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The demonstration of lumpy skin disease virus in semen of experimentally infected bulls using different diagnostic techniques

By

Victor P. Bagla

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DEDICATION

To my mum, late Mrs. Princess Mamie Bagla, may the lord grant you a perfect rest.

DECLARATION

I hereby declare that the work presented in this, apart from the assistance received as reported in the acknowledgement and in the appropriate places in the text, this dissertation represent the original work of the author.

No part of the dissertation has been previously submitted and is not being submitted in candidature for any other degree at any other University.

Signature of candidate **Date / / 2005**

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ABSTRACT

Lumpy skin disease virus (LSDV), a poxvirus that belongs to the genus *Capripoxvirus* is an important pathogen that can be shed in the semen of infected bulls. The screening of semen for infectious virus prior to artificial insemination requires a sensitive diagnostic method. The isolation of the virus on cell cultures and/or the use of polymerase chain reaction (PCR) are sensitive diagnostic tests which can be used to screen semen for LSD viral DNA prior to artificial insemination. Although cell culture is a sensitive method and detects infectious virus, its use has major limitations due to the toxic effect of semen on the cells. This study was therefore aimed at finding a method that decreases the toxic effect of semen on cell culture and enhances LSDV isolation. Secondly, the efficiency of this method in enhancing the isolation of LSDV in field samples was tested.

In order to eliminate the toxic effect of semen on cell culture, a pilot study was conducted in which semen samples from LSDV sero-negative bulls were collected and infected with a field isolate of LSDV, strain 248/93 with a titre of 6.5 log $TCID_{50}$. The semen samples were subjected to one of four different methods, viz centrifugation, serial dilution, filtration and chemical treatment with kaolin. The centrifugation, serial dilution, and filtration methods were supplemented with additional amounts of gentamycin.

The toxic effects of semen on cell culture were completely eliminated when supernatants of semen samples, centrifuged at 2000 rpm for 1, 3 and 5 mins and serial diluted was used to inoculate confluent monolayers of bovine dermis cells. Semen diluted in MEM with or without additional antibiotics was the most sensitive method of demonstrating virus at higher dilutions, followed by pellets of samples centrifuged for 1 and 3 minutes. The toxicity recorded when the pellet fraction of semen samples were centrifuged for 5 mins at 2000 rpm was comparable to results obtained from serially diluted samples supplemented with gentamycin. The use of filtration and kaolin treatment of semen samples could not remove the toxic effect of semen on cells.

To evaluate the presence of LSDV in semen of experimentally infected bulls, six seronegative post-pubertal bulls housed in an insect proof facility were infected with LSDV via the intravenous route. The experimentally infected bulls were monitored for clinical sign of the disease. Two bulls showed severe, two a mild and two an inapparent infection. Blood samples were collected for virus isolation and semen samples for virus isolation and

PCR. Vesicular fluid and preputial washes were also investigated for the presence of LSD viral nucleic acid using PCR. The infectious titre of the virus shed in semen of these bulls was also calculated.

The incubation period in infected bulls varied from 7 to 14 days. The length of viraemia varied between groups and did not correlate with the severity of clinical disease. The virus was isolated from blood samples of bulls in the severely infected group on several occasions. Bulls in the mildly infected group had the lowest rate of isolated virus when compared to those with inapparent infection.

The use of supernatants of centrifuged serial diluted semen samples, as shown in the pilot study, have considerably reduced the toxic effect of semen on cell culture. This method was used to test field samples for its sensitivity to isolated LSDV in semen of experimentally infected bulls with PCR as a gold standard.

In all the semen samples tested using supernatants of semen samples LSDV was isolated in 53.1% of the samples on cell culture while in the serial diluted samples, only 28.1% of samples were positive with a median time of detection on cell culture of 4 and 8 days, respectively. The use of the supernatant fraction was able to detect infectious LSDV in semen samples for prolonged periods with reduced time of development of cytopathic effect, than previously reported.

In order to compare the sensitivity of PCR and virus isolation, PCR positive and a few negative samples were subjected to virus isolation using the centrifugation method developed in the pilot study. The PCR was able to detect LSD viral nucleic acids in some semen samples even when virus could not be isolated on cell culture.

The PCR was also able to detect viral nucleic acid in vesicular fluid and preputial washes of infected bulls. The titre of the virus shed in the semen at a certain stage of the infection was calculated to be 3 log $TCID_{50}$.

In conclusion, this study provides evidence of a complete reduction of the toxic effect of semen on cell culture and increase chances of LSDV isolation with reduced detection time when semen samples are processed using the centrifugation method as described in the pilot study. Furthermore, it showed PCR was more sensitive than virus isolation in the

detection of LSD viral nucleic acid in semen samples and can be used for routine diagnosis. However, virus isolation must be used when the infective nature of virus shed in semen is desirable. This study provides the first evidence of the shedding of LSDV nucleic acid in vesicular fluid and preputial washes of experimentally infected bulls. It also represents the first report that a considerable amount of LSDV is shed in semen of experimentally infected bulls, which may be infective at certain stages of clinical disease.

LIST OF ABBREVIATIONS

AGIP	Agar-gel immunodiffussion test
AI	Artificial insemination
AKV	Adult vervet monkey kidney cell
BD	Bovine dermis
BHK-21	Baby hamster kidney cell
bp	Base pair
BVDV	Bovine viral diarrhea virus
CPE	Cytopathic effect
dATP	Deoxy adenine triphosphate
dCTP	Deoxy cytocine triphosphate
dGTP	Deoxy guanidine triphosphate
DNA	Deoxiribonucleic acid
DTT	Dithiothreitol
dUTP	Deoxy uracil triphosphate
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylendiamine tetra-acetic acid
ELIZA	Enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organization
FAT	Fluorescent antibody test
FCS	Foetal calf serum
FMDV	Foot and mouth disease virus
HIV	Human immunodeficiency virus
hrs	Hours
IBRV	Infectious Bovine rhinotraechietis virus
KCl	Potassium chloride
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
mins	Minutes
mM	Milli molar
NaCl	Sodium chloride
ng	Nano gram
nm	Nano metre
OIE	Office International des Epizooties
p.i.	Post inoculation
PBS+	Phosphate buffered saline containing Mg^{2+} and Ca^{2+}
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PRRSV	Porcine reproductive and respiratory syndrome virus
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
sec	Seconds
SNT	Serum neutralization test
SOP	Standard operative procedure
Taq polymerase	*Thermus aquaticus* DNA polymerase
tc	Toxicity
$TCID_{50}$	Tissue culture infective dose 50
Tris-HCl	Tris hydrochloride

UDG	Uracil DNA glycosylase
UPBRC	University of Pretoria Biomedical Research Centre
USA	United States of America
VNT	Virus neutralization test
µl	Micro litre
µM	Micro molar

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CHAPTER 1

1. INTRODUCTION

Lumpy skin disease (LSD) is an infectious, eruptive, occasionally fatal disease of cattle caused by a virus that belongs to the family *Poxviridae* and shares the same genus *Capripoxvirus* with sheep pox and goat poxviruses. The disease is characterized by nodules in the skin, mucousal membranes, enlarged superficial lymph nodes and occasionally, death (Kitching and Taylor 1985b; Davies 1991).

The outbreak of the disease has been reported in a wide range of ecotypes in Africa and appears to have spread to virtually all countries on the continent. Since its emergence in 1929, the first case outside Africa was recorded in Israel in 1989 (Abraham and Zissman 1991)

The cycle of sporadic disease and epizootics results in severe economic losses. Losses are mainly due to morbidity. Recorded morbidity rates have varied greatly from as low as 5% to 100% while mortality rates rarely exceed 5% (Merck veterinary manual 1998). Fever and general malaise cause weight loss and purulent mastitis accentuates the fall in milk yield. Abortion in pregnant cows and permanent or temporary sterility in bulls has been observed. The skin lesions of LSD cause permanent damage to hides with financial losses through their rejection or reduced value (Green 1959). The disease limits the productivity of high producing breeds introduced from outside the endemic area. The prevention of cattle trade and movement restrictions causes significant economic losses.

Lumpy skin disease was considered a “List A” disease by the Office International des Epizooties (OIE) that lists a disease according to its potential for rapid spread and ability to cause severe economic losses. It has also been added to the European Economic Community council directive 82/894/EEC on the notification of animal diseases within the community and as such any incidence of the disease is to be reported within 24 hrs of a primary outbreak to the commission and member states (Carn 1993). Vaccination with live attenuated virus has been commonly employed. The South African Neethling and Kenyan sheep pox and goat poxvirus vaccines are widely used in Africa (Capstick and Coackley, 1961; Weiss, 1968).

1.1 History of lumpy skin disease

The clinical signs of LSD were first described in Zambia in 1929 (MacDonalds 1931; Morris 1931). Initially, it was considered to be the result either of poisoning or hypersensitivity due to insect bites. Le Roux first referred to the disease as "pseudo-urticaria" in 1945 (cited by Weiss 1968) and believed the disease was caused by a plant poison and gave it the name "lumpy disease". The infectious nature of lumpy skin disease was first recognized by Von Backstrom (1945) when an outbreak occurred in Ngamiland in 1943. However, Thomas and Maré (1945) were the first to demonstrate the transmissibility of the infectious agent by subinoculation of suspensions of skin nodules. The virus causing lumpy skin disease was first isolated in tissue culture by Alexander *et al.* (1957).

1.2 Aetiology

Early attempts to isolate and characterize the etiological agent of LSD incriminated a virus as the causative agent (Thomas and Mare 1945). More than one virus was isolated from typical skin lesions on a number of occasions; these were divided into three groups:

Group 1. Represented by an orphan virus bovine herpesvirus 4

Group 2. Allerton virus - bovine herpes mammilitis or bovine herpesvirus 2

Group 3. Contains a virus that resembled vaccinia virus (Weiss 1963; Munz and Owen 1966).

Table 1: Poxviruses of vertebrates (Carn 1993)

Capripoxvirus	Sheeppox virus, goatpox virus and bovine lumpy skin disease virus
Parapoxvirus	Pseudocowpox virus, bovine papular stomatitis virus and contagious pustular dermatitis virus
Orthopoxvirus	Cowpox virus and vaccinia virus
Suipoxvirus	Swinepox virus
Avipoxvirus	Fowlpox, canarypox, juncopox viruses
Leporipovirus	Hare fibroma, myxoma, rabbit fibroma viruses
Molluscipoxvirus	Molluscum contagiosum virus
Yatapoxvirus	Yaba and tanapox viruses

One of the first purified group 3 virus isolates was a South African isolate named the Neethling-isolate which became officially known in Africa as LSDV, type Neethling (Alexander *et al*. 1957; Weiss 1963).

Lumpy skin disease virus belongs to the family *Poxviridae* which is divided into two subfamilies: *Entomopoxvirinae* (Poxviruses of insects) and *Chordopoxvirinae* (Poxviruses of vertebrates). Genera within the vertebrate members of the *Poxviridae* family are listed in Table 1.

1.2.1. Lumpy skin disease virus

The vertebrate poxviruses share a group specific antigen, called NP antigen, which is obtained from viruses by alkaline extraction of the virus particles (Woodroofe and Fenner, 1962). Poxviruses have numerous antigens in common, but every species have their own species-specific polypeptides (Fenner *et al.* 1987).

The morphology of the capripoxviruses causing sheeppox (Abdussalam 1957), goatpox (Tantawi and AlFulliji 1979) and lumpy skin disease has been described (Kitching and Smale 1986; Munz and Owen 1966). The virions are ovoid and classified as “C” (capsule or “C” form) or “M” if surrounded by a membrane or covered in cordlike elements (mulberry or M form).

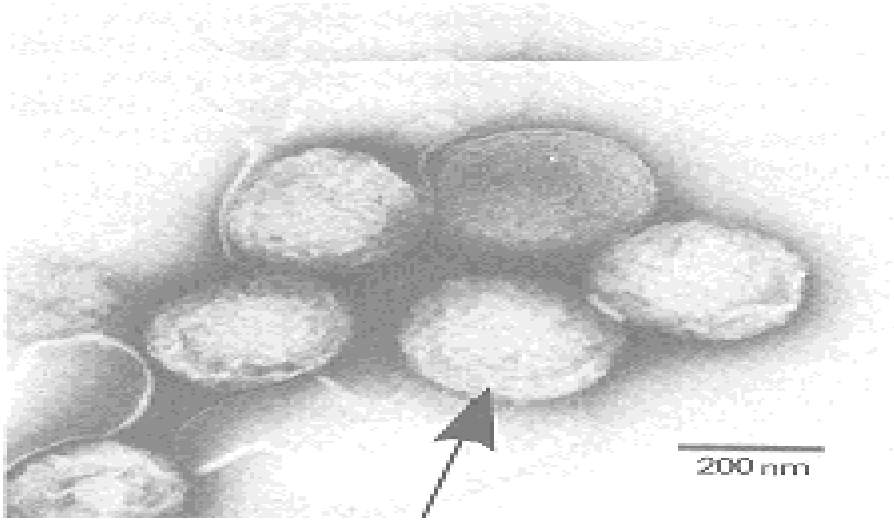

Figure 1: Electron micrograph of a capripoxvirus isolate. Arrow shows “ M” form surrounded by membrane. (Kitching *et al.* 1986)

Poxviruses are the largest of all animal viruses, with the average size for capripoxviruses being 260-320 nm. The virus is stable between pH 6.6 and 8.6, ether sensitive and readily inactivated by sodium dodecyl sulphate (SDS) and chloroform (Plowright and Ferris 1959b).

Poxviruses contain a double stranded DNA. They appear to be genetically stable on the basis of restriction endonuclease digestion analysis of their DNA (Kitching *et al.* 1989). Lumpy skin disease virus contains a number of host range genes. These genes may play a significant role in the modulation or evasion of host immune responses, inhibition of host cell apoptosis and in cell or tissue tropism. In the disruption or modulation of the host immune response, six LSDV proteins are potentially secreted. These include homologues of cellular and viral interleukin-10 (IL-10), gama interferon (IFN-γ) receptor, IL-1R, IFN-α/β binding protein and IL-18 binding protein. Lumpy skin disease virus is the poxvirus believed to encode two proteins in addition to the poxvirus IFN-α/β binding protein and contains four potentially membrane localized immunomodulatory proteins with functions of potentially binding extracellular factors or influence intracellular signal transduction mechanisms to affect immune mechanisms or host range (Cameron *et al*. 1999; Lalani *et al*. 1999; Massung *et al*. 1993; Sanderson *et al*. 1996).

Lumpy skin disease virus also encodes six of other poxvirus proteins, which affects virus virulence, growth in specific cell types or cellular apoptotic response. These proteins include homologues of the epidermal growth factor, VV C7L host range, N1L virulence and A14.5L virulence proteins, MYX M004 and M011L anti-apoptosis proteins, and the rabbit fibroma virus N1R/ectromelia virus p28 host range factor. It also encodes five other proteins containing ankyrin repeat motifs, two of which are likely orthologues of proteins in leporipoxvirus and sheep poxvirus (Cameron *et al*. 1999; Willer *et al*. 1999). It is believed that specific complements of ankyrin genes dictate poxvirus host range and the same is most likely for LSDV (Antoine *et al*. 1998; Shcelkunov *et al*. 1998).

Entrance of poxvirus into host cells is accompanied by uncoating of virion particles and penetration of the cytoplasm of host cell and replication is independent of the host nucleus in discrete areas of the cytoplasm (Cairns 1960). In a study to determine the early and late transcriptional phases in the replication of LSDV, Fick and Viljoen (1994) observed that LSD virions produced infective foci on cell culture 4-5 days post inoculation (p.i).

Recombination occurs at high levels in poxvirus infected cells and is linked to DNA replication (Ball 1987; Evans *et al*. 1988). From studies on vaccinia virus, it is clear that gene expression is divided into an early and a late phase (Moss 1990; Moss 1992). Early transcripts are synthesized and encoded for functional proteins required for DNA replication. The LSDV mRNA transcriptional switch from early to late transcript was observed to be 9 hours p.i with DNA replication believed to have occurred earlier (Fick and Viljoen 1994). As demonstrated for other poxviruses (Moss 1990), DNA replication is required for late transcription to be initiated (Fick and Viljoen 1994).

1.3. Epidemiology

1.3.1. Occurrence and pathogenicity

Lumpy skin disease was first described in 1929 in Zambia (Morris 1931). The disease reoccurred irregularly in Zambia until 1943 when it spread to Botswana (Von Bactstrom 1945), Zimbabwe, (Houston 1945) and the former Transvaal Province in South Africa (Thomas and Mare 1945). The spread of the disease in South Africa continued and by 1946 it had entered the Free State, Swaziland, Kwazulu Natal and Mozambique. In the following year, 1947, it entered Lesotho and finally the Eastern Cape Province (De Sousa Dias and Limpo-Serra 1956; Diesel 1949).

It subsequently spread across most parts of Africa and occurs in sub-Saharan Africa and Egypt (Ali *et al*. 1990). In 1957 LSD was identified in Kenya, in East Africa (MacOwen 1959) and epizootics were reported north of Sudan in the 1970s (OIE Manual 1996). Between the years 1970-1985 LSD occurred in most Central and West African countries i.e. Nigeria in 1974 (Nawathe *et al.* 1978), Chad and Niger in 1973, Ivory Coast in 1976 and Somalia in 1983 (Davies 1991). The disease is now enzootic throughout sub-Saharan Africa including Madagascar. It was reported in Egypt in 1988 (Ali *et al.* 1990), and an extension occurred eastward into Israel in 1989 probably by the movement of insect vectors (Abraham and Zissman 1991; Yeruham *et al.* 1995). In other Middle East countries, it occurs sporadically (Greth *et al.* 1992). Although outbreak reports in Bahrain and Reunion in 1993 have not been confirmed (OIE Manual 1996), further spread westward may occur despite all the control strategies. Sporadic outbreaks have for example occurred in European countries like Italy in 1993 and Greece in 1989/1990 (Carn 1993). There was a marked increase in clinical disease in southern Africa from 1990 to 1999 (Hunter and Wallace 2001).

Lumpy skin disease can appear sporadically or epidemically. Frequently, new foci of infection appear in areas far removed from the initial outbreaks. The epidemics in different countries have also shown much variation in morbidity and mortality. Mortality rates are usually low but may be as high as 10 % (Thomas and Maré 1945). Variation in morbidity rates was suggested to be the result of strains of different pathogenicity (Carn and Kitching 1995b). Although some authors suggested breed variations in the susceptibility of cattle to LSD (Ayre-Smith 1960; Le Roux 1945), Weiss (1968) noted that all breeds appear to be equally susceptible. Furthermore, all age groups of cattle are affected with calves developing the characteristic lesions 24 - 48 hours earlier than their dams (Le Roux 1945).

Lumpy skin disease is more prevalent during the wet summer and autumn months and occurs particularly in low-lying areas and along watercourses (Ali and Obeid 1977; Diesel 1949; Haig 1957; MacDonalds 1931; Morris 1931; Nawathe 1982; Von Backstrom 1945), but outbreaks may also occur during the dry season (Haig 1957; Nawathe *et al.* 1982).

1.3.2. Transmission

The mode of transmission of LSD has not been clearly established. However, evidence suggests that the disease can be transmitted by biting arthropods (Barnard *et al.* 1994). In addition, the skin lesions contain high titres of virus, which are sufficient to contaminate the mouthparts of biting insects (Carn and Kitching 1995a). A report by Davies and Otema (1981) alluded to the possibility of the involvement of arthropod vectors but also suggested that husbandry methods where cattle are crowded together would predispose them to aerosol transmission. However, recent reports (Chihota *et al*. 2001) incriminated a species of mosquito, *Aedes aegypti* in the mechanical transmission of the disease. The failure of disease control measures e.g. movement restrictions across international boundaries, further indicates that a vector(s) may be involved in the transmission of the disease (Weiss 1968; Nawathe et al. 1982).

Experimental transmission of Capripoxvirus by *Stomoxys calcitrans* has been demonstrated by various authors (Kitching and Mellor 1986; Weiss 1968). Infection can also occur following intravenous, intradermal or subcutaneous inoculation. Kitching and Taylor (1985a) also demonstrated that sheep could be infected by means of an artificially produced virus aerosol.

Contact transmission between cattle housed in the absence of insects has also been observed (Carn and Kitching 1995; Weiss 1968), and in this case, saliva and shared water troughs have been implicated in the transmission (Haig 1957). The excretion of the virus in body secretions during the short viraemic stage following infection may play a role in the transmission of lumpy skin disease virus (Burdin and Prydie 1959; Haig 1957; MacOwen 1959). The disease can be transmitted to suckling calves through infected milk but there is no evidence of a carrier state in recovered animals (Haig 1957).

1.3.3. Host range

Lumpy skin disease virus, a Capripoxvirus differs from *Orthopoxviruses* by having a narrow vertebrate host range, infecting only sheep, goats and cattle with the possible exception of a report of five cases in Asian water buffalo (*Bubalus bubalis*) in Egypt (Ali *et al*. 1990), an Arabian Oryx (*aepyceros melampus*) in Saudi Arabia (Arnaud *et al*. 1992) and experimental infection of impala and giraffe (*giraffa camelopardis*) (Young *et al*. 1970).

Antibodies against LSD have been detected in blue wildebeest (*Connochaetes taurinus*), springbok (*Aepyceros melampus*), eland (*Taurotragus oryx*) and black wildebeest (*Connochaetes gnou*). The low prevalence of antibodies to the virus in these animals supports the hypothesis that game is not maintenance hosts of the disease (Barnard 1997; Hedger *et al.* 1983).

1.3.4. Economic importance of the disease

With LSD extending beyond its traditional boundaries, there is growing concern that the morbidity and mortality rates are rising. Although the mortality rate of the disease is low, recorded morbidity rates can be as high as 100% (Merck Veterinary Manual, 1998). The loss in productivity in the form of fever, general malaise causing weight loss, decrease in milk production in lactating cows, abortion, infertility in cows, mastitis and infertility in the bull cause severe economic loss. In severely affected animals, damage to hides greatly reduces the value and in turn affects the leather industries (Green, 1959; Weiss, 1968).

1.4. Clinical signs

Lumpy skin disease virus causes inapparent to severe diseases in cattle of all ages (Ayre-Smith 1960; Carn and Kitching 1995b; Davies 1991; MacDonald 1931; Von Backstrom 1945). In the field the incubation period is 2 to 4 weeks (Haig 1957), while that following experimental inoculation is between 7 and 14 days (Carn and Kitching 1995b). A biphasic fever of 40 - 41.5 °C can occur. Animals remain febrile for a total period of 4 - 14 days during which inappetence, salivation, lachrymation and mucoid or mucopurulent nasal discharges may develop. Lachrymation may be followed by conjunctivitis resulting in subsequent corneal opacity and blindness. Generally within 2 days after the appearance of the second febrile reaction, swellings or nodules 10 - 50 mm in diameter appear on the skin.

Skin nodules characteristic of the disease appear before or during a second rise in body temperature 4 – 10 days after the initial fever response. The skin nodules are 10-50 mm in diameter, well circumscribed, firm, round and raised and are particularly conspicuous in short haired cattle unlike long haired animals in which it is only recognized when the skin is palpated or moistened. In some cases nodules and ulcers are seen in the mucous membranes of the reproductive and respiratory tract. Subcutaneous swelling of the legs may also occur (Barnard *et al*. 1994; Thomas and Mare 1945). Nodules may form in the skin of the udder and teats, which become swollen and tender and may lead to mastitis. Pregnant cows may abort or have prolonged anoestrus (Weiss 1968). Davies (1991) has reported intrauterine infection of late-term fetuses in which calves are born with LSD lesions. Temporary or permanent sterility in bulls can result from the fever or lesions in the testes.

Secondary infected necrotic tissue in the upper respiratory tract may be inhaled resulting in pneumonia. Stenosis of the trachea following healing of lesions has been described (De Boom 1948). Lesions may persist in various stages over a course of 4 – 6 weeks. Their final resolution may take 2 – 6 months, but in some animals nodules can remain visible for 1 – 2 years.

1.5. Pathology

The gross lesions of LSD are well described (Burdin 1959; De Boom 1948; Haig 1957; Prozesky and Barnard 1982; Thomas and Mare 1945; Weiss 1968). Skin nodules are congested, hemorrhagic, edematous and necrotic. All layers of the epidermis, dermis and subcutaneous tissue are involved, and in some cases, often the adjacent musculature is

affected. Skin nodules appear whitish-grey on cut section. Circumscribed necrotic lesions appear on the muzzle, and mucous membrane of the mouth, respiratory tract, vulva and prepuce, which may ulcerate. Pox lesions are not easily visualized in the lungs in which focal areas of atelectasis and edema may be present. In severe cases, pleuritis may occur with enlargement of the mediastinal lymph nodes. Synovitis and tendosynovitis with fibrin in the synovial fluid can occur. Lesions may also be present in the testes and urinary bladder.

Histologically, sections of early skin lesions of the epidermis show an epitheliod cell (the cells clavelleuse of Borrel) infiltration, which is also characteristic of the lesions of LSD as well as sheep and goatpox. Lymphocytes, macrophages, plasma cells and fibroblast proliferation appear in the later stages. If secondary infection occurs, necrosis, polymorphonuclear leukocytes and red cells are seen. There is a cuffing of blood vessels by leukocytes. Typical eosinophilic, intracytoplasmic pox inclusion bodies may be seen in the epithelioid cells, and cells of hair follicles, smooth muscle and skin glands (Thomas and Mare 1945; Burdin, 1959; Prozesky and Barnard 1982).

1.6. Immunity

Immunity to poxviruses is mainly cell-mediated (Carn 1993). Tissue reaction within nodules is largely due to infiltration by lymphocytes and macrophages, which further suggest the involvement of cell mediated immunity to a greater extent (Capstick and Coakley 1961). Passive transfer of serum from sheep previously exposed to a capripox-virus protects against challenge from virulent strains of capripoxvirus although the immunity is of short duration (Kitching 1986). Also, high levels of antibody, attained through immunization with an inactivated antigen preparation will at best provide a short-term protection (Boulter *et al*. 1971). There are instances where recovered animals from apparent or inapparent natural infection, developed antibodies capable of neutralizing the virus and are also resistant to reinfection (Weiss 1968). The persistence of neutralizing antibodies, last for at least 2 to 3 years after vaccination. Antibodies appear 10 days post vaccination and reach a peak level on Day 30. The levels of neutralizing antibodies are not an indication of the immune status of a previously infected or vaccinated animal (Kitching 1986).

The three members of the genus *Capripoxvirus* are antigenically similar due to the presence of a common precipitating antigen that permits the use of heterologous virus for protection (Carn 1993). There are indications from serological evidence (Davies and Otema 1981), cross infection and cross protection studies (Capstick *et al.*1959; Kitching and Taylor 1985b) that the Capripoxviruses cross-react immunologically.

Calves born to immunized cows are likely to have passive immunity that persists for about 6 months (Weiss 1968). Colostrum also provides passive protection and may interfere with the response to vaccination before 6 months of age. Circulating antibodies limit the spread of the virus in animals but do not prevent replication of the virus at sites of infection (Carn 1993). Furthermore animals that have been vaccinated or show mild clinical disease develop low levels of neutralizing antibodies (Kitching and Hammond 1992).

1.7. Control

In countries where capripoxvirus do not occur, restrictions on the importation of livestock and animal products from affected areas can prevent the introduction of disease (Carn 1993). In countries remote from enzootic areas, immediate slaughter in an event of an outbreak, severe movement restrictions and ring vaccination in a radius of 25 – 50 km can result in the elimination of the disease, while in enzootic areas the disease can be controlled by slaughter and vaccination policies.

The use of an attenuated vaccine for LSD was first introduced by Weiss (1968). This vaccine however, produced a local reaction at the site of inoculation. Capstick and Coackley (1961) described the first use of a heterologous virus for protection of cattle with the use of the Kedong and Isiolo strain of capripoxvirus isolated from sheep with no local reaction at the site of inoculation. The use of heterologous virus strains in outbreaks in Egypt and Isreal, which proved to be immunogenic in the field, has also been reported (Davies 1991).

Recently, new recombinant vaccines have been developed using capripoxviruses as vectors, for the expression of rinderpest or rabies virus genes (Aspden *et al*. 2002; Ngichabe *et al*. 2002). Clinical trials of the LSDV – rinderpest vaccine do not indicate any adverse reaction or local reaction at the site of inoculation (Ngichabe *et al*. 1997). Two different vaccines have been widely used for the prevention of LSD in Africa with remarkable success. In

southern Africa, the Neethling strain is used as a freeze-dried product and in Kenya a local strain of sheep and goat poxvirus have been used to immunize cattle (Capstick and Coackley 1961).

1.8. Laboratory diagnosis

The initial diagnosis of the disease is made on clinical grounds followed by laboratory confirmation. Electron microscopy, being technically simple, provides a rapid method for detecting LSDV in tissue samples (Kitching and Smale 1986) but in large parts of endemic areas in Africa this facility is not available.

Histopathology of skin lesions demonstrates the presence of intracytoplasmic inclusion bodies containing LSDV in infected cells, and antigen identification using immunoperoxidase staining can be achieved in acute and chronic skin lesions. Fluorescent antibody staining of frozen skin sections reveals brilliant, stippled cytoplasmic staining of virus - infected cells often also containing large intracytoplasmic inclusion bodies (Kitching and Smale 1986).

The difficulties encountered with conventional tissue culture dependant techniques have been described (Carn *et al.* 1994). Isolation of LSDV (Plowright and Witcomb 1959a) is tedious due to the slow growth of the virus in tissue culture and cythopathic effect, if visible, may take up to 14 days to develop. The virus can be cultured in a variety of cell cultures: cultures of lamb and calf adrenal cells, thyroid cells, calf kidney cells, chicken embryo fibroblasts, adult vervet monkey kidney cell line (AKV58), sheep embryonic kidney cells, foetal lamb and calf muscle cells, rabbit foetal kidney, and skin cells and baby hamster kidney cells (BHK/21) have been used (Alexander *et al.* 1957; Prydie and Coackley 1959). The virus has also been cultured in primary cell cultures of bovine dermis and equine lung cells (SOP, DVTD). Primary lamb testis cell cultures have been reported to be the most sensitive (Binepal *et al.* 2001). Some field strains adapt poorly to tissue culture and need to be passaged blindly two or three times. Bacterial and fungal contamination is frequent especially in cultures that require such prolonged incubation (Plowright and Ferris 1959a). Multiplication of the virus also occurs in chick embryos and on the chorio-allantoic membrane of embryonated eggs (Van Rooyen *et al.* 1969).

Serology is limited in its application due to the often low antibody response following infection (Kitchning and Hammond 1992). However, an antigen trapping enzyme-linked immunosorbent assay (ELISA) for the detection of capripox virus in biopsy material has been developed (Carn 1995). This test uses a recombinant capripox virus-specific antigen and has a diagnostic sensitivity comparable to that of virus isolation in cell culture. However, both these methods fail to detect virus particles that are bound to neutralizing antibody (Kitching cited by Ireland and Binepal 1998).

The agar gel immunodiffusion (AGID) and fluorescent antibody (FAT) methods are difficult to interpret because of the existence of a common antigen between capripox and parapox viruses (Kitching *et al.* 1986) and the lack of a monoclonal antibody against a capripox virus-specific antigen. The fluorescent antibody test, however, may indicate the presence of LSDV antigens in the early stages of the disease (Davies *et al.* 1971).

The virus neutralization test (VNT) is relatively reliable, but because immunity to LSD infection is predominantly cell mediated, it is not sensitive enough to identify animals that have been in contact with the virus but have developed only low levels of neutraling antibodies (Kitching and Hammond 1992).

Detection of viruses that are shed in semen has been achieved by virus isolation in cell cultures. However, the cytotoxic effect of semen has been reported for many years (Carbrey *et al.* 1978; Da Silver *et al*. 1995; Revell *et al.* 1988; Rola *et al*.2003; Schultz *et al*. 1982; Weiblen *et al*. 1992). Thus, the isolation of virus in semen using cell cultures is said to be difficult because of the toxicity of semen to cell cultures, occurrence of false negative results when virus concentration in semen is low, and the extensive time required for the process. This has been reported in a study by Rola *et al*. (2003) on the isolation of bovine herpes virus –1 (BHV-1) in semen of bulls.

However, different methods of semen processing have been used by various authors in their attempt to reduce or completely eliminate the toxicity of semen on cell culture and enhance the isolation of viruses that are shed in semen (Howard *et al.* 1985; Larska and Rola 2003; Pietro *et al.* 1996; Van Oirschot *et al.* 1993; Xia *et al.* 1995,).

In studies by Voges *et al.* (1998), it was observed that unprocessed semen was toxic to tissue culture cells but toxicity was reduced after dilution of samples prior to inoculation onto cell culture. However, they observed that the recovery of virus from unprocessed semen samples after dilution was insufficient when compared to semen processed using egg yolk diluent. While Meyling and Jensen (1988) observed no difference in virus detection when using unprocessed and processed semen, Revell *et al.* (1988) reported that unprocessed semen might not be suitable for virus isolation. Furthermore, Kirkland *et al.* (1991) observed that virus recovery is more efficient from processed semen than from unprocessed semen at equivalent final dilutions. However, although the dilution of semen prior to inoculation on cell culture may reduce toxicity to some degree, it is said to reduce the chances of isolation of the virus in samples which have a low virus concentration (Lang and Kummer1975).

Some authors have also used fractionation of semen into supernatant and pellet fractions by centrifugation before inoculation onto cell culture with the aim of reducing the toxic effect of semen on cultures. In a study by Lang *el al.* (1974), low speed centrifugation of cytomegalovirus infected semen revealed a higher virus concentration in the supernatant fluid than in the cellular fraction, but toxicity still remained a major problem. Prieto and others (2003) were also able to isolate porcine reproductive and respiratory syndrome virus (PRRSV) from the supernatant of centrifuged semen samples. In contrast to this report Howell *et al.* (1986) used pellet fractions of semen samples, which they found to be less toxic to culture cells when compared to supernatant fractions in a study to enhance the detection of cytomegalovirus in semen from patients with acquired immune deficiency syndrome. However, Kim *et al*. (2001) were unable to isolate porcine circovirus – PCV 1 and 2 on cultures when pellet fractions of centrifuged samples were used for virus isolation. The effect of this fraction on culture was however not emphasized in that study. In another study, Van Rijn *et al.* (2004) also attempted the isolation of foot and mouth disease virus (FMDV) in the pellet fraction of a semen sample with no success despite the use of extra antibiotics.

The use of a combination of different antibiotics in the culture of semen has been employed by various authors to reduce bacterial contamination of cell cultures with favorable results (Irons, *et al.* 2005; Van Rijn *et al.* 2004; Pietro *et al.* 1996). This is consistent with observations by Breckon *et al*. (1980) on the use of adequate concentration of antibiotics to reduce bacterial contamination of semen specimens.

The filtration of semen samples to reduce its cytotoxic effect on culture has also been used. Pietro *et al*. (1996), in their study to develop a sensitive method for HIV detection on cell culture, used low protein binding Millex filter membranes, with a 0.450 µm pore size. Less significant results were obtained when supernatants of centrifuged samples was used.
The use of kaolin to remove toxic factors associated with semen on cell culture has also been attempted. Darcel and Coulter (1976) reported that kaolin was able to remove both toxicity and the neutralizing activity of infectious bovine rhinotreachietis virus (IBR) in seminal fluid. However, it was noted that kaolin has the unfortunate property of removing considerable amount of the virus. In a similar study by Richmond (1978) cytotoxic factor(s) was reduced in semen samples adsorbed with kaolin before inoculation onto cell culture thereby increasing CPE.

Nucleic acid hybridization techniques based on the polymerase chain reaction (PCR) (Saiki *et al*. 1988) are now widely used for detection and characterization of many viruses (Browning and Begg 1996; Forsyth and Barrett 1995; Uwatoko *et al.* 1996). The presence of immune complexes has no effect on the detection of viral DNA or RNA. A PCR - based test for the detection of capripoxvirus in biopsy samples described by Ireland and Binepal, (1998) have better analytical and diagnostic sensitivity than the antigen trapping ELISA described by Carn, (1995). Polymerase chain reaction has an added advantage over ELISA in that it can be used later in the course of the disease when virus-specific antibodies are present.

In studies by Irons *et al*. (2005), PCR was able to detect viral nucleic acids in semen samples more efficiently than virus isolation. In this study, the presence of infectious virus being present even when it could not be isolated on culture was not ruled out. In a similar study in an attempt to isolate bovine viral diarrhea virus in semen samples, Givens *et al*. (2003) were able to detect viral nucleic acid for longer periods using a reverse transcriptase nested PCR than they could isolate the virus on cell culture. These authors also show that infective virus was present in the semen despite the lack of success to isolate it. The sensitivity of PCR in detecting herpes viruses in semen and other specimens over virus isolation has also been reported by various authors (Lawrence *et al*. 1994; Sharma *et al*. 1992; Rocha *et al*. 1998; Wald *et al*.1999; Xia *et al*. 1995b).

The detection of LSDV nucleic acid in vesicular fluid and preputial washes of infected bulls has not been reported. However, in a study by Kirland *et al*. (1980) the authors

demonstrated that the seminal vesicles and prostrate gland may be productive sites for the replication of bovine viral diarrhea virus. Xia *et al*. (1995) in another study were able to isolate bovine herpes virus-1 on cell culture from preputial swabs and semen samples after preputial inoculation of experimental bulls. There is also evidence that bovine herpes virus and bovine leukaemia virus can be shed in semen due to the leakage of infected lymphocytes from the traumatized genital tract (Kahrs *et al.* 1980a; Afshar and Eaglesome 1990).

Virus contaminated semen have been incriminated as one of the possible routes of transmission of bovine herpes-1 virus and bovine viral diarrhea virus (McGowan *et al*. 1995; Phipott 1993). Reports by Carn and Kitching (1995) indicated that LSDV with a titre higher than 1log $TCID_{50}$ can establish an infection by intradermal inoculation and that 3 – 3.3 log $TCID_{50}$ could produce a generalized infection by intravenous and intranasal inoculation. To demonstrate if BVD virus shed in semen could cause infection, Givens *et al.* (2003) reported the infective nature of BVD virus from PCR - positive semen by inoculation into susceptible calves. Brunner *et al*. (1988) was also able to demonstrate a titre of 5 log $TCID_{50}$ of BHV-1 in a semen sample.

From the available literature it is evident that there are difficulties associated with virus isolation from semen on cell culture, and in particular, specific information is lacking on a diagnostic method that can be used to isolate LSDV from semen on tissue culture. Furthermore the presence of LSDV in vesicular fluid, preputial washes and the infective titre of the virus shed in semen have not been reported. This study will therefore seek to develop a more effective and rapid culture technique that will eliminate the toxic effects of semen on culture and enhance the isolation on cell culture of LSDV from semen samples. This method will then be applied and compared with PCR testing on field samples obtained from bulls experimentally infected with LSDV. The use of PCR to investigate the presence of LSDV in vesicular fluid, preputial washes as well as the determination of the $TCID_{50}$ of virus in semen will be undertaken.

1.9. Problems or hypothesis

- The isolation of LSDV from semen using cell cultures has many limitations due to the toxicity of semen to cell cultures.

- The different diagnostic methods have variable sensitivity in detecting LSDV in semen.

- The titre of infective virus in the semen may be an indication that the disease may be transmitted through the use of semen.

- Lumpy skin disease virus may be shed in vesicular fluid and preputial excretions.

1.10. Objectives of the study

- To develop a method which will decrease the toxicity of semen and enhance the isolation of LSDV on cell culture (Pilot study)

- To detect the presence of LSDV in semen of experimentally infected bulls using the method as determined by the pilot study for virus isolation and PCR.

- To compare the sensitivity of the virus isolation method with PCR in the detection of LSDV in the semen.

- To determine the titre ($TCID_{50}$) of LSDV, shed in the semen of bulls, following experimental infection.

- To detect the presence of LSDV in preputial wash and vesicular fluid of experimentally infected bulls using PCR.

CHAPTER 2

PILOT STUDY: Lumpy skin disease virus (LSDV) in experimentally infected semen samples.

2.1. Introduction

Available data suggests a growing demand for the screening of semen to ensure that semen used in artificial insemination and for export are free from infective agents. However, the achievement of these goals lies in the availability of a sensitive diagnostic method that can be used to isolate virus from semen on cell culture. The isolation technique will also give an insight into the viability of these pathogens in the semen.

Various authors have identified the difficulties associated with virus isolation from semen such reported by Da Silver *et al*. (1995). Revell *et al.* (1988) and Schultz *et al*. (1982) also noted that seminal plasma exhibits virucidal properties, cell culture cytotoxicity and inhibition of the reverse transcriptase enzyme. Virus isolation is said to be difficult because of the natural cytotoxicity of semen and its overshadowing effect on the viral cytopathic effect (Weiblen *et al*. 1992). Rola and co-workers (2003), in their attempt to isolate bovine herpes virus – 1 in semen of bulls concurred that isolation using cell cultures is difficult due to the toxicity of semen on cell cultures, occurrence of false negative results when virus concentration in semen is low and the extensive time required for the process.

Although the duration of shedding of LSDV in semen has been reported (Weiss 1968; Irons 2005), no published data exists on a suitable diagnostic method to isolate the virus from semen using cell cultures.

The objective of the study therefore was to determine the most sensitive method to isolate LSDV from semen using cell cultures. Semen samples were collected from LSD seronegative bulls and spiked with a virulent, well-characterised,South African field isolate of LSDV. Before inoculation onto cell cultures semen samples were subjected to the following methods of treatment:

- Centrifugation
- Serial dilutions with the inclusion of antibiotics
- Filtration
- Chemical treatment using kaolin.

The best method that could considerably reduce the toxicity of semen on cell culture and thereby increase the chances of virus isolation was then used to detect the presence of LSDV in field samples as described in sections 3.4.2.1 and 3.4.2.2.

2.2. Materials and Methods

2.2.1. Maintenance of cells

Bovine dermis (BD) cells, a primary cell culture prepared in the Department of Veterinary Tropical Diseases (DVTD) from an aborted calf's ear were passaged at a split ratio of 1:3. At the start of this project cells were at passage level 6. Cells were maintained in minimum essential medium (MEM) with L-glutamine (Highveld Biological), 0.2 % sodium bicarbonate (Highveld biological), 5 % foetal calf serum (Adcock Ingram) and 0.05 mg gentamycin [Genta 50 Phenix, stock 50 mg / ml]. Culture flasks were incubated at 37 °C in an atmosphere of 5 % CO_2. Flasks were observed daily and were passaged when about 80 % confluency was reached normally on Day 7 following passage.

2.2.2. Growing of virus and harvesting

Culture flasks containing BD cells that had attained 80 % confluency were inoculated with 0.1 ml of a field isolate of LSDV strain 248/93 and incubated at 37 °C in an atmosphere of 5 % CO_2. Samples were observed daily for evidence of cytopathic effect (CPE). Development of CPE following inoculation with LSDV (s248/93) took 8 days to attain a 4+ infectivity level. When a 4+ CPE was reached, culture flasks were frozen at –70 °C for 1 hr and thawed at room temperature to release cell bound virus particles.

2.2.3. Counting of bovine dermis cells

The virus titre was determined using 96 well plates. Bovine dermis cells were used at a concentration of 480.000 cells per well. In order to obtain this, cells were counted as follows: Bovine dermis cells were passaged and dissolved in 10 ml of MEM [L-glutamine (Highveld Biological), 0.2 % sodium bicarbonate (Highveld biological), 5 % foetal calf serum (FCS) (Adcock Ingram) and gentamycin 0.05 mg [(Genta 50 Phenix, stock 50 mg / ml)], loaded onto slide chambers of a hemocytometer and the cells were counted.

2.2.4. Titration of virus

A 10-fold series of dilutions were prepared and 100 µl of each dilution was added per well of a 96 well plate. Minimum essential medium, was used as described in section 2.2.1. Growth medium (100 µl) was added to seven wells and 200 µl to the cell control wells. Bovine dermis cells at a concentration of 480.000 cells / ml was added into each well and the plate was covered and incubated at 37 °C in an atmosphere of 5 % CO_2. Cells were monitored daily for evidence of CPE. The CPE in the various wells was recorded and is represented in Table 2.

Table 2: Determination of $TCID_{50}$ of LSDV field strain on BD cells

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
A	+	+	+	+	+	+	-
B	+	+	+	+	+	+	-
C	+	+	+	+	+	+	-
D	+	+	+	+	+	-	-
E	+	+	+	+	+	-	-

+ = CPE, - = negative

The $TCID_{50}$ of the virus was calculated by the method of Reed and Muench (1938) to be 6.5 log $TCID_{50}$.

2.2.5. Semen collection

Semen samples were collected from LSDV seronegative bulls using an electro - ejaculator. An aseptic procedure to reduce most of the bacterial contamination associated with sample collection by exogenous means was performed. Briefly, the hair around the preputial area was clipped and the area was then washed using clean water, disinfected and thoroughly dried before commencement of sample collection.

2.2.6. Methods of treatment of spiked semen samples

2.2.6.1. Centrifugation of spiked semen samples

Semen samples obtained from LSDV seronegative bulls were aliquoted into 3 tubes of 1 ml each and spiked with 0.1 ml of a field isolate of LSDV strain 248/93 at a titre of 6.5 log

$TCID_{50}$. A volume of 10 mg gentamycin [Genta 50, Phenix, 50 mg / ml] was added to the semen/virus mixture and incubated at 37 °C for 2 hrs in an atmosphere of 5 % CO_2. The semen samples were then centrifuged at 2000 rpm, at different time intervals. Tube 1 for 1 min, tube 2 for 3 min and tube 3 for 5 min. The pellet fraction of each tube was re-suspended in 2 ml of MEM and 0.5 ml of both the supernatant and pellet fractions were serially diluted in MEM. A 0.5 ml volume of each dilution was inoculated onto BD cells in 25 cm^2 culture flasks. Cells were maintained in MEM containing 8 % FCS and 0.05 mg gentamycin [50 mg / ml] and flasks were incubated at 37 °C in an atmosphere of 5 % CO_2. Cells were observed daily for appearance of CPE and evidence of toxicity.

2.2.6.2. Serial dilution of spiked semen samples

Semen samples were aliquoted into two tubes of 1 ml each from LSD seronegative bulls and spiked with 0.1 ml LSDV strain 248/93 at a titre of 6.5 log $TCID_{50}$. Semen samples in tube 1 were serially diluted using a 10-fold dilution in tubes containing MEM and 0.5 ml of each dilution was inoculated onto BD cells. In tube 2, an extra volume of 10 mg gentamycin [50 mg / ml] was added and incubated for 2 hrs in an atmosphere of 5 % CO_2. The sample was then 10-fold serially diluted in MEM and 0.5 ml of each dilution inoculated onto BD cells. All inoculated cultures were incubated at 37 °C in an atmosphere of 5 % CO_2 and observed daily for appearance of CPE. And evidence of toxicity.

2.2.6.3. Filtration of spiked semen samples

Five millilitres of semen samples from LSD seronegative bulls were liquoted into 7 tubes. This volume of semen was used to compensate for the lost of liquid volume during the filtration process. A 10-fold serial dilution of 0.5 ml LSDV strain 248/93 at a titre of 6.5 log $TCID_{50}$ was prepared in tubes containing 4.5 ml MEM. A 0.1 ml volume of each serially diluted virus was used to spike each semen sample. The semen/virus mixture was filtered using a high protein binding 0.8 µm pore size Millipore filter (Millipore Corporation, Bedford, USA). The filtrate of each dilution was collected in different tubes and refiltered using a high protein binding 0.45 µm pore size Millipore filter. A volume of 10 mg gentamycin at [50 mg / ml] was added to 1 ml of the filtrate and incubated for 2 hrs in an atmosphere of 5 % CO_2. Thereafter, 0.5 ml of the antibiotic/filtrate mixture was inoculated onto BD cells. Inoculated cells were incubated at 37 °C in an atmosphere of 5 % CO_2. Samples were observed daily for evidence of toxicity

2.2.6.4. Kaolin treatment of spiked semen samples

A 10-fold serial dilution of 0.5 ml of LSDV at a titre of 6.5 log $TCID_{50}$ was prepared in 4 tubes containing 4.5 ml of MEM. Volumes of 0.1 ml of each dilution of viral samples were added to 3 ml of semen. The semen/virus mixture was incubated for 1 hr at 37 °C in an atmosphere of 5 % CO_2. Two millilitres of 10 % kaolin (Separations) was then added and stirred at room temperature for 20 min. The samples were centrifuged at 2000 rpm for 20 min and separated into supernatant and pellet fractions.

The pellets were resuspended in 3 ml of MEM. Volumes of 0.5 ml each of the supernatant and pellet fractions were used to inoculate BD cells. Cells were observed daily for appearance of CPE and evidence of toxicity.

The positive controls used in the different methods comprised of BD cells inoculated with 0.5 ml LSDV at a titre of 6.5 log $TCID_{50}$ while negative controls were BD cell without virus.

2.3. Results

2.3.1. Centrifugation of spiked semen samples

Supernatants of semen samples centrifuged at 2000 rpm for 1 min were positive on Day 3 post inoculation (p.i.) in dilutions 10^{-1} to 10^{-3} (Table 3) with cells attaining a 3+ CPE on Day 5 p.i. at 10^{-1} and 10^{-2} dilutions and on Day 7 p.i. at dilution of 10^{-3}. At the higher dilutions of 10^{-4} to 10^{-6}, no CPE for LSDV was observed.

In the pellets of these samples, toxicity was recorded at dilutions 10^{-1} and 10^{-2} (Table 4) by Day 2 p.i. At a dilution of 10^{-3}, CPE was observed on Day 3 p.i. with cells attaining a 3+ infectivity level on Day 6, and at dilutions of 10^{-4} and 10^{-5} cultures were positive on Day 4 with cells attaining a 3+ CPE on Days 7 and 8, respectively. No CPE or toxicity was recorded at a dilution of 10^{-6}.

Table 3: Supernatants of semen samples with additional antibiotics centrifuged for 1 min at 2000 rpm

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	+	+	+	-	-	-
4	++	++	+	-	-	-
5	+++	+++	++	-	-	-
6	+++	+++	++	-	-	-
7	+++	+++	+++	-	-	-
8	+++	+++	+++	-	-	-
9	+++	+++	+++	-	-	-
10	+++	+++	+++	-	-	-

+ = CPE, - = negative

Table 4: Pellets of semen samples with additional antibiotics centrifuged for 1 min at 2000 rpm

Days No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	tc	tc	-	-	-	-
3	tc	tc	+	-	-	-
4	tc	tc	+	+	+	-
5	tc	tc	++	+	+	-
6	tc	tc	+++	++	++	-
7	tc	tc	+++	+++	++	-
8	tc	tc	+++	+++	+++	-
9	tc	tc	+++	+++	+++	-
10	tc	tc	+++	+++	+++	-

tc = toxicity, + = CPE, - = negative

In semen samples that were centrifuged at 2000 rpm for 3 min, the supernatants showed evidence of CPE in dilutions 10^{-1} to 10^{-3} on Day 3 p.i. with attainment of a 3+ CPE on Days 7, 9, and 10 p.i. respectively, and at a dilution of 10^{-4} on Day 4, with a 3+ CPE on Day 10 p.i. At dilutions 10^{-5} and 10^{-6} cells remained viable with no indication of CPE (Table 5).

Table 5: Supernatants of semen samples with additional antibiotics centrifuged for 3 mins at 2000 rpm

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	+	+	+	-	-	-
4	+	+	+	+	-	-
5	++	+	+	+	-	-
6	++	++	++	++	-	-
7	+++	++	++	++	-	-
8	+++	++	++	++	-	-
9	+++	+++	++	++	-	-
10	+++	+++	+++	+++	-	-

- = negative, + = CPE

The development of CPE on BD cell culture when supernatants of centrifuged semen samples supplemented with additional antibiotics were used is shown in figure 2.

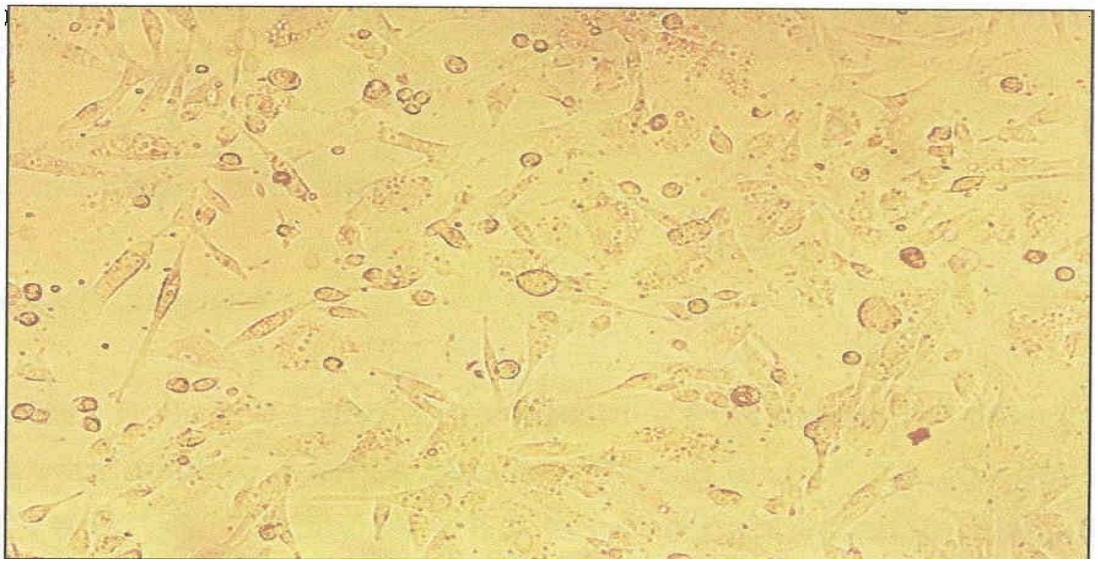

Figure 2: Development of a 4+ CPE on BD cells inoculated with supernatant of centrifuged semen samples at a dilution of 10^{-1} .

However, in the pellets of these semen samples, toxicity to cell culture was recorded on Day 2, p.i in dilutions of 10^{-1} and 10^{-2}. At dilutions of 10^{-3} and 10^{-4}, CPE was observed on Day 3 p.i. attaining a 3+ infectivity level in cultures on Day 8 and 9 p.i. respectively, and at a dilution of 10^{-5}, CPE was recorded on Day 4 and only attained a 2+ infectivity level on culture. Samples at a dilution of 10^{-6} remained negative for LSDV (Table 6).

Table 6: Pellets of semen samples with additional antibiotics centrifuged for 3 mins at 2000 rpm

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	tc	tc	-	-	-	-
3	tc	tc	+	+	-	-
4	tc	tc	+	+	+	-
5	tc	tc	++	+	+	-
6	tc	tc	++	++	++	-
7	tc	tc	++	++	++	-
8	tc	tc	+++	++	++	-
9	tc	tc	+++	+++	++	-
10	tc	tc	+++	+++	++	-

tc = toxicity, - = negative, + = CPE

Semen samples that were centrifuged at 2000 rpm for 5 min, supernatants showed evidence of CPE at dilutions of 10^{-1} to 10^{-3} on Day 3 p.i. with a 3+ CPE on Days 5 and 9, p.i. while at a dilution of 10^{-4} a 3+ CPE was not attained (Table 7).

Table 7: Supernatant of semen samples with additional antibiotics centrifuged for 5 mins at 2000 rpm

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	+	+	+	+	-	-
4	++	++	+	+	-	-
5	+++	+++	++	+	-	-
6	+++	+++	++	+	-	-
7	+++	+++	++	+	-	-
8	+++	+++	++	+	-	-
9	+++	+++	+++	+	-	-
10	+++	+++	+++	++	-	-

- = negative, + = CPE

The pellets of these dilutions, recorded toxicity on cell culture at a dilution of 10^{-1} on Day 2 p.i. At dilutions 10^{-2} and 10^{-4}, CPE was observed on Day 3. A 3+-infectivity level on cultures was attained on Day 5 p.i. at dilution 10^{-2} and on Day 8 at dilutions 10^{-3} and 10^{-4} respectively. At dilutions 10^{-5} and 10^{-6} culture flasks remained negative with no evidence of CPE (Table 8).

Table 8: Pellets of semen samples with additional antibiotics centrifuged for 5 mins at 2000 rpm

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	-	-	-	-	-	-
2	tc	-	-	-	-	-
3	tc	+	+	+	-	-
4	tc	++	+	+	-	-
5	tc	+++	++	+	-	-
6	tc	+++	++	++	-	-
7	tc	+++	++	++	-	-
8	tc	+++	+++	+++	-	-
9	tc	+++	+++	+++	-	-
10	tc	+++	+++	+++	-	-

tc = toxicity, - = negative, + = CPE

2.3.2. Serial dilution of spiked semen samples

Semen samples serially diluted in MEM showed toxicity on cell culture at dilutions 10^{-1} to 10^{-3} on Day 1 p.i. At dilutions of 10^{-4} to 10^{-6}, CPE was observed on Days 4, 7 and 7 p.i. respectively. At these dilutions the level of cell culture damage did not attain a 3+ CPE (Table 9).

Table 9: Semen samples serially diluted in MEM

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	tc	tc	tc	-	-	-
2	tc	tc	tc	-	-	-
3	tc	tc	tc	-	-	-
4	tc	tc	tc	+	-	-
5	tc	tc	tc	+	-	-
6	tc	tc	tc	+	-	-
7	tc	tc	tc	+	+	+
8	tc	tc	tc	++	+	+
9	tc	tc	tc	++	+	+
10	tc	tc	tc	++	++	++

tc = toxicity, - = negative, + = positive

However, in semen samples containing an extra volume of 10 mg gentamycin [50 mg / ml], toxicity of the cell culture was recorded at a dilution of 10^{-1} on Day 1 p.i. At dilutions 10^{-2} to 10^{-6}, CPE was recorded on Days 3, 4, 7 and 10 p.i respectively. The CPE of 3+ was attained on Day 7 p.i. at dilutions 10^{-2} and 10^{-3} and on Day 9 p.i. at dilution 10^{-4}. At dilutions of 10^{-5} only a 1+ CPE was recorded on cultures (Table 10).

Table 10: Semen samples with additional antibiotic serially diluted in MEM

Day No.	Dilutions					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	tc	-	-	-	-	-
2	tc	-	-	-	-	-
3	tc	+	+	-	-	-
4	tc	++	++	+	-	-
5	tc	++	++	+	-	-
6	tc	++	++	+	-	-
7	tc	+++	+++	+	+	-
++	+	+++	+++	++	+	-
9	tc	+++	+++	+++	+	-
10	tc	+++	+++	+++		

tc = toxicity, - = negative, + = positive

2.3.3. Filtration of spiked semen samples

Evidence of toxicity in the cell cultures inoculated with semen filtered through 0.8 µm and 0.45 µm pore size Millipore filter was recorded on Day 1 p.i. at all levels of dilution (Table 11).

Table 11: Semen sample with additional antibiotics filtered using 0.8 µm and 0.45 µm pore size Millipore filters

Day No.	Dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	tc	tc	tc	tc

tc = toxicity

2.3.4. Kaolin treatment of spiked semen samples

In supernatant fractions of semen samples treated with kaolin, cell culture toxicity was evident on Day 4 p.i. in all dilutions. The trend of toxicity in the pellet fraction at the various dilutions was similar to that recorded for the supernatant (Tables 12 & 13). However, with the pellets, toxicity was recorded earlier, on Day 2 p.i. (Table 13).

Table 12: Supernatants of kaolin treated semen sample centrifuged at 2000 rpm for 20 mins

Day No.	Dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	tc	tc	tc	tc

- = negative, tc = toxicity

Table 13: Pellets of kaolin treated semen samples centrifuged at 2000 rpm for 20 mins

Day No.	Dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	-	-	-	-
2	tc	tc	tc	tc

- = negative, tc = toxicity

Table 14: Summary of results of the four methods used in the pilot study illustrating toxicity, CPE and cell culture infectivity.

Treatment		Toxicity	CPE	Cell culture infectivity level
Centrifugation	Supernatant (1, 3, 5 min)	none	$10^{-1} - 10^{-4}$	10^{-3}
				10^{-4}
				10^{-4}
	Pellet (1, 3, 5 min)	$10^{-1} - 10^{-2}$	$10^{-3} - 10^{-5}$	10^{-5}
		$10^{-1} - 10^{-2}$	$10^{-3} - 10^{-5}$	10^{-5}
		10^{-1}	$10^{-2} - 10^{-4}$	10^{-4}
Serial dilution	+ AB	10^{-1}	$10^{-2} - 10^{-6}$	10^{-6}
	- AB	$10^{-1} - 10^{-3}$	$10^{-4} - 10^{-6}$	10^{-6}
Filtration	Filtrate	all	none	none
Kaolin	Supernatant	all	none	none
	Pellet			

+AB = Presence of antibiotic, -AB = Absence of antibiotics

2.4. Discussion

The isolation of viruses from semen using cell cultures has proved to be difficult (Da Silver *et al*. 1995; Revell *et al.* 1988; Rola, *et al*. 2003; Schultz *et al*. 1982; Weiblen *et al*. 1992). One reason for this is the cytotoxic effect that bovine semen has on tissue culture (Kahrs, *et al.* 1977). It is very important to effectively eliminate this toxic effect in order to isolate viruses shed in semen by using tissue culture methods, the success of which can establish the infectious nature of these viruses. There are, however, no published data available on a technique that completely eliminates this toxic effect of bull semen in order to enhance virus isolation. In this study, four different methods were used in an attempt to eliminate the toxicity of bull semen spiked with LSDV but yet would still allow the isolation of the virus by cell culture techniques. These were centrifugation, serial dilution, filtration and chemical treatment using kaolin.

Viral CPE on cell culture was observed in all supernatants of spiked and centrifuged semen samples at different time intervals ranging from 1- 5 min and serially diluted (Tables 3, 5, 7 & 14). This method proved more successful in reducing the toxic effect of semen on cell cultures and in enhancing virus isolation as compared to the results obtained in other studies in which different isolation techniques were used (De Smit *et al*. 1999; Kahrs *et al*.

1977; Lang *et al*. 1974; Prieto *et al*. 2003). Prieto *et al*. (2003) used supernatant of centrifuged porcine semen to inoculate cell cultures. The authors allowed an absorption time of 1.5 hours following inoculation onto cells and thereafter, cultures were washed and replaced with growth medium in their attempt to isolate porcine reproductive and respiratory syndrome virus on cell culture. Although the method used by these authors effectively reduced the toxic effect of semen on cell culture, the sensitivity of the method to isolate the virus on cell culture was considerably reduced.

In this experiment, after centrifugation for 3 and 5 min of bull semen spiked with LSDV and then supernatants diluted and inoculated onto cell cultures, CPE was observed in the cells up to a dilution of 10^{-4}, a higher dilution than was recorded at 1 min (10^{-3}). It is however interesting to note that the cell culture infectivity at one dilution higher did not attain a 3+ CPE in supernatants of samples centrifuged for 5 min. The presence of virus at a dilution of 10^{-4} may have resulted through cross contamination during the titration process or a low number of virus particles were present at this level of titration. The possibility that virus growth inhibitory substances present in semen were spun out with the longer centrifugation time cannot be ruled out. The addition of antibiotics to the supernatant fluid in combination with serial dilution after centrifugation improved the retention of the virus in the cultures and eliminated toxic substances in the supernatant fraction.

The sensitivity of virus isolation was reduced when pellets obtained by centrifuging semen samples for 5 mins were used to infect monolayer cells. However, this length of time of centrifugation did considerably reduce the toxicity to the cells when compared to samples centrifuged for 1 and 3 mins (Tables 4, 6 & 14). Howell *et al.* (1986) inoculated pellet fractions of human semen samples onto cell cultures to detect cytomegalovirus in the semen of patients infected with human immunodeficiency virus and obtained little or no toxicity. Results in the study described here indicate a high toxicity of the pellet fractions at certain dilutions (Tables 4, 6, 8 & 14). The discrepancy between the results obtained in this study and that of Howell *et al* (1986). may be a result of the fact that there were less chances of contamination during sample collection in humans. The latter correlates with the observations of Breckon *et al*. (1980) who recommended the use of a proper aseptic procedure in sample collection. It is also possible that toxicity may be associated with enzymes toxic to cell culture present in bovine semen (Kahrs *et al*. 1977) which may be associated with the pellet fraction. In another study, Junghun *et al.* (2001) were not able to isolate porcine circovirus 1 and 2 from boar semen when pellet fractions were used. In this

study, although the pellet fractions were toxic at certain dilutions, viral CPE was observed in cells of tissue cultures inoculated with either the supernatant or the pellet fractions. The high titre of 6.5 log $TCID_{50}$ of the virus used to spike the semen samples may not be a true reflection of the number of virus particles that are present in field samples. This may have contributed to the development of CPE in the pellet fraction at the higher dilutions. In addition, the virus inoculum that was used to spike semen samples comprised of infected cell culture fluid which could have made the adaptation to cell culture easier. It is also possible that virus adhesion to cell culture debris or the relatively large size of capripoxvirus of 320-260 nm may have been responsible for the presence of virus in the pellet fraction after centrifugation. The concentration of virus in the various fractions was not determined in this study.

Previous studies have shown that serial dilutions of semen samples prior to inoculation on cell culture do reduce toxicity to some extent (Lang *et al*. 1974; Tuppurainen *et al*. 2005). This is in agreement with results obtained in this study, in which toxicity to cell cultures was observed at some dilutions in samples not supplemented with antibiotics (Tables 9 & 14) with destruction of the architectural integrity of the cells. Although the cells were preserved at higher dilutions, with CPE recorded at dilutions of 10^{-6}, the toxic effect of semen on cultures at lower dilutions was very evident.

Similarly, although toxicity was also observed in those samples that were supplemented with antibiotics, cell destruction was considerably reduced (Tables 10 & 14). The level of toxicity observed in samples supplemented with antibiotics is consistent with those observed in studies by De Smit *et al.* (1999) where raw semen samples were 10-fold diluted and simultaneously inoculated together with culture cells onto culture plates in order to isolate classical swine fever virus in semen.

It is, however, clear that the addition of adequate concentration of antibiotics does reduce most of the bacterial contamination of semen specimens (Breckon *et al.* 1980), but it is not known by what mechanism antibiotics reduce the deleterious effect of toxic substances present in semen.

In this study the use of filtration did not play a significant role in removing the cytotoxic factors associated with semen on cell culture (Tables 11 & 14). This agrees with the

findings of Prieto *et al*. (2003) who used low protein binding Millex filters in an attempt to reduce the toxic effect of semen on cell culture with no success.

Although Darcel and Coulter (1976) reported that kaolin removed virus neutralising activity in semen and prevented the cytotoxic effect of semen on cell culture, the effect of kaolin on cultures in this study made it impossible to determine the presence of virus prior to commencement of toxicity in both fractions (Tables 12, 13 & 14).

Previous published data as well as this study (Table 9) showed that the use of untreated semen is toxic to cell cultures. This work however, provides evidence of a decrease in the toxic effect of semen on cell culture when supernatants of centrifuged semen samples were used to inoculate confluent monolayer cells (Tables 3, 5 & 7). The time for the development of CPE was also reduced, when compared to the slow growth rate of LSDV in tissue culture of up to 14 days for CPE to develop as reported by Plowright and Witcomb. (1959). Whole semen diluted in MEM with or without additional antibiotics was the most sensitive method of demonstrating virus at higher dilutions, followed by pellets of samples centrifuged for 1 and 3 mins. However, further study is needed to determine a more definite time and speed of centrifugation to reduce the chances of virus being lost in the pellet fraction. The determination of virus concentration in both fractions should also be undertaken.

CHAPTER 3

3.1 Introduction

This chapter describes the use of experimental animals, mode of infection, virus strain and titre used to infect the experimental animals, type of samples collected and samples tested in this study. It also describes the recording of vital parameters of infected bulls and how the infective virus titre shed in the semen was calculated following manifestation of clinical disease.

In the isolation of LSDV on cell culture from semen of experimentally infected bulls, the centrifugation and serial dilution methods, established from the pilot study to have favorably reduced the toxic effect of semen on cell cultures were used to test field samples. In order to compare the sensitivity of the two isolation methods in detecting LSDV in semen on cell culture, PCR was used as a gold standard. The PCR was also used to detect the presence of LSD viral nucleic acid in vesicular fluid and preputial wash samples.

3.2. Materials and Methods

3.2.1. Experimental animals

Six post-pubertal Dexter breed bulls aged between 13 and 16 months from a herd where vaccination against LSD was not practiced were used as experimental animals. Serum samples were collected before and after purchase and tested to be LSD seronegative using the SNT according to SOP of DVTD. The animals were kept in the insect proof facility of the University of Pretoria Biomedical Research Centre (UPBRC), Faculty of Veterinary Science, University of Pretoria throughout the period of the experiment. Before the commencement of the experimental trials, the animals were allowed a two week acclimatisation period. They were numbered using ear-tags inscribed A, B, C, D, E and F.

3.2.2. Preparation of virus suspension and animal inoculation

A virulent South African 4 times-passage field isolate strain 248/93 of LSDV was used to infect the experimental animals. The preparation of virus for animal inoculation was previously described (sections 2.2.2 and 2.2.4). The intravenous route of infection was used.

3.2 3. Clinical observation

Routine clinical examination of bulls was conducted and rectal temperatures were taken twice a day until the end of the period of sample collection. Following infection, blood and semen samples were collected. Blood in heparin was collected in tubes at 2 day intervals up to Day 40 p.i and stored at –70 °C.

3.2.4. Sample collection and processing

3 2 .4.1. Semen samples

Semen samples for PCR and virus isolation were collected using an electro-ejaculator as described previously in section 2.2.5 at 2 day intervals up to Day 40 p.i. The semen samples were immediately submitted to the laboratory and were aliquoted in 1.8 ml cryotubes. Samples for PCR were stored at –20 °C and those for virus isolation at –70 °C until used.

3.2 4.2. Vesicular fluid

Vesicular fluid from experimentally infected bulls was collected by rectal massage of the vesicular gland into 5 ml tubes at 2 day intervals for 40 days following infection. The samples were aliquoted into 1.8 ml tubes and preserved at –20 °C for PCR.

3.2 4.3. Preputial wash

Preputial washes were collected by flooding of the preputial cavity with distilled water and the wash collected by the use of a receptacle into tubes. Aliquots were placed into 1.8 ml cryotubes and stored at –20 °C for testing by PRC.

3.2 5. Virus isolation

3.2 5 1. Heparinized blood samples

Bovine dermis cells at ± 50 % confluency were inoculated with 0.5 ml of heparinized blood and incubated at 37 °C in an atmosphere containing 5 % CO_2. After 24 hours, the cells were washed twice with phosphate buffered saline containing Mg^{2+} and Ca^{2+} (PBS+) and 0.05 mg gentamycin [50 mg / ml]. The medium was replaced with MEM containing 8 % FCS

and 0.05 mg gentamycin [50 mg / ml]. The cells were reincubated and observed daily for evidence of CPE. After 14 days negative cultures were frozen for 20 mins at –70 °C and thawed. Flasks were shaken gently to break up the cell material and to release the cell bound virus. A second passage was done and observed daily for evidence of CPE. Positive results from cultures were recorded.

3.2 5.2. Semen samples

Before attempting to isolate virus on BD cells, semen samples were treated using the centrifugation and serial dilution methods.

Centrifugation

After thawing of semen samples at room temperature, 1 ml aliquots were taken, 10 mg gentamycin [50 mg / ml] was added and incubation at 37 °C was performed for two hours in an atmosphere containing 5 % CO_2. The semen samples were centrifuged at 2000 rpm for 1 minute. Supernatant and pellet fractions were collected in different tubes. The pellet fraction was discarded. The supernatant fractions were serially diluted into tubes. Bovine dermis cells in a 25 cm^2 culture flask that have attained 80 % confluency were inoculated using 0.5 ml from the serially diluted supernatant fractions. Cells were maintained in MEM containing 8 % FCS and 0.05 mg gentamycin [50 mg / ml]. Culture flasks were incubated at 37 °C in an atmosphere containing 5 % CO_2 and observed daily for evidence of CPE. After 14 days a second blind passage (section 3.4.1) of negative cultures was performed and results recorded.

Serial dilution of semen with antibiotics

After thawing of the semen samples, 1 ml aliquots of semen samples were collected in tubes and extra antibiotic, 10 mg gentamycin [50 mg / ml] was added and the tubes were incubated for 2 hrs at 37 °C in an atmosphere containing 5 % CO_2. The samples were 10-fold serially diluted in tubes containing MEM, 8 % FCS and 0.05 mg gentamycin [50 mg / ml] and 0.5 ml of each diluted sample was then inoculated onto BD cells. All inoculated cultures were incubated at 37 °C in an atmosphere containing 5 % CO_2 and observed daily for evidence of CPE. Negative cultures were blind passaged (section 3.4.1) and results recorded.

3.2.6. Determination of $TCID_{50}$ of virus in semen

A semen sample from a bull in the severely affected group was collected on day 15 p.i. and centrifuged at 2000 rpm for 1 min, supernatant collected and serial diluted in seven tubes. The $TCID_{50}$ of the virus in semen was determined as previously described (section 2.2.4) and calculated using the method of Reed and Muench (1938).

3.2.7. Polymerase chain reaction

3.2.7.1. Semen samples, vesicular fluid and preputial washes

The QIAamp DNA extraction kit (Qiagen, Southern Cross Biotechnology) was used to extract LSDV DNA from semen samples. Briefly, 200 µl semen, vesicular fluid or preputial washes were suspended in 200 µl, 2x buffer containing 20 Mm Tris-HCl (pH 8.0), 20 mM EDTA, 200 mM NaCl, 80 mM DTT and 10 % SDS. Proteins were digested by adding 20 µl of Proteinase K to samples and incubated at 56 °C for 16 hrs. The samples were then re-incubated for another 10 mins after the addition of 200 µl of lysis buffer. Thereafter, 200 µl ethanol was added to the samples and incubated at room temperature for 5 mins. Samples were washed in a QIAamp minElute column using AW1 and AW2 buffers. In the final elution step, 100 µl AE buffer was added and DNA collected after centrifugation at 14,000 rpm for 3 mins. As a positive control, 180 µl semen samples spiked with 20 µl LSDV at a titre 6.5 log $TCID_{50}$ was used, while the negative control comprised of 200 µl semen obtained from LSDV seronegative bulls.

For the amplification of LSDV nucleic acid in semen samples, a platinum qualitative PCR Supermix-UDG (2x) reaction mixture (Life Technologies) was used. The PCR Supermix-UDG (2x) reaction mixture constitutes 60 µl / ml platinum Tag DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl_2, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, 40 µ/ml UDG (uracil DNA glycosylase) and stabilizers. For the amplification of nucleic acid in vesicular fluid and preputial washes (SIGMA), a ready mix PCR reaction mix containing $MgCl_2$ was used. All samples were amplified using primers developed from the gene for the viral attachment protein (Ireland and Binepal, 1998). The PCR generated a 147 bp gene product.

Forward primer 5’ – TTTCCTGATTTTTCTTACTAT 3'
Reverse primer 3’ – AAATTATATACGTAAATAAC 5'

Amplification of DNA was conducted in a 25 µl final volume containing

12.5 µl platinum Quantitative PCR Supermix-UDG / REDTaq 12.5 µl
0.5 µl of 20 mM of each primer
2.5 µl (+/- 70 ng DNA)
High quality water was used to adjust the final volume to 25 µl.

A Perkin – Elmer Gen Amp PCR system 9600 was used for the PCR reaction. The reaction had an initial cycle of 94 °C for 5 mins, 50 °C for 30 sec, 72 °C for 1 min followed by 34 cycles of 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min and a final elongation step of 72 ºC for 5 mins to elongate the complete extension of the primers (Ireland and Binepal 1998). Amplified products were electrophoresed. Positive amplicons were confirmed according to a 147 bp size on a commercially available appropriate DNA molecular weight marker of 300-1000 bp size range (Promega, USA) e 2 % agarose gel containing 2 ul / ml ethiduim bromide (10 mg / ml) in Tris-EDTA buffer.

CHAPTER 4

RESULTS

4.1. Clinical signs

Before the commencement of the experiment, all the bulls were observed to be in a good physical condition. Following experimental infection, bulls were divided into three groups according to the severity of clinical signs as illustrated in Table 15. Bulls in group 1 showed severe generalised lesions, in group 2, a mild clinical disease and in group 3 an inapparent infection. The day of infection of bulls in this study was counted as Day 1 p.i.

Table 15: Grouping of animals according to the severity of clinical signs

Group no.	Clinical signs	Animal no.	Age (months)	Weight (kg)	Breed
1	Severe	D	15	252	Dexter
		E	14	246	Dexter
2	Mild	B	16	342	Dexter
		C	13	207	Dexter
3	Inapparent	F	13	206	Dexter
		A	13	215	Dexter

Group 1. An intermittent rise in rectal temperature was observed in animals following inoculation. A fever reaction started on Day 7 p.i. and persisted for up to Day 18 p.i. in bull D and Day 34 p.i. in bull E, with a decline in rectal temperature at Day 23 p.i. in bull D. Temperatures as high as 40.3 °C on Day 12 p.i. for bull D and 40.7 °C at Day 11 p.i. for bull E were recorded. After the height of fever reaction, bull D had a slight fluctuation in rectal temperature of 40.0 °C on Day 13, 40.2 °C on Day 14 and 15 p.i. and 40.1°C on Day 16 p.i., after which it declined steadily. In bull E, after the height of fever reaction, a temperature below 39 °C was not recorded. Temperatures remained high in bull E until the end of sample collection (Figure 2). In addition, bull D developed circumscribed cutenious nodules on the flank, penis and scrotum on Day 8 p.i. after the fever reaction on Day 7 p.i., which became generalised 5 days later with corneal opacity developing on Day 23 p.i. In bull E, skin nodules were observed on the flank and perineum on Day 8 p.i., on the same

day as the fever reaction, which became suppurative as the infection progressed with ulceration around the muzzle and bucal mucosa. Corneal ulcerations were observed on Day 29 p.i. following the fever reaction in this bull. There was also an enlargement of the superficial lymph nodes in both bulls.

Group 2. Animals showed a mild form of clinical disease. An intermittent temperature rise was observed in the bulls. The highest temperature recorded in bull B was 39.1 °C on Day 7 p.i., with fever lasting till Day 8 p.i. and for bull C a temperatures of 39.3 °C on Day 9 p.i. A gradual decrease in rectal temperature was observed after the height of the fever reaction with subsequent fluctuation in individual bulls at varying days (Figure 3). In bull B ulcers were observed on the muzzle and a few on the bucal mucosa on Day 15 p.i., seven days after the fever reaction, while in bull C a few scattered nodules were observed along the flank.

Group 3. Animals showed an inapparent infection. An intermittent temperature rise was observed in the bulls in this group. The peak temperature in bull A was 38.9 °C on Day 15 p.i. and that for bull F was 39.4 °C on Day 7 p.i. A decrease in temperature was observed after the peak temperature rise (Figure 3). Skin nodules were observed on the tail, which did not progress beyond that region in bull A, on Day 8 p.i. No skin lesions were observed in bull F.

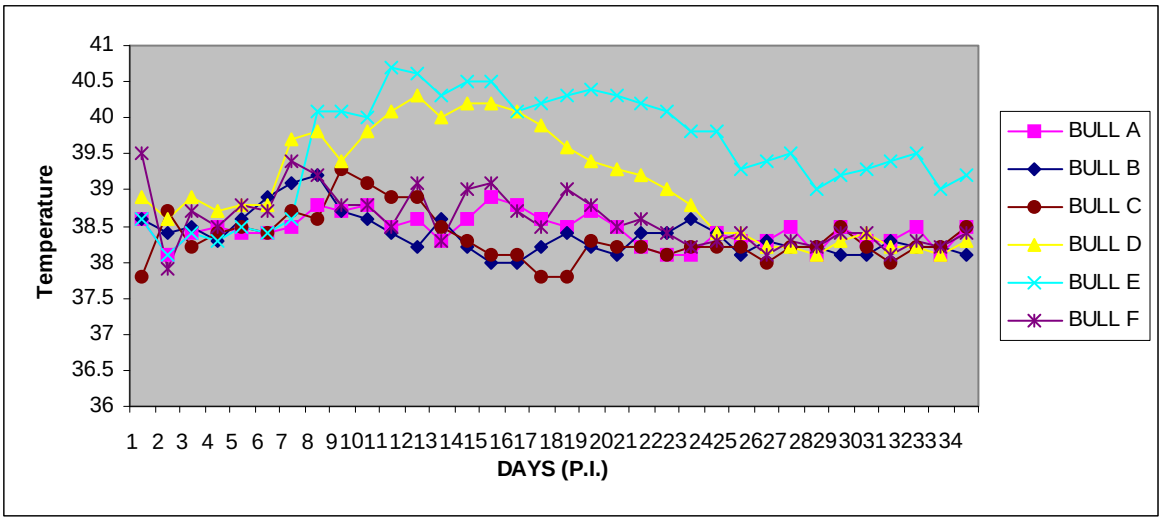

Figure 3: Temperature reaction in LSDV infected bulls

4.2. Virus isolation

4.2.1. Virus isolation on bovine dermis cells from heparinized blood samples

Group 1. Virus was isolated from blood samples in bull D on seven occasions, on Day 9, 11, 13, 15, 17, 19 and 21 p.i. Virus was isolated from blood 2 days after the initial rise in rectal temperature on Day 9 p.i. (Figure 4). In bull E rectal temperature began to rise on Day 8 p.i. and virus was isolated from blood one day after. In this bull virus was isolated on eight occasions from Days 9, 11, 13, 15, 17, 19, 21 and 23 p.i. (Figure 4).

Group 2. In bull B, virus was not isolated on culture from blood samples, while in bull C virus was isolated only on one occasion on Day 7 p.i. This was 2 days before the height of fever reaction on Day 9 p.i.

Group 3. In bull A virus was isolated from blood samples on three occasions on Day 15, 17 and 19 p.i. The isolation of virus from the blood of bull A corresponds with the height of fever reaction on Day 15 p.i. In bull F virus was isolated on three occasions from blood samples on Day 13, 17 and 25 p.i. In this bull virus was detected in blood samples in cell culture 6 days after the height of fever reaction on Day 13 p.i. (Figure 4).

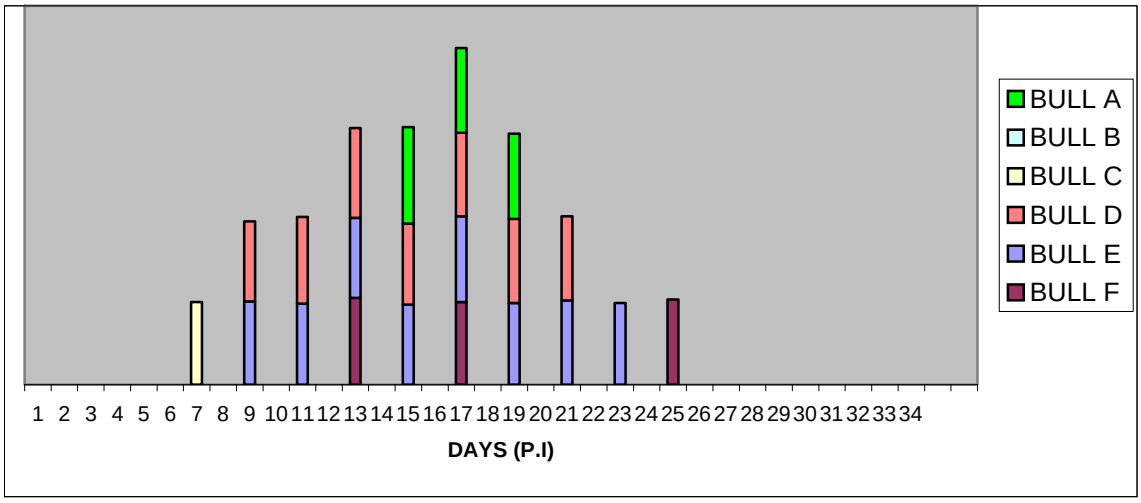

Figure 4: Virus isolation on bovine dermis cells from heparinized blood samples

4.2.2. Virus isolation on cell culture from supernatants of centrifuged semen samples

Group 1. Virus in semen was isolated on cell culture in bulls D and E on seven occasions on Days 11, 13, 15, 17, 23, 25 and 27 p.i. (Figure 5). All other samples were negative. In bull D the first day of virus isolation on cell culture was one day before the height of fever reaction and two days earlier than the isolation of virus from blood samples on cell culture on Day 9 p.i. In bull E the first isolation of virus on cell culture on Day 9 p.i. corresponds with the height of fever reaction (Day 11 p.i.), while virus was isolated from blood samples on culture 2 days earlier on Day 9 p.i.

Group 2. In bull B virus in semen was positive only once on culture on Day 27 p.i., which corresponds with the height of fever reaction on Day 8 p.i. (Figure 5). All other samples were negative. In bull C virus was isolated on cell culture on Day 13 and 15 p.i. (Figure 5), three and five days later than the height of fever reaction on Day 9 p.i. and detection of virus in blood on cell culture on Day 7 p.i., respectively. All other samples were negative.

Group 3 No virus could be isolated on cell culture from the semen collected from bulls A or F (Figure 5).

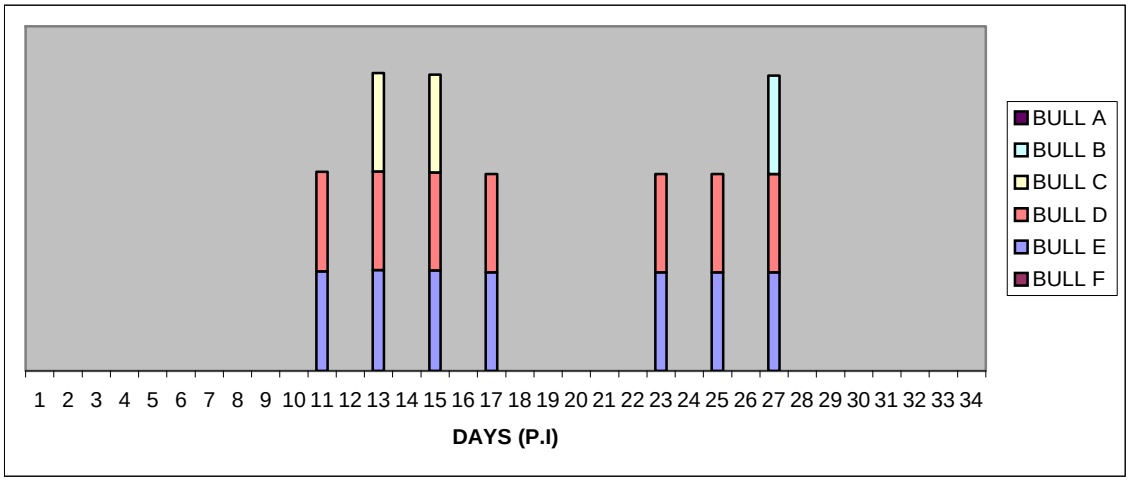

Figure 5: Virus isolation on cell culture from supernatants of centrifuged semen samples

4.2 3. *The detection of LSD viral nucleic acid in semen samples by PCR*

Group 1. Viral nucleic acid could be detected by PCR in semen samples of bulls D and E on Day 11 p.i., a day before the height of fever reaction on Day 12 p.i. in bull D and 2 days

earlier than the detection of virus in blood samples on culture on Day 9 p.i. (Figure 6). In bull E, the detection of viral nucleic acid in semen corresponds with the height of fever reaction on Day 11 p.i. (Figure 6) while virus in blood was isolated on cell culture two days earlier on Day 9 p.i. Viral nucleic acid was detected in semen samples of bulls D and E on ten occasions, on Days 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29 p.i. All other samples were negative (Figure 6).

Group 2. In bull B the height of fever reaction was on Day 8 p.i. and semen samples were PCR positive once, on Day 27 p.i. (Figure 6). All other samples were negative. Virus was also not isolated on culture from blood samples of this bull. In bull C the highest temperature recorded was on Day 9 p.i. and semen samples were PCR positive on Days 13 and 15 p.i. (Figure 6). All other samples were negative. In this bull virus was isolated on cell culture once from a blood sample, on Day 7 p.i.

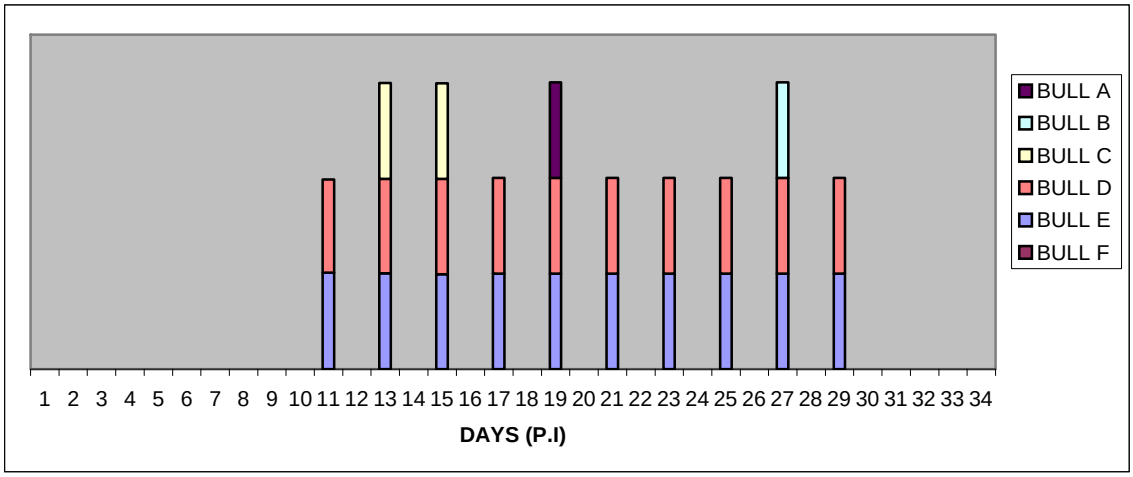

Figure 6: The detection of LSD viral nucleic acid in semen samples by PCR

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Group 3. One semen sample was PCR positive in bull A on Day 19 p.i. (Figure 6), four days after the first isolation of virus on cell culture from blood samples and the height of fever reaction on Day 15 p.i. In bull F all the semen samples were PCR negative.

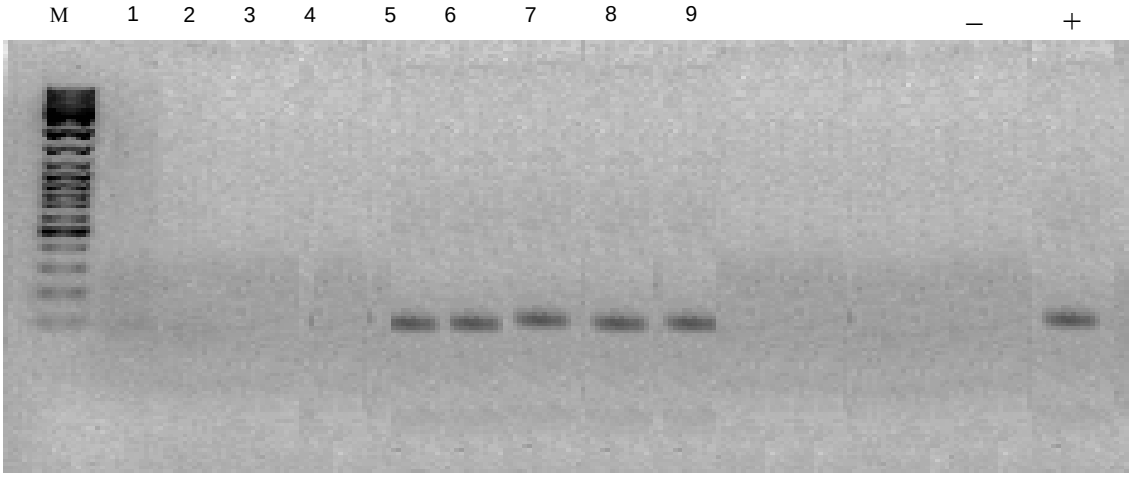

Figure 7. A 2 % agarose gel showing positive semen samples of infected bulls

M = molecular marker, lanes 1-4 = negative samples, lanes 5-9 = positive samples, – = negative control, + = positive control

4.2 4. Virus isolation on cell culture from supernatants of centrifuged and serially diluted semen samples

When supernatants of centrifuged semen samples were used to infect cultured cells (section 3.4.2.1), 17 out of 32 (53.1 %) were positive. The average number of days for the first appearance of signs of viral induced CPE following inoculation was 4 days. No toxicity was recorded on culture in these samples.

In serially diluted semen samples (section 3.4.2.2), 9 (28.1 %) were positive, 12 (37.5 %) negative and 11 (34.3 %) toxic on cell culture. The average number of days for the first appearance of signs of viral induced CPE following inoculation was 8 days following inoculation of BD cell cultures. The summary of the data is represented in Table 16.

Table 16: Infectivity and toxicity of LSDV infected semen samples processed by centrifugation and serial dilution

Test Method	No. of positive cultures identified	No. of negative cultures identified	No. of toxic cultures	Median day to detect LSDV
Supernatant	17	15	0	4
Serial dilution	9	12	11	8

4.2 5. Comparison of the sensitivity of PCR and virus isolation

Group 1. Supernatants of centrifuged semen samples of bull D and E were PCR positive on ten occasions on Days 11, 13, 15, 17, 19, 21, 23, 25, 27 and 29 p.i. Virus was isolated from the supernatants on cell culture on seven occasions on Days 11, 13, 15, 17 p.i. and again on Days 23, 25 and 27 p.i. (Figure 8). Virus was not isolated in three semen samples on Days 19, 21 and 29 p.i. that tested positive by PCR (Figure 8).

Group 2. Viral nucleic acid was detected by PCR in semen samples of bull B only once on Day 27 p.i and virus was isolated on cell culture on the same day. In bull C samples were positive for virus isolation and PCR on Days 13 and 15 p.i. (Figures 5 and 6)

Group 3. Viral nucleic acid was detected by PCR in semen samples of bull A only once on Day 19 (p.i.). Virus was not isolated on cell culture from this bull. Bull F was negative for PCR and virus isolation (Figures 5 and 6).

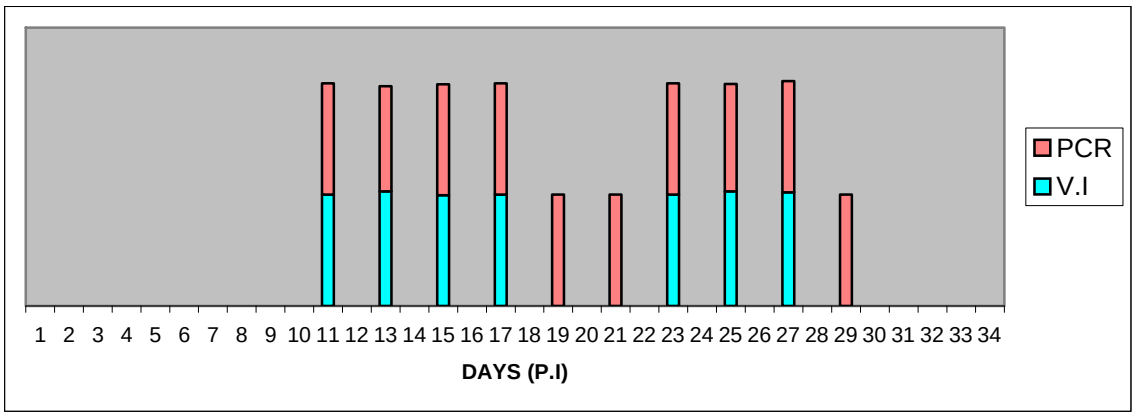

Figure 8: Comparison of the sensitivity of PCR and virus isolation in detecting LSDV in semen samples of bulls in the severely affected group

4.2 6. Detection of LSD viral nucleic acid in preputial wash by PCR

Group 1. The preputial wash of bull D was PCR positive for LSDV on seven occasions on Days 11,13, 15 p.i. and again on Days 21, 23, 25 and 27 p.i. (Figure 9). All other samples were negative. Bull E was PCR positive on six occasions on Days 11, 13, 15, 17 p.i. and again on Days 21 and 23 p.i. All other samples remained negative (Figure 9).

Group 2. The preputial wash of bull B was PCR positive only on one occasion on Day 19 p.i. (Figure 9). All other samples remained negative. Bull C was PCR positive only on Day 17 p.i. with all other samples remaining negative (Figure 9).

Group 3. The preputial wash of bull A was PCR negative in all the samples tested. Bull F was PCR positive on three occasions on Day 13 p.i. and again on Days 17 and 19 p.i. (Figure 9). All other samples were negative.

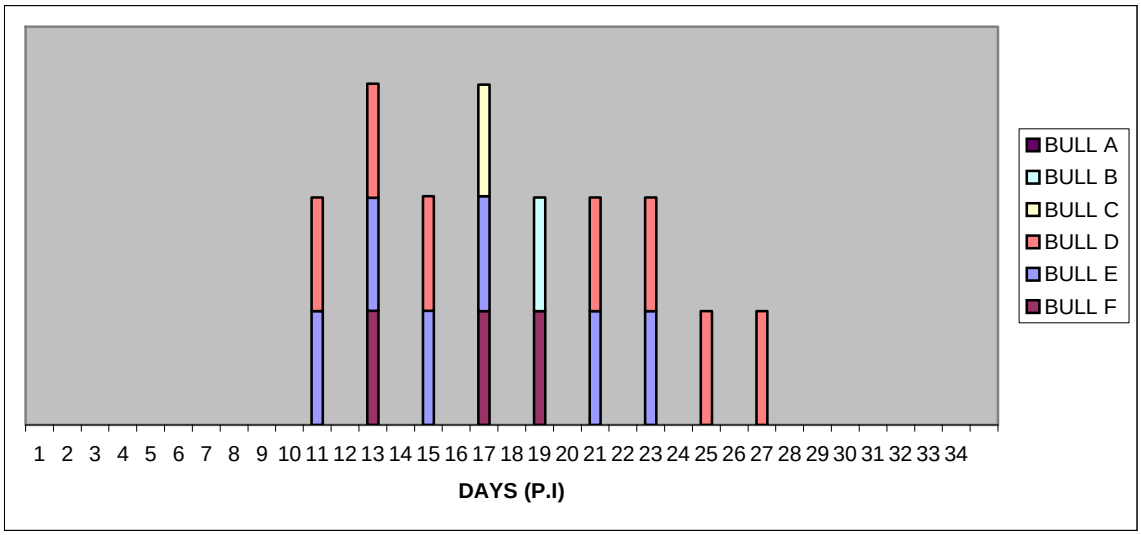

Figure 9: Detection of LSD viral nucleic acid in preputial washes of different bulls by PCR

4.2 7. Detection of LSD viral nucleic acid in vesicular fluid by PCR

Group 1. The vesicular fluid of bull D was PCR positive on eight occasions on Days 11, 13, 15, 17 p.i. and again on Days 21, 23, 25 and 27 p.i. (Figure 10). In bull E samples were PCR positive on five occasions on Days 17, 19, 21 p.i. and again on Days 25 and 27 p.i. (Figure 10). In both bulls all other samples were negative.

Group 2. The vesicular fluid of bull B was PCR negative in all the samples tested. Bull C was PCR positive on three occasions on Day 15 p.i. and again on Days 23 and 27 p.i. (Figure 10). All other samples remained negative.

Group 3. The vesicular fluid of bull A was PCR negative in all the samples tested. Bull F was PCR positive on one occasion on Day 17 p.i. (Figure 10). All other samples remained negative.

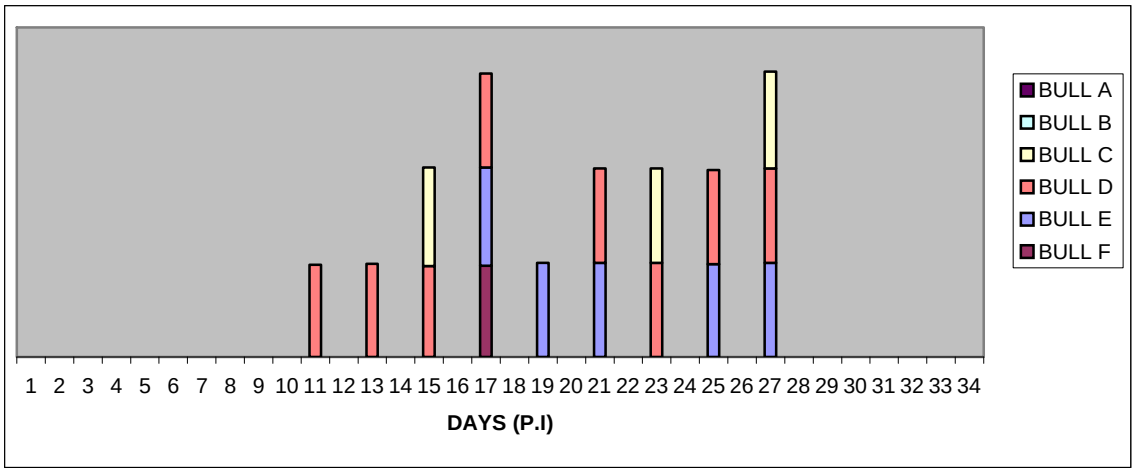

Figure 10: Detection of LSD viral nucleic acid in vesicular fluid of different bulls by PCR

4.2 8. *Quantification of infective virus in supernatants of centrifuged semen samples on cell culture*

Cell culture infectivity of the LSDV strain 248/93 in a semen sample from bull E collected on Day 15 p.i. was recorded at dilutions 10^{-1} to 10^{-3}. The $TCID_{50}$ of the virus was calculated to be 3 log $TCID_{50}$.

CHAPTER 5

.DISSCUSION

The prevention of the introduction of LSDV into non-endemic areas through the use of infected semen samples in AI is of utmost importance. It requires therefore a sensitive diagnostic method to confirm the infective nature of the virus shed in the semen.

Recent studies have focused on the duration of shedding of the virus in semen (Irons *et al*. 2005), persistency of the virus in blood and skin lesions (Tuppurainen *et al.* 2005), development of recombinant vaccines (Ngichabe *et al.* 1997; Ngichabe *et al.* 2002; Aspden *et al.*, 2002; Wallace and Viljoen 2005) and transmission (Carn and Taylor, 1985; Kitching and Mellor, 1986; Carn and Kitching1995a; Chihota *et al*. 2001).

Reports on the sensitivity of diagnostic techniques were also published. The use of PCR in the detection of LSD viral nucleic acid in semen was reported to be more efficient than virus isolation (Irons *et al.* 2005). The sensitivity of PCR compared to virus isolation in detecting herpes viruses in semen and other specimens has also been reported by various authors (Sharma *et al*. 1992; Lawrence *et al*. 1994; Xia *et al*. 1995; Rocha *et al*. 1998; Wald *et al*. 1999). However, no studies have been performed on the comparative sensitivity of a virus isolation method that eliminates the cytotoxic effect of semen and enhances the isolation of LSDV on cell culture with PCR. Furthermore, the infectivity level of LSDV shed in semen, and the presence of the virus in ejaculatory fluid other than semen, has also not been reported.

Six Dexter bulls were experimentally infected with a field isolate of LSDV by the intravenous route. The same volume and titre of virus suspension was used to infect all the bulls.

5.1. Clinical signs

The appearance of clinical signs was consistent with existing data in which 10 – 50 % of animals fail to develop generalised disease in the field or following experimental infection (Carn and Kitching 1995; Capstick, 1959; Tuppurainen *et al.* 2005) two bulls in this study developed characteristic clinical disease, two a mild infection and two an inapparent infection. This may possibly be attributed to the genetic resistance determined by the major

histocompatibility complex present on cell surfaces of individual animals described by Amills *et al.* (1998).

The incubation period of the virus of 7 to 14 days in the severely affected group following experimental infection of bulls in this study correlates with those reported in previous studies (Carn and Kitching 1995b; Capstick 1959; Haig 1957; Irons *et al.* 2005; Prozesky and Barnard 1982; Tuppurainen *et al.* 2005). The duration of fever varied within and between groups. In the severely affected group (Group 1) fever in bull D started on Day 7 p.i. and lasted for 10 days (Day 18 p.i.). In bull E fever started on Day 8 p.i. and lasted until the end of sample collection (Day 34 p.i.). In the mildly affected group (Group 2) only 2 days of fever reaction was recorded. In bull B fever started on Day 7 p.i. and declined 2 days later. In bull C, fever started on Day 9 p.i. and lasted 2 days. This short duration of fever reaction in this group can be correlated with observations by Kitching and Hammond (1992) and the authors further showed that animals with mild clinical disease develop low levels of neutralising antibodies. In bulls that developed inapparent infection (Group 3), a slight intermittent fever reaction was recorded, which further buttress the involvement of cell-mediated immunity in LSD infection (Carn 1993).

In group 1 bulls appearance of skin lesions following fever reaction in bull D and E was recorded on Day 8 p.i., one day after the fever reaction. These findings correlate with observations by Weiss (1968), where skin lesions appear 48 hrs after the initial rise in temperature. In bull D the lesions became generalised 5 days after the temperature rise and cornea opacity was observed 2 weeks after the appearance of skin lesions. In bull E skin lesions extended around the muzzle and bucal mucosa with cornea opacity developing 3 weeks after the appearance of skin lesions. In group 2 bulls a few ulcers were observed on the muzzle and bucal mucosa in bull B and only on the flank in bull C on Day 16 after the fever reaction. The appearance of skin lesion in these bulls correlates with previous studies by Prozesky and Barnard (1982), where skin nodules were observed in some bulls 2-3 weeks after inoculation. In group 3 bull, a few skin nodules were observed on the tail in bull A on Day 8 p.i. that did not develop or progress beyond that region. In bull F no skin lesions were observed.

5.2. Viraemia

In this study virus isolation from heparinized blood samples was performed on BD cell culture. The choice of culture cell type was based on the susceptibility of BD cells to LSDV and the development of typical CPE in these cultures (SOP, DVTD). The appearance of CPE in some cultures took 4 days to develop while in others, CPE was only observed on Day 7 after the second passage. Negative samples were blind passaged three times and were discarded when no CPE was evident.

The isolation of LSDV from blood samples the bulls used in this study was largely dependant on the individual response to infection. The virus was isolated from blood samples of severely affected bulls (group 1) on several occasions (Figure 4). Virus was isolated from blood samples after the onset of fever reaction and appearance of skin lesions in both bulls. The time of isolation of virus from blood samples in these bulls correlated with recent studies by Tuppurainen *et al.* (2005) where virus was isolated one day before, the same day or one day after the onset of fever in some bulls. In these bulls virus was isolated for up to Days 21 and 23 p.i. in bull D and E, respectively. The period of viraemia in bull E is consistent with Day 16 p.i. reported by Carn and Kitching (1995b).

In group 2 bulls virus was not isolated from blood on culture in bull B. The inability to isolate virus even though a few lesions were evident in this bull is noteworthy. One possible explanation for this maybe the low level of virus in the blood beyond the limit of detection even after blind passaging. In bull C virus was isolated only once, two days preceding the height of fever reaction. The number of times virus was isolated in this bull is consistent with results obtained from a bull that developed mild infection in a study by Tuppurainen *et al.* (2005).

In group 3 bulls virus was isolated from blood samples for longer periods (3 occasions) in both bulls when compared to bulls that developed mild clinical disease (Figure 4). Again consistent with observations by Tuppurainen *et al.* (2005) where viraemia in bulls that developed inapparent disease persisted much longer than in bulls that showed mild clinical disease. In that study Tuppurainen *et al.* (2005) were able to isolate virus on seven occasions from blood samples of a bull with an inapparent infection.

5.3. Virus isolation from semen samples on cell culture

From the pilot study discussed in Chapter 2, the two methods that were able to reduce the toxic effect of semen on cell cultures and still allow the isolation of LSDV from semen of experimentally infected bulls was used to test field samples. Semen samples were therefore processed by the centrifugation method that completely eliminated the toxic effect of semen on cell culture as well as the serial dilution method.

Of all the semen samples inoculated on cell culture using the centrifugation method (section 3.4.2.1) virus was isolated from 17 of them (53.1%) with the median time of detection of 4 days following inoculation of confluent BD monolayers with no toxicity observed (Table 14). Semen toxicity on cell cultures was completely eliminated when samples were processed using this technique, including an increased recovery rate of isolation of LSDV than has been previously reported (Irons *et al.* 2005). The appearance of CPE on cell culture is also quicker than have been reported in attempts to isolate other viruses from semen (Kim *et al.* 2001; Larska & Rola 2003). Larska & Rola (2003) were only able to isolate equine arteritis virus from semen samples after one or two passages of inoculated cell culture. Similarly, Kim *et al* (2001) could only isolate porcine circovirus from boar semen after two successive passages and an incubation period of five days. The use of supernatants of centrifuged samples identified more positive samples when compared with PCR than the serial dilution method in detecting LSDV shed in semen on culture.

In semen samples that were serially diluted (section 3.4.2.2) virus was isolated from 9 samples (28.1%) and 11 samples (34.3%) were toxic on cell culture with the median day of detection of 8 days following inoculation on BD cells (Table 16). Serial dilution did reduce the toxic effect of semen on cell culture in some samples as previously reported by Lang *et al*. (1974) but toxicity remained a major problem in the majority of samples with this method. Some cultures did require washing of cells, replacement of growth medium and further blind passaged before viral CPE became evident. It was clearly shown that virus isolation from the supernatants of centrifuged semen sample was more sensitive than the serial dilution method.

5.4. Comparison of the sensitivity of PCR and virus isolation in the detection of LSDV in semen samples

In order to compare the sensitivity of PCR and virus isolation, semen samples from experimentally infected bulls were first tested using PCR. The PCR positive and a few negative samples were then subjected to virus isolation using the centrifugation method developed in the pilot study.

In group 1 bulls virus was isolated from semen from both animals on Day 11 p.i, on the same day viral nucleic acid was detected by PCR. Semen samples were PCR positive on 10 occasions while virus was detectable on cell culture on only 7 occasions from these samples (Figure 8). Virus could not be detected on culture on 3 occasions from semen samples that were PCR positive. The inability to isolate virus from PCR positive samples may be due to an intermittent shedding of viable virus in semen or only fragments of viral nucleic acid were present. It is also possible that low concentrations of virus were shed at that stage of the infection, which was below the detection threshold of the isolation technique employed in this study. In group 2 bulls virus was isolated on culture from the same number of semen samples that tested positive for PCR while in group 3 bulls PCR was able to detect viral nucleic acid only once, in bull A. No virus was isolated in any of the bulls in this group. However, the possibility that infective virus was present in these samples even though it could not be isolated, cannot be ruled out. In previous studies, Givens *et al.* (2003) could demonstrate the infective nature of PCR-positive semen where virus could not be isolated on cell culture, by inoculation into susceptible calves. In that study, the authors showed that infective BVD virus was present in the semen samples even though they were not able to isolate it on cell culture. It may be that although the isolation technique was able to eliminate the toxicity problems associated with semen on cell culture, inhibitors of virus replication as suggested by Kirkland *et al.* (1991) could still reduce the efficiency of virus isolation.

In this study PCR was able to detect LSD viral nucleic acids in semen samples even when virus could not be isolated on cell culture. This finding is consistent with previous reports by various authors of the sensitivity of PCR over virus isolation in detecting viruses in semen and other specimens (Irons *et al*. 2005; Lawrence *et al*. 1994; Rocha *et al*. 1998; Sharma *et al*. 1992; Tuppurainen *et al*. 2005; Wald *et al*.1999; Xia *et al*. 1995). The PCR procedure can be completed in a few hours, whereas at least 4 days was necessary before LSDV could be isolated on cell culture using the isolation technique employed in this study

(section 3.4.2.1). This method of sample processing enhanced LSDV isolation on culture from semen samples with reduced detection time, when compared to the slow growth rate of the virus in tissue culture of up to 14 days for CPE to develop as reported by Plowright and Witcomb (1959) and is efficient in the isolation of viable LSDV from bull semen. This result shows that PCR for the detection of LSDV nucleic acid in bull semen is sensitive and efficient and can be used for routine screening of semen samples.

5.5. Detection of LSD viral nucleic acid in preputial wash by PCR

To detect LSDV nucleic acid in preputial washes, PCR was also used. In group 1 bulls LSD viral nucleic acid in preputial washes of bull D was detected on 7 occasions and in bull E on 5 occasions. In bull D preputial washes were PCR negative for LSDV on 3 occasions while in bull E on 4 occasions (Figure 9). On all of these days semen samples were PCR positive on the same days in both bulls.

In studies by Xia *et al.* (1995), bovine herpes virus-1 was isolated on cell culture from preputial swabs and semen samples were also PCR positive at day 4 after preputial inoculation of experimental bulls. In this study, the possibility that the presence of LSD viral nucleic acid detected in preputial washes is associated with extended lesions observed on the penis into the preputial mucosa or intrinsic through viral shedding in preputial excretions may not be ruled out. A similar mode of shedding of bovine herpes virus and bovine leukaemia virus in semen via abraded genital mucosa has been reported (Kahrs *et al.* 1980; Afshar and Eaglesome 1990).

However, in group 2, preputial wash and semen samples of bull B were PCR positive only on one occasion on different days. In bull C preputial wash was PCR positive only once on Day 17 p.i. and virus in semen twice on Days 13 and 15 p.i., four days before the detection in the preputial wash (Figure 9). Although LSD viral nucleic acid was detected in preputial washes no obvious lesions were observed on the penis of these bulls. The presence of viral nucleic acid in the preputial washes in the absence of localised lesions further buttress the hypothesis of a possible preputial excretion involvement or contamination from a previous ejaculate.

Furthermore, in group 3 preputial washes of bull A were PCR negative in all the samples tested while semen samples tested positive only once. In bull F preputial washes were PCR

positive on three occasions while all the semen samples were PCR negative (Figure 6 & 9). In this group, like bulls of group 2, no lesions were observed on the penis or scrotum nor the perineum.

Although the mechanism by which LSDV is shed in the preputial wash is not well understood, results from this study suggests that there is no correlation between the presence of LSDV nucleic acid detected in preputial washes and semen samples. Further work is required to investigate the source of the virus shed in the preputial wash.

5.6. Detection of LSD viral nucleic acid in vesicular fluid by PCR

In group 1 LSD viral nucleic acid in vesicular fluid was detected on 8 occasions in bull D and on 5 occasions in bull E. The shedding of virus in vesicular fluid in bull D correlates with the detection of viral nucleic acid in semen samples except on Days 19 and 29 p.i on which vesicular fluid was PCR-negative (Figure 10). Viral nucleic acid was also detected in preputial washes on seven occasions in this bull (Figure 9). In bull E detection of viral nucleic acid in vesicular fluid coincided with the same days of detection in semen samples except on 5 occasions on which vesicular fluid samples were PCR negative (Figure 10). Preputial washes of bull E were PCR - positive on 6 occasions. In these bulls some vesicular fluid samples were PCR negative even when viral nucleic acid could be detected in semen samples. This suggests that semen contamination is not responsible for the presence of LSD viral nucleic acid in vesicular fluid. Previous studies by Kirkland *et al.* (1991) on the replication of BVD virus in the bovine reproductive tract suggested that the seminal vesicles and prostrate gland may be productive sites for the replication of the virus. Similarly, Prieto *et al.* (2003) were able to isolate PRRSV from vesicular and prostrate glands of experimentally infected boars. Whether the presence of LSD viral nucleic acid in the vesicular fluid in this group of bulls is associated with contamination from the prepuce, or an isolated shedding from the vesicular gland, is unclear.

In group 2 bulls vesicular fluid in bull B was PCR - negative in all the samples collected while viral nucleic acid was detected only once in semen samples and preputial washes on different days (Figures 6, 8 & 9). In bull C viral nucleic acid was detected in vesicular fluid more than it could be detected in peputial wash and semen samples (Figures 6, 9 & 10).

In group 3 vesicular fluid and preputial wash of bull A were PCR negative in all the samples tested while semen sample tested positive only on 1 occasion (Figures 6, 8 & 10).

In bull F, vesicular fluid was PCR positive only once and in preputial wash on 3 occasions (Figures 9 & 10). No virus was detected in the semen of this bull.

The presence of viral nucleic acid in vesicular fluids in these bulls may not be associated with the presence of virus in semen or preputial wash. Although the contamination of vesicular fluid may occur by either means, the negative results from vesicular fluids in bulls A and B and its detection in bulls C and F, suggests that there could be periods during which no virus is shed in the vesicular fluid after infection or viral shedding may not be dependant on the severity of clinical disease. It is however, important to investigate the source of the virus shed in the vesicular fluid.

5.7. Tissue culture infective dose ($TCID_{50}$) of LSDV in semen of experimentally infected bulls

Reports by Carn and Kitching (1995) indicated that LSDV with a titre higher than 1log $TCID_{50}$ can establish an infection by intradermal inoculation and that 3 – 3.3 log $TCID_{50}$ could produce a generalized infection by intravenous and intranasal inoculation. In this study, the choice of semen sample that was evaluated was based on the assumption that at that stage of clinical disease, a significant amount of virus may be shed in semen. The $TCID_{50}$ of LSDV in a semen sample collected from bull E in the severely affected group on Day 15 p.i., four days after its first isolation on cell culture and the detection of LSD viral nucleic acid by PCR was determined to be 3 log $TCID_{50}$. This indicates the presence of a considerable amount of LSDV in semen of experimentally infected bulls at this stage of clinical disease.

Virus-contaminated semen has been incriminated as one of the possible routes of transmission of bovine herpes-1 virus and BVD virus (McGowan *et al*. 1995; Phipott 1993). It is however, not certain if the infectivity of the LSDV shed in the semen at that stage of the infection can infect susceptible cows through artificial insemination or the natural mating process. Further studies are therefore required to inseminate susceptible heifers with LSD infected semen samples.

In conclusion, this study provides evidence of a complete reduction of the toxic effect of semen on cell culture and increase chances of LSDV isolation with reduced detection time when semen samples were processed using the centrifugation method as described before. Furthermore, it showed PCR was more sensitive than virus isolation in the detection of

LSD viral nucleic acid in semen samples. Although PCR was more sensitive than virus isolation in the isolation of LSDV on cell culture, the use of supernatants of centrifuged semen samples gave more positive results when compared to other virus isolation techniques previously employed. This study provides the first evidence of the shedding of LSDV nucleic acid in vesicular fluid and preputial washes of experimentally infected bulls. It also indicated that the LSDV shed in semen of experimentally infected bulls may be infective at certain stages of clinical disease.

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