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skin disease virus in the genital tract of experimentally infected bulls;

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Running title: LSDVsemen

Abstract

The objectives of this work were to determine the site of persistence of lumpy skin disease virus (LSDV) in bulls shedding the virus in semen for a period longer than 28 days, to determine if the virus is present in all fractions of semen and to study lesions that developed in the genital tract. Six serologically negative postpubertal bulls were experimentally infected with a virulent field isolate of LSDV. The polymerase chain reaction (PCR) was performed on sheath washes, vesicular fluid, supernatant and cell-rich fractions of semen from Day 10 to Day 26 post infection (p.i.) Bulls that were positive by PCR on the whole semen sample collected on Day 28 p.i. were slaughtered and tissue samples from their genital tracts submitted for histopathological evaluation, immunoperoxidase staining, virus isolation and PCR.

Two of the bulls developed severe lumpy skin disease (LSD) and were found to be shedding viral DNA in their semen on Day 28 p.i. Viral DNA was identified in all semen fractions from all bulls, but mostly from the cell-rich fraction and from the severely affected bulls. The PCR assay was positive on post mortem samples of testes and epididymides from the two severely affected bulls. Virus could be recovered from the testes of these two bulls and from the epididymis of one of them. Immunoperoxidase staining was observed in sections of testes and epididymides exhibiting necrosis.

This study suggests that the testis and epididymis are the sites of persistence of LSDV in bulls shedding virus in semen for prolonged periods and revealed that viral DNA is present in all fractions of the ejaculate.

Keywords: Lumpy skin disease virus; *Capripoxvirus*; bull; semen

Introduction

Lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*, causes an acute, subacute or chronic disease of cattle, characterized by fever and the formation of multiple firm, circumscribed nodules in the skin of affected animals and necrotic plaques in the mucous membranes, as well as generalized lymphadenopathy (Coetzer 2004). The disease has significant economic implications not only in subSaharan Africa and Egypt, where it is endemic, but also internationally (Agag et al. 1992, OIE 2001). It is therefore classified as a disease that is notifiable to the OIE (2001). Economic losses are incurred due to damage to hides, reduced feed intake, pneumonia, mastitis and infertility. Although mortality rarely exceeds 3%, it can be as high as 40% (Coetzer 2004). The deleterious effect that the disease has in affected bulls on their ability to mate and to produce fertile sperm is often not appreciated, but has important economic implications, especially in the context of subsistence farming and in farming systems where single sires are used. A recent review article (Hunter and Wallace 2001) has identified the need for research into the epidemiology and transmission of lumpy skin disease (LSD) in South Africa due to its resurgence over the last decade of the 20th century.

The exact method of transmission of LSDV remains unknown but circumstantial evidence suggests that biting flies play a role as vectors (Woods 1988). Direct or fomite-mediated transmission of LSDV between animals is inefficient (Carn and Kitching 1995b). The potential for transmission via semen has serious implications, particularly for the international movement of bovine semen (Hentzen 2000). The presence of LSDV in semen can be detected by virus isolation

(VI) and by polymerase chain reaction (PCR), and recently several methods of improving the diagnostic sensitivity of these methods have been published (Bagla et al. 2006, Tuppurainen et al. 2005). Although Weiss (1968) stated that LSDV is shed in semen for 22 days after the fever reaction, Irons (Irons et al. 2005) isolated viable virus in semen 42 days after infection and identified viral DNA up to 5 months after infection. The risk of the presence of LSDV in semen poses a potential threat to movement of semen from countries where LSD is endemic (Hentzen 2000). There is currently no information on the ability of semen infected with LSDV to establish clinical disease in cows inseminated with it, nor is there any data available as to the pathobiological mechanisms involved in the shedding of virus and viral particles into the ejaculate.

The objectives of this work were to determine the site of virus persistence in bulls shedding virus in semen for a period longer than 28 days, to determine if the virus is present in all fractions of the semen, to study the development of lesions in the genital tract and to compare such lesions with the presence of virus in different fractions of the semen.

Materials and methods

Animals and housing

Six healthy Dexter bulls, which had not been vaccinated for LSD, and with no detectable neutralizing serum antibodies to LSDV were selected. All bulls were reared in semi-extensive conditions on a commercial farm and were between 13 and

17 months of age. Semen containing motile spermatozoa was collected from them by electrical stimulation prior to their selection for the trial. They were housed in vector-protected stalls at the University of Pretoria's Biomedical Research Centre (UPBRC) for the duration of the experiment. The experiment was approved by the University of Pretoria Animal Use and Care Committee (V37/04).

Experimental infection, observations and sampling

After an acclimatization period of 14 days, the bulls were artificially infected by intravenous injection of 2 ml of virus suspension. Virus was prepared by culture of a pathogenic field isolate of LSDV of known history (strain V248/93) on bovine dermis cell monolayers. After harvesting it, its infectivity was established by titration and adjusting the infective dose to a titre of 10^5 TCID₅₀/ml. From the day of infection (Day 0) the animals were observed daily for the development of clinical signs. Rectal temperatures were measured twice daily. A reproductive examination was performed every other day, which comprised measuring the scrotal circumference, palpation of the testes and epididymides, and an ultrasound examination of the testes and accessory sex glands using an Aloka SSD-500 ultrasound machine* and 5 MHz linear probe. A rigid 15 mm Perspex tube, cut longitudinally along its 40 cm length, was attached to the ultrasound probe and enabled evaluation of the accessory sex glands without introduction of the arm of the operator into the rectum of the animal. Blood samples were collected every other day for the duration of the trial and were subjected to virus isolation from Day 6 p.i. On

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Day 28, serum samples were collected and subjected to a serum neutralization test to confirm whether or not seroconversion had occurred.

After infection of the bulls preputial washes were performed every other day for the duration of the trial by infusing 50 ml phosphate buffered saline (PBS) into the preputial cavity and then massaging the preputium vigorously for one minute before collecting the samples. The vesicular glands were massaged per rectum prior to insertion of the probe of the electro-ejaculator and the fluid emanating from the preputial opening was collected. Semen was collected by electrical stimulation into new graduated collection tubes to which latex cones were attached. It was then centrifuged at 250g for 10 min to separate it into a cell-rich fraction and supernatant. Each bull was assigned a collection cone that was used only for that bull for the duration of the trial and the collection cones were washed, scrubbed and rinsed with distilled water between uses to prevent cross-contamination of samples. Unfractionated semen samples were collected on the last day of the trial and subjected to PCR to identify the bulls that were still shedding viral DNA in their semen at that stage. A PCR was done on the sheath washes, vesicular fluid, supernatant and cell-rich fractions of the semen from Day 10 p.i. to Day 26 p.i. Virus isolation was not performed on semen samples as it has previously been determined that this method has a low sensitivity for the detection of LSDV in semen (Irons et al. 2005). The presence of blood in the semen was assessed by microscopy of semen smears stained with Diff-Quick[®].

Bulls that were positive by PCR on the whole semen sample collected on Day 28, were slaughtered and a diagnostic post mortem was performed. The genital tracts and tissue samples from them were taken and treated accordingly for histopathological evaluation, virus isolation and PCR. Tissue samples that showed evidence of viral-

induced damage, from which virus could be isolated or in which viral DNA could be detected, were then subjected to immunoperoxidase staining and transmission electron microscopy.

Polymerase chain reaction test

The PCR was performed using a QIAmp[®] extraction kit with commercially available primers for LSDV. The forward and reverse primers had the sequences 5'-TTTCCTGATTTTTCTTACTAT-3' and 5'-AAATTATATACGTAAATAAC-3' respectively, rendering an amplicon of 192 bp (Ireland and Binopal 1998). A positive control consisting of bovine semen spiked with LSDV and a negative bovine semen control as well as a water control were included in the PCR. Amplified products were analysed using a 100 bp DNA ladder as a molecular marker on 1.5% agarose gels. Amplicons were visualized using an UV transilluminator at a wavelength of 590 nm and positive reactions were confirmed according to size.

Virus isolation

Virus isolation was performed on heparinized blood samples from Day 6 p.i. and on tissue samples collected at necropsy. The cell cultures used comprised bovine dermis (BD) cells, maintained in minimum essential media (MEM) containing 4% foetal calf serum (FCS) and 1 ml gentamycin (0.1 mg/ml). Flasks were incubated at 37°C in an atmosphere of 5% CO₂. Virus isolation from the blood samples was performed by inoculation of 0.5 ml from each sample into the cell cultures. The cell cultures were observed daily for cytopathic effects (CPE). After 14 days, negative cultures were

frozen briefly at -70°C and then thawed. A second passage was done and each culture was observed for a further 14 days. Virus isolation from tissue samples was accomplished by mincing the tissue with sterile scissors and then grinding it with a pestle in a mortar containing sterile sand. Ten ml PBS containing gentamycin (0.1 mg/ml), ampicillin (0.05 mg/ml) and amphotericin B ($5\mu\text{g/ml}$) were added to the ground up tissue and the resulting suspension was left to stand overnight at 4°C . The following day suspensions were partially clarified by centrifugation at 2000 rpm for 3-5 min to remove gross particles and 0.5 ml of them was then inoculated into the BD cell cultures in 25 cm^3 flasks. Negative controls were included in each batch of specimens tested. These consisted of a cell monolayer without any virus and a positive control comprising a cell monolayer inoculated with 0.2-0.5 ml LSDV (strain V248/93) suspension at a titre of 4 log TCID_{50} .

Histopathology and electron microscopy

Tissue sections for histopathology were prepared using routine procedures and were stained with haematoxylin and eosin (H&E). Immunohistochemistry, using immunoperoxidase staining, was performed on tissue sections prepared from parts of the genital tract that, on microscopic examination of H&E stained sections, revealed evidence of viral-induced damage. The primary antibody used for immunohistochemistry was developed in New Zealand White rabbits following two subcutaneous inoculations of the South African strain of LSDV, 21 days apart. The rabbits were then inoculated with gamma radiation-inactivated virus on day 31 and euthanased and exsanguinated on day 41 post-inoculation. The serum was prepared using standard procedures and stored at -20°C . The results of immunoperoxidase

staining were validated against samples from the testes of bulls known to be free of LSD. Transmission electron microscopy (TEM) was performed on positively stained preparations of testis samples.

Results

Clinical signs and serology

Fever appeared on Day 5 or 6 in all the animals, while skin lesions became apparent on Day 7 in the animals that did develop them. After an incubation period of 6 days, two of the bulls, Bull S1 and Bull S2, developed severe clinical signs of LSD. They became depressed, were inappetent and remained febrile for 15 and 22 days, respectively. Generalized lymphadenopathy of the superficial lymph nodes developed in both of them from 10 days p.i. and was still present at the day of their slaughter. Multiple skin lesions consisting of raised nodules of 5 to 20 mm in diameter typical of LSD appeared mainly on the flanks, paralumbar fossae, ventrum and scrotum. These lesions became ulcerated and healed leaving areas of scarring towards the latter part of the experiment. These two bulls also developed corneal opacities in the last 2 weeks of the trial and showed some respiratory distress during this time. They were treated with anti-inflammatory drugs and antimicrobials for pneumonia. Semen from these bulls was PCR positive for LSDV viral DNA on Day 28 and they were subsequently slaughtered.

Bull M1 and Bull M2 showed mild clinical signs of LSD consisting of fever for 4-7 days, a small number of skin lesions and mild enlargement of superficial lymph

nodes, while infection in the remaining two bulls (I1 and I2) was inapparent with the exception of a transient fever.

All the bulls were serologically positive at the end of the experiment as determined by the serum neutralization test.

Scrotal circumference, clinical findings in testes and epididymides

The two bulls that showed severe clinical signs of LSD showed pronounced changes in their scrotal circumference measurements. Their scrotal circumference initially increased, but in Bull 2 this was followed by a decline to below pre-infection values, while the scrotal circumference of Bull S1 returned to the value it was at the commencement of the trial. The scrotal circumference measurements for the other four bulls showed far less variation but it did increase progressively during the trial period. Abnormalities were only detected in the scrotums, epididymides and testes of the two severely affected bulls. These include LSD nodules in the skin of the scrotums, scrotal oedema and the presence of nodules which could be palpated predominantly in the tails of the epididymides. The consistency of the testes of these two bulls decreased progressively from Day 10 p.i. and was very soft by the time they were slaughtered.

Ultrasonographic changes

Ultrasonographic changes in the reproductive organs were limited to the two severely affected bulls. A transient decreased echogenicity of the parenchyma of the vesicular glands and oedema of the mucosa of the glands could be demonstrated from Day 10

to Day 18 p.i. In Bull S1, a focal hyperechoic area of 8 mm in diameter could be demonstrated in the left *ampulla ductus deferens* from Day 22 – 28 p.i. Ultrasonographic changes of the scrotum and scrotal contents included a thickened scrotal skin, scattered hyperechoic foci in the testes (corresponding to areas of infarction seen on post mortem examination) and anechoic foci in the epididymides (subsequently found to be epididymal granulomas). These changes were first visible on Day 10 p.i. and persisted for the remainder of the experiment.

Post mortem examination and histopathology

The macroscopic pathology of Bulls S1 and S2 was typical of that of severe LSD and included multiple nodular skin lesions, corneal opacities, generalized lymphadenopathy and chronic-active interstitial pneumonia. Areas of fibrosis, necrosis and infarction could be visualized in the testes and epididymides.

Microscopically, in the testes of both bulls, a mild diffuse orchitis accompanied by a segmental necrosis of seminiferous tubules was visualized. The germinal compartment of the testes of Bull S1 revealed the presence of normal Sertoli cells, but very few spermatogonia and spermatocytes. Some tubules in sections of the testes of this bull were totally devoid of spermatogenic cells. In contrast, sections of the testes of Bull S2 showed relatively more seminiferous tubules in which spermatogonia still in contact with the basement membrane were retained. Multifocal to coalescing granulomatous epididymitis could be demonstrated in sections of the epididymides from Bull S1 and Bull S2. In some of the more severe lesions, the entire epididymal ductule was obliterated by granulomas containing necrotic tissue.

Microscopic pathology of the accessory sex glands was restricted to the *ampullae ductus deferentes* in both bulls and was characterized by chronic inflammation, obstruction and secondary dilation of glandular lumina by casts of necrotic spermatids, spermatozoa and neutrophils.

Immunohistochemistry

Necrotic tissue in the testes of Bulls S1 and S2 stained a pale diffuse to dark granular orange indicating the presence of LSDV antigen. The granulomas containing necrotic tissue in the epididymides of both bulls showed similar positive staining, some pigmented granules occurring in the cytoplasm of necrotic cells.

Electron microscopy

Lumpy skin disease virions could be seen in a positive stained section of the testis from Bull S1 but not Bull S2. Poxvirus particles could be demonstrated in wax-embedded testicular sections from the bull that showed LSDV-positive staining on immunohistochemistry.

Virus isolation

All bulls except Bull I1 had evidence of viraemia, which was of an intermittent nature and persisted for 10 – 14 days in Bulls S1 and S2 and 4 days in Bull M1 and Bull M2, while virus was isolated from the blood of Bull I2 only on one occasion. Virus could

not be recovered from tissue samples of the ampullae, vesicular glands and prostate glands of Bull S1 and Bull S2. Virus was only recovered from the testes of Bulls S1 and S2 and the epididymis of Bull S1, but not from the epididymis of Bull S2.

PCR Assay

The PCR amplified viral DNA in all of the fractions of the ejaculate from all the bulls at some stage p.i.. The cell-rich fractions of Bull S1 and Bull S2 were PCR positive for all samples analyzed from day 10 p.i. Viral DNA could be identified in 66.7% (6/9) of sheath wash samples; 88.9% (8/9) of vesicular fluid samples; and 44.4% (4/9) of supernatant samples collected from Bull S1. This is compared with 55.6% (5/9) for all these samples collected from Bull S2 during the same time. The PCR assay was consistently positive when performed on the testicular tissue from Bulls S1 and S2 and epididymis of Bull S1, and gave a faint positive reaction after a second PCR was performed on dilutions of extracted DNA of the epididymis of Bull S2. Samples from the ampullae and vesicular and prostate glands of Bulls S1 and S2 were negative by PCR.

Insert table 1 here

Discussion

This work further confirms the phenomenon of persistent shedding of LSDV in the semen of infected bulls as described by Irons *et al.* (2005). Lumpy skin disease viral

DNA was found in all fractions of semen and in sheath wash samples from the bulls that were shedding virus in the semen for protracted periods of time.

The scrotal circumference of all the bulls increased shortly after the onset of fever, reached a maximum 2 weeks p.i. and then declined progressively until the end of the trial. The scrotal circumference of Bull S1 was the same value as it was at the commencement of the experiment, while that of Bull S2 was 15% lower than at the start. The softness of the testes and the decreased scrotal circumference suggested severe testicular degeneration, which was confirmed at post mortem examination. Histopathological examination of the tissue specimens of the testes of Bulls S1 and S2 in this experiment revealed that necrosis was the main underlying pathological change. Although not commenting specifically on testicular changes, Prozesky and Barnard (1982) concluded that vasculitis and thrombosis, leading to oedema and necrosis was central to the pathogenesis of the lesions in LSD. The initial increase in scrotal circumference can thus possibly be ascribed to an inflammatory oedema. The extent of testicular degeneration in Bull S2 was more severe than in Bull S1. The reason for this is unknown. A possible explanation for different animal responses to challenge with LSDV is genetic resistance as determined by major histocompatibility complexes (MHC) found on cell surfaces of individuals (Amills et al. 1998).

The testicular and epididymal pathology reported here is more severe than that described by Nagi (1990) as numerous seminiferous tubules were totally devoid of primary spermatogonia and extensive necrosis with obliteration of epididymal ducts could be demonstrated. Possible reasons for these differences could be the route of infection and/or the protracted illness that the bulls in the present study suffered from.

The tissue specimens in the report by Nagi may have originated from animals with a generalized infection as well as from those that showed only localized skin and mucous membrane lesions. It has been shown experimentally that the first appearance and severity of such localized lesions is related to dose of virus that the animal received (Carn and Kitching 1995a). It is possible that the intravenous route of infection in the present study led to a higher generalized viral load, which had a greater effect on the spermatogonia. Spermatogenesis is determined by the amount of functional seminiferous tissue (Johnson 1991). Severe pathology of the epididymal ducts as was present in these two bulls may also have resulted in permanent occlusion of them, which would also have resulted in a decline in fertility. It is therefore likely that these bulls would have suffered from reduced sperm production if they had recovered from the infection, and not been slaughtered.

In Bull S1 there was a good association between lesions in the accessory sex glands, as determined by ultrasonography, and the identification of viral DNA in vesicular fluid samples by PCR. Only on one occasion were ultrasonographic lesions observed without the vesicular fluid samples simultaneously being positive by PCR. This relationship, however, was not well established in Bull S2. In this bull viral DNA was only found in vesicular fluid samples on three occasions on which ultrasonographic lesions were concurrently identified. In animals suffering from LSD therefore, the usefulness of ultrasonographic changes as a predictor of viral shedding in genital fluid is questionable.

The results obtained in this experiment suggest that LSDV found in the semen of affected animals is not due to contamination of the semen with blood. The semen was

positive by PCR after the time when virus was no longer detectable by virus isolation in the blood. Furthermore, semen samples were tested for the presence of blood and found to be free.

The fact that viral DNA could only be recovered from testicular and epididymal tissues in both of the bulls which were still shedding virus 28 days p.i., combined with the fact that viral antigen could be demonstrated by immunoperoxidase staining in necrotic tissue in these organs, suggests that the testis and epididymis are the sites of persistence of the virus. While initially virus was found in both the supernatant as well as the cell-rich fraction of the semen, indicating that it was not strictly associated with the spermatozoa, it is possible that in the later stages of the persistent shedding state virus particles are associated with the sperm cell. Further research is required to investigate this aspect. It is considered that the results of the PCR performed on vesicular fluid samples should be interpreted with caution as the possibility that the samples were contaminated *en transit* through the preputial cavity cannot be excluded.

The demonstration of viral antigen by immunoperoxidase staining in necrotic testicular tissue is significant. A general characteristic of members of the *Poxviridae* is that they are relatively resistant to unfavourable conditions, and are not dependant on the presence of live cells for their survival; LSDV has, for example, been shown to survive in skin scabs for up to 33 days (Tuppurainen et al. 2005). If virus can persist for this length of time in necrotic skin, it is likely that it survives for at least as long in necrotic testicular tissue. The voiding of such necrotic tissue into patent seminiferous tubules, would explain the presence of LSDV in semen for prolonged periods and as

no evidence was encountered of viral activity in other parts of the genital tract, this is deemed to be the most likely explanation for this phenomenon. Further studies are required to confirm or refute this possible phenomenon.

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Table 1. A summary of the results on ante-mortem testing for the two severely affected bulls.

Days	post-	6	8	10	12	14	16	18	20	22	24	26	28	
infection														
Bull	Parameter													
S1	Clinical signs	+	+	+	+	+	+	+	+	+	+	+	+	
	VI blood*		+		+	+	+		+					
	PCR-SW*			+	+				+	+	+	+		
	PCR-VF*			+	+	+	+		+	+	+	+		
	PCR-SN*					+	+		+	+				
	PCR-CR*			+	+	+	+	+	+	+	+	+	+	#
S2	Clinical signs	+	+	+	+	+	+	+	+	+	+	+	+	
	VI blood		+		+		+	+		+				
	PCR-SW			+	+		+		+	+	+	+		
	PCR-VF						+		+	+				
	PCR-SN				+	+			+	+		+		
	PCR-CR			+	+	+	+	+	+	+	+	+	+	#

Note: * VI blood: Virus isolation on blood; SW Sheath wash; VF vesicular fluid; SN supernatant; CR cell rich

Semen collected on Day 28 p.i. was not fractionated

Figure 1: Electron micrograph of lumpy skin disease virus from the testis of Bull S1

