free of bovine viral diarrhoea virus (BVDV) using a RT-nPCR (Qiagen Cador BVDV Type 1/2 real-time RT-PCR Kit, Qiagen, Hilden, Germany). Embryos were washed as described in the Manual of the International Embryo Transfer Society (IETS) (Stringfellow and Seidel, 1998) and tested for the presence of LSDV by PCR. The samples that were positive by PCR were subjected to virus isolation. The Embryo heifers stayed in the experiment until Day 27, after which time they were removed.

On Day 28, ultrasound pregnancy diagnosis per rectum was performed on the AI heifers. Pregnant heifers were retained until Day 42, but non-pregnant heifers were removed from the trial.

**Laboratory testing**

To detect the presence of LSDV DNA in EDTA blood samples, embryos, flushing media and organs, PCR was performed using the primers of Ireland and Binepaul (1998). Virus isolation was carried out on bovine dermis cell monolayers according to the method described by Tuppurainen et al. (2005). To determine seroconversion, an SNT was performed using a 96-well plate format according to the standard protocol of the Virology Section, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria (OIE, 2010). Grading of embryos flushed from Embryo heifers was conducted according to the guidelines of the Manual of the LSDV (Stringfellow and Seidel, 1998). Electron microscopy was performed on skin lesions by the EM-Section, Faculty of Veterinary Science, University of Pretoria. Immunoperoxidase staining of the uterine tissues were carried out by the section of Pathology, Faculty of Veterinary Science, University of Pretoria.

**Results**

**AI heifers**

In the AI group, three of the seven animals tested positive for viral DNA in blood between 10 and 17 dpi, and five animals had seroconverted by the end of the trial. The three clinically affected heifers in the AI group showed severe clinical signs, especially affecting the reproductive tract. Due to extreme, non-responsive stranguria and extensive necrosis of their caudal reproductive tracts, two of these heifers were humanely destroyed and postmortem examinations were performed.

**Embryo heifers**

Embryos were harvested from two infected heifers, and these embryos were found positive for LSDV DNA by PCR. Virus could also be isolated from both embryos using cell cultures. Stepwise washing as per IETS guidelines rendered the embryos free of LSDV DNA (Stringfellow and Seidel, 1998). One heifer tested PCR positive and VI positive on Day 16 and Day 23 respectively, while the other heifer...
remained negative for the duration of the trial (Table 1). Neither PCR nor virus isolation could identify LSDV infection in the flushing media recovered from the other heifers. Three of the five experimental heifers in the EM group had seroconverted by the end of the trial (Table 1).

In all affected animals, five in total, the first clinical signs of LSD infection were visible 10 days after experimental infection in the form of a severely swollen vulva. Fever reactions appeared from 11 dpi and were accompanied by characteristic skin nodules. Generalized lymphadenopathy was observed from 13 dpi. Severe generalized LSD could be seen in three of the heifers, mild LSD in another two heifers, while the other heifers showed no obvious clinical signs. None of the heifers was found to be pregnant when ultrasonographic examination was performed 28 days after AI. None of the control animals tested positive by PCR or had seroconverted by the end of the trial (Table 1).

Necropsy findings

Sub-acute to chronic-active, severe diffuse haemorrhagic, deeply necrotic to sloughing vulvovaginitis and metritis were observed macroscopically during necropsy. The ovaries appeared normal with follicles in different developmental stages visible. Microscopically, severe multifocal thromboses with concurrent infarction, ischaemia and tissue devitalization characterized the uterus. Severe vasculitis combined with a lymphoplasmacytic cell infiltrate was present. Immunoperoxidase staining of the uterine tissues revealed positive, red granular cytoplasmic staining with an apparent pre-dilection for macrophages. LSD viral DNA and virus could be demonstrated by PCR and VI in the reproductive organs (excluding ovaries) of both heifers. Transmission electron microscopy demonstrated LSD virus in skin samples.

Discussion

The risk of transmitting LSDV via semen is not only a major concern mainly for the export industry in South Africa, but also by determining the significance of the presence of the virus in semen, light will be shed on the epidemiology of the disease. The persistent shedding of LSDV in the semen of infected bulls was described by both Irons et al. (2005) and Tuppurainen et al. (2005), while the presence of viral DNA was demonstrated in all fractions of semen and in sheath wash samples from bulls that were shedding virus in semen for protracted periods of time (Annandale et al., 2010). This trial is the first experimental demonstration of seminal transmission of LSDV in heifers, thereby confirming the biosecurity risk posed by semen infected with LSDV.

Although many viruses are known to be shed in the semen of bulls (Afshar and Eaglesome, 1990), the true significance of the presence of virus in semen seems unclear. Burgess (1973) inseminated heifers with bovine ephemeral virus and failed to produce clinical disease, but did manage to show the development of antibodies two weeks after insemination. Similarly, Kupferschmied et al. (1986) demonstrated humoral immunity subsequent to AI with semen spiked with bovine herpes virus 1 but was only able to induce very slight clinical signs of infectious bovine rhinotracheitis (IBR). Seminal transmission of bovine viral diarrhea virus (BVDV) was demonstrated, as well as foetal infection via placental circulation (Meyling and Jensen, 1988). Reports of persistently infected calves following insemination with BVDV-infected semen (Niskanen et al., 2002) support the epidemiological role of BVDV in the spread of the disease and justifies the control measures that are in place.

In the current trial, seroconversion could be demonstrated in eight of the 11 heifers inseminated with semen spiked with LSDV. Babiuk et al. (2008) established that the shedding of LSDV is low in bodily secretions (semen was not investigated specifically), and that the highest concentration of viral particles could be found in skin lesions. We postulate therefore that it is unlikely that similar infection rates would be achieved in herds where natural mating takes place or in herds where AI is practiced, considering the expected lower infective dose. The natural infective dose in semen still remains to be established. Serologically, the detection of an SNT positive sample on 9 dpi is unusual. Irons et al. (2005) detected the first positive SNT sample on 12 dpi. Possible explanations for the difference in results of the two trials could be the higher viral load used in the current trial and the intra-uterine route of infection, allowing different exposures to the immune system than the intravenous or intradermal route of infection.

The low pregnancy rates in this trial are noteworthy. While the exact reason for this was not investigated, the effect of viraemia or a direct virus effect cannot be excluded.

Necropsy results are similar to what has been previously reported in the literature (Prozesky and Barnard, 1982; Nagi, 1990; Annandale et al., 2010). In the current trial, LSD skin lesions seemed less severe. However, lesions on mucous membranes, especially of the conjunctiva, nose, mouth and reproductive tract seemed more pronounced than what can normally be seen in the field (Coetzer, 2004). The difference in severity of clinical disease between the AI group and Embryo group suggests that embryo flushing on Day 6 had a protective effect on the reproductive tracts of the heifers in the ET group. Of the two animals that were necropsied, one had outspoken necrosis in the entire reproductive tract while the other one had only
vaginal and vestibular necrosis. This finding might indicate
that the clinical signs associated with LSDV infection via
natural mating may differ from that of AI, as the cervix
serves as a natural barrier during mating. This finding war-
rants further investigation. It has to be noted though that
the small number of experimental animals in this trial pre-
cludes statistical analysis and definitive conclusions.

The current study confirms transmission of LSDV to
heifers and embryos through AI. The high titre of virus
used to spike semen in the current trial precludes conclu-
sions on the quantification of the biosecurity risks associ-
ated with the presence of LSDV in bovine semen under
field conditions. Further studies are indicated to investigate
the biosecurity risk under field conditions. The effect of the
route of infection on the clinical course of clinical LSD sim-
ilarly needs further investigation.

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