Mechanisms by which lumpy skin disease virus is shed in semen of artificially infected bulls

by

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Dedicated to my loving parents, John and Rita Annandale, who have always given wings to my dreams

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DECLARATION

I, Cornelius Henry Annandale, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in partial fulfillment of the requirements for the degree MMedVet (Gyn).

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Signed ……..

Cornelius Henry Annandale

Date ……..

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FIGURES AND TABLES

ABSTRACT

Lumpy skin disease (LSD) is a disease of significant economic importance in Africa. It causes considerable production losses and its presence in semen is a constraint to international trade. Recent findings that LSDV viral DNA can be found in the semen of artificially infected bulls for up to five months, while viable virus could be isolated 42 days after infection, indicated the need for studies into the mechanism by which this protracted shedding occurs.

Six healthy, seronegative, postpubertal Dexter bulls were housed in vector-free stables and challenged with LSD virus by intravenous injection. Sheath washes, vesicular fluid and semen collection was performed every other day and subjected to PCR. On these days, blood was collected for serum neutralization tests and virus isolation, and ultrasonography of the reproductive tracts performed. Semen was centrifuged to separate cell-rich and seminal plasma fractions, and tested by PCR. Clinical parameters were recorded twice daily. Bulls shedding viral DNA 28 days after challenge were slaughtered, their reproductive tracts were harvested and diagnostic post mortem was performed. Histopathology, immunoperoxidase staining, electron microscopy, virus isolation and PCR were done on tissue samples.

Of the six bulls, two showed no clinical signs, two showed mild and two showed severe clinical signs. Fever appeared five to seven days and lesions eight to ten days post challenge. Bulls were viraemic and febrile during the same time. Viral DNA was detected in all semen fractions of all bulls, but mostly from the cell-rich fraction and from the bulls showing the most severe clinical signs. Ultrasonography showed infarction in the testes and epididymides of the two bulls that were most severely affected. Necropsy of the two bulls that were still shedding after 28 days showed testicular degeneration and infarction, as well as epididymal granuloma formation. None of the accessory sex organs showed significant pathology. Histopathological changes seen were necrogranulomata in testes and peididymides. IMP staining of reproductive tissues showed that staining was restricted to areas in the testes and epididymides that were associated with necrosis. Virus could be seen on negative staining EM of sections of the testes.

Our results show that LSDV is not limited to specific fractions of the ejaculate and that the testes and epididymides are most profoundly affected. Blood contamination is not responsible for the presence of viral DNA in semen, and it is unlikely that the virus is sperm-associated. Results suggest that the ejaculate is contaminated with viral DNA as it is shed from necrotic lesions in the genital tract. Further research is indicated into the ability of infected semen to produce disease as well as treatment protocols that could render semen free of viral DNA.

SAMEVATTING

Knopvelsiekte (KVS) is ekonomies belangrik in Afrika. Dit is die oorsaak van merkbare produksieverliese en die teenwoordigheid daarvan in semen strem internasionale handel. Onlangse bevindinge dat KVS virus partikels vir tot vyf maande gevind kan word in die semen van eksperimenteel besmette bulle, terwyl lewensvatbare virus 42 dae na besmetting geisoleer kan word, het die noodsaaklikheid van studies na die meganisme betrokke in verlengde afskeiding onderstreep.

Ses gesonde, seronegatiewe, postpubertale Dexter bulle is in vektorvrye stalle gehuisves en binneaars met KVS virus gedaag. Skedewasse, vesikulêre vloeistof- en semenversameling is elke ander dag uitgevoer en onderwerp aan polimerase kettingreaksie (PKR). Bloed vir serum neutralisasietoetse en virus isolasie is ook op hierdie dae versamel en die geslagstelsel is met ultraklank ondersoek. Semen is uitgeswaai om dit te skei in selryke- en seminale plasmafraksies, en onderwerp aan PKR. Kliniese parameters is tweemaal daagliks geneem. Bulle wat 28 dae na daging virale DNA in hul semen uitgeskei het, is geslag en diagnostiese nadoodse ondersoek is op hul geslagstelsels uitgevoer. Histopatologie, immunoperoxidase (IMP) kleuring en elektronmikroskopie, sowel as virus isolasie en PKR is op weefselmonsters uitgevoer.

Van die ses bulle het twee geen kliniese tekens getoon nie, twee slegs matige en twee erge kliniese tekens. Koors is gemerk vyf tot sewe dae en letsels agt tot tien dae na daging. Bulle was tergelykertyd viremies en koorsig. Virale DNA is waargeneem in alle fraksies van semen op een of ander stadium, maar meestal vanaf die selryke fraksie en van die bulle wat erge kliniese tekens getoon het. Ultrasonografie het infarksie van die testes en epididimides van die erg- geaffekteerde bulle getoon. Nadoodse ondersoek van die twee bulle wat 28 dae na daging nog virale DNA uitgeskei het, het testikulêre degenerasie en ganuloomvorming in die epididimis getoon terwyl histopatologie hierdie veranderinge as nekrogranulomata geidentifiseer het. Geen veranderinge kon in die bygeslagskliere gevind word nie. IMP kleuring van die geslagsweefsel het getoon dat kleuring beperk was tot areas van nekrose in die epididimides en testes. Negatiewe kleuring elektronmikroskopie kon virus identifiseer in snitte van die testes.

Ons resultate toon dat LSDV nie beperk is tot ‘n spesifieke fraksie van semen nie en dat die mees uitgesproke effek op die testes en epididimis is. Besmetting met bloed is nie verantwoordelik vir die teenwoordigheid van virale DNA in semen nie, en dit is onwaarskynlik dat die virus spermsel-geasosieerd is. Resultate dui daarop dat die ejakulaat besmet word soos virale DNA loskom van nekrotiese areas af. Verdere studies is aangedui om te bepaal of semen wat besmet is met virale DNA in staat is om siekte te veroorsaak en welke behandelingsmetodes toegepas kan word om semen van virale DNA te bevry.

Chapter 1

Introduction

Economic farming of livestock in Africa is challenging. Not only is the environment one of extremes, but there are also a number of diseases which pose a significant health risk to humans and animals alike. Lumpy skin disease (LSD) is one example of a disease endemic to Africa, which frequently affects bovine production systems.

Lumpy skin disease is an acute, subacute to chronic viral disease of cattle, characterized by fever and the formation of multiple firm, circumscribed nodules in the skin of affected animals and necrotic plaques in the mucous membranes, as well as generalized lymphadenopathy (Coetzer 2004). The direct effects on affected animals range from damage to hides, reduced feed intake, pneumonia, mastitis and infertility to death. It is of considerable economic importance in southern Africa, although the disease has been reported in other African countries, Israel and Egypt (Agag *et al.* 1992). An outbreak of LSD has been reported in Egypt in April 2006 (OIE 2006). The Office International des Epizooties (OIE) has identified the disease as one with the ability to spread rapidly across continents and to have significant economic implications to international trade. It is therefore classified as a disease that is notifiable to the OIE (List A disease (2001)).

The effect on ability to mate and to produce fertile sperm is often not appreciated, but has important economic implications, especially in the context of subsistence farming and in farming systems where single sires are used. A recent review article (Hunter and Wallace 2001) has identified the need for research into the epidemiology and transmission of LSD in South Africa due to resurgence of the disease over the last decade of the twentieth century.

The export of ruminant semen from southern Africa holds the potential to be a form of trade from which economic benefits can accrue. Currently, the risk of the presence of Lumpy skin disease virus (LSDV) in semen is halting the approval of export protocols to some countries (Hentzen 2000). Recent work (Irons *et al.* 2005) has highlighted numerous aspects of the shedding of LSDV in semen that had previously been unknown. This mainly involved the shedding of LSDV particles in semen for much longer periods than previously reported, and the shedding of such viral particles at times when clinical signs of LSD where no longer evident. Determining the mechanisms by which LSDV is shed in semen of infected bulls for protracted periods could elucidate the possible involvement of certain genital organs in the development of this prolonged shedding state and allow more accurate prediction of the course of the infection from a reproductive point of view. This would enable safer selection of potential semen donors and contribute to meaningful quantification of risks associated with semen. A further benefit would be the formulation of preventative measures and the application of

processing methods to render semen safe from viral contamination. These steps would greatly facilitate the approval of export protocols.

1.1 Literature review

Literature is replete with examples of viruses that are excreted in semen during different stages of infection (Kahrs *et al.* 1980; Afshar and Eaglesome 1990). Some examples include the human immunodeficiency virus (HIV), bovine herpesvirus-1 (BHV-1), bluetongue virus (BTV), bovine viral diarrhoea virus (BVDV), bovine leukosis virus (BLV) and ephemeral fever virus (BEFV) in cattle, porcine reproductive and respiratory syndrome virus (PRRSV) and classical swine fever virus (CSFV) in swine, feline immunodeficiency virus (FIV) in cats, equine arteritis virus (EAV) in horses and foot-and-mouth disease virus (FMDV) in a number of species.

The pathobiological mechanisms responsible for the presence of virus in semen can be divided as follows:

- Presence in semen due to viraemia
- Presence due to contamination with blood during the collection process
- Venereal transmission / viral replication in tissues of the genital tract

It is with this division borne in mind that the following viruses identified as being shed in semen, are discussed.

1.1.1 Shedding of virus in bovine semen

As a product which is frequently shipped to destinations all over the world, semen has the potential to multiply male genetics manifold. It similarly has the potential to spread disease across borders. This is of particular concern in areas where large naïve populations, with no prior exposure to certain infectious agents known to be shed in semen, occur. The identification of viruses in semen is therefore of understandable importance.

The techniques that are most frequently employed to detect viruses in semen are virus isolation (VI) and nucleic acid amplification with the use of the polymerase chain reaction (PCR). There are significant technical difficulties in using semen as the inoculum on cell cultures; these include semen's toxicity to cells, high concentration of trypsin-like acrosomal enzymes and the occurrence of bacterial contaminants in the semen (Bowen *et al.* 1983). Positive PCR results do not confirm the ability of such a virus to replicate in host tissue, as it merely signifies the presence of viral nucleic acid. PCR and virus isolation can be used in concert or separately and the significance of the results obtained is dependent on the biology and nature of the virus under investigation. The presence of virus in

semen does not imply the ability to establish disease in animals inseminated with such semen, nor does it preclude such ability.

1.1.1.1 Lumpy skin disease virus

Taxonomically, LSDV resides in the genus Capripoxvirus of the *Poxviridae* family of viruses. Other members of this genus are sheep – and goatpox virus. Poxviruses are generally epitheliotrophic and can cause localized or systemic disease. Initial multiplication of the virus occurs at the entry site of the virus into the body. In systemic infections, further viral replication takes place in the draining lymph nodes, followed by viraemia and further viral multiplication in many different organs including the liver, spleen and lungs (Fenner *et al.* 1987). The latter multiplication leads to establishment of secondary viraemia and subsequent infection and development of disseminated focal lesions in the skin. Viral replication takes place in the cytoplasm of cells. Viral particles are enveloped when mature virus particles move to the Golgi complex; most particles are however non-enveloped and are released by cell disruption. Both enveloped and non-enveloped particles are infectious (Fenner *et al.* 1987). Few studies are available on the pathology and pathogenesis of LSDV infection. The earliest description by Thomas and Maré (1945) highlighted the histological changes. Their findings were confirmed to a large extent by Prozesky and Barnard (1982) who found that LSDV exerts its pathogenic effects by infiltrating a variety of cell types, including epithelial and endothelial cells, pericytes and fibroblasts,

resulting in lymphangitis and vasculitis. The latter report highlighted the difference in histological changes associated with acute or chronic lesions. During the acute stage vasculitis and lymphangitis with concomitant thrombosis and infarction resulted in oedema and necrosis (Prozesky and Barnard 1982). The lesions were initially infiltrated by neutrophils and macrophages, and later on these cells were gradually replaced by lymphocytes, plasma cells and macrophages, as well as fibroblasts (Prozesky and Barnard 1982). The exact pathogenesis of the development of the lesions associated with lumpy skin disease is not as well understood as the pathogenesis of sheeppoxvirus, prototype member of the capripoxvirus genus (Fenner *et al.* 1987). It was established that sheeppox infection spreads in macrophages around the body and becomes localized in a variety of organs, including kidneys, testes and lungs (Kitching 2004). Coagulation necrosis is the result of thrombi in blood vessels. It seems not to be determined whether a single cell type is responsible for the spread of LSDV around the body and its localization in various organs.

Nagi (1990) investigated cutaneous and testicular lesions in an outbreak of LSD in Egypt in 1989. It was noted that diffuse degenerative and inflammatory changes could be observed in the seminiferous tubules and blood vessels. The seminiferous tubules were devoid of primary and secondary spermatocytes and of spermatids, although the spermatogonia and Sertoli cells appeared resistant. The author speculated that the possible infertility due to LSDV infection may be transient as the regeneration of the germinal epithelium depends mainly upon the

persistence of spermatogonia and Sertoli cells, although extensive fibrosis may preclude the return to fertility. There are no other published reports on the pathology of LSDV in testicular tissue.

Experimental infection of cattle with LSDV in several studies has outlined the clinical course of lumpy skin disease. Carn and Kitching (1995) inoculated British cattle with 10^4 – 10^5 $TCID_{50}$ of a virulent field strain of LSDV using either the conjunctiva, nasal passages, skin or jugular vein as the route of infection. The researchers found that pyrexia was not a consistent feature of the disease, that generalized lesions were seen 9-14 days post infection and that the development of generalized infections did not seem to be dose-related. An interesting finding was a transient viraemia detected in two of eleven animals that did not show generalized signs. From their findings, it was concluded that less than 50% of cattle experimentally infected with LSDV or naturally exposed during an outbreak develop generalized infection. This compares favourably to the findings of Prozesky and Barnard (1982) where only three of seven cattle experimentally challenged developed generalized infection. Interestingly, in this study virus could be isolated from only two of the three cattle showing generalized infection. It would thus seem that development of generalized infection is not a reliable indicator of whether or not viraemia will be detected in the animals.

Several researchers have investigated the transmission of LSDV but the exact method of transmission of LSDV remains obscure. The epidemiology of lumpy

skin disease is characterized by a resurgence in appearance of the disease following seasons of particularly good rainfall which leads to the emergence of large numbers of vectors (Woods 1988), suggesting that transmission is vector-borne. Further evidence that transmission is arthropod-borne came to the fore when Carn and Kitching (1995) compared different routes of infection of experimental animals with LSDV. None of the animals inoculated via the conjunctival route seroconverted nor did they show any clinical signs. Animals infected via intradermal inoculation developed localized lesions and a small percentage of these animals (4 from 25 infected) developed generalized disease. Eight of eleven animals infected via the intravenous route developed generalized disease. Unaffected animals kept with infected animals failed to develop any lesions and also did not seroconvert. These findings led the researchers to conclude that transmission of LSDV between animals was inefficient and that blood feeding arthropods were an important factor in the natural spread of the disease. Chihota *et al.* (Chihota *et al.* 2001) proved that *Aedes aegypti* acts as a mechanical vector of LSDV but failed to implicate other biting insects as similar agents of transmission (Chihota *et al.* 2003). The latter finding was unexpected as viral particles were demonstrated by PCR in the gut contents of the insects, yet no transmission to susceptible cattle was observed.

Antibodies to LSDV have been demonstrated in some African wildlife species but their role in the epidemiology of LSD is thought to be insignificant (Hedger and Hamblin 1983). Giraffe (*Giraffa camelopardalis*) and impala (*Aepyceros*

melampus) are highly susceptible to experimental infection but the African buffalo (*Syncerus caffer*) and black wildebeest (*Connochaetes gnou*) appear to be resistant or only slightly susceptible (Young *et al.* 1970).

In countries where LSD is endemic, rapid and accurate laboratory techniques is essential for confirmation of a tentative clinical diagnosis. Various laboratory techniques and procedures have been published for the diagnosis of LSD. In studies performed on cattle affected by the LSD outbreak in Egypt in 1989, it was demonstrated that severe biochemical and trace element changes occur between 7 and 14 days following the first appearance of clinical signs (Agag *et al.* 1992). These changes were however not specific to LSD and hence not of diagnostic value. The authors also detected neutralizing antibodies between 21 and 42 days after infection. More recently, some reports have been published on attempts to optimize laboratory procedures. Kenyan workers have found fetal bovine muscle cells to be an adequate alternative for lamb testis cells (Binepal *et al.* 2001) in a virus isolation system. The long time required to read the cell cultures makes virus isolation an impractical diagnostic tool, but it is still regarded as essential to establish the infectivity of the virus isolated. South African workers (Tuppurainen *et al.* 2005) evaluated virus isolation, PCR and electron microscopy for their ability to meet the diagnostic demand of a fast and reliable test. They concluded that PCR was a fast and sensitive method to demonstrate viral DNA in blood and skin samples, but that virus isolation, although sensitive and reliable, may be too time-consuming. It is stressed that virus isolation would

be essential to determine the infectivity of the virus. It has to be emphasized that the majority of work on diagnostic techniques has focused on blood and tissue samples and not on semen.

Little data exists as to the risk posed by semen in the transmission of LSDV. One reference by Weiss (1968) to unpublished work stated that the virus is excreted in semen for 22 days from the fever reaction following experimental infection. Few procedures to detect the presence of LSDV in semen and none to render semen safe by biosecurity procedures or laboratory processing methods have been published. In an experiment to confirm the excretion of LSDV in semen and establish the duration and infectivity in relation to clinical signs, serological status and viraemia, Irons *et al.* (2005) showed that PCR and virus isolation can both be successfully employed to detect the presence of virus or viral particles in semen, that PCR identifies virus for longer periods than virus isolation and that shedding of viral particles in the semen of bulls occurred for up to five months following experimental infection. This is much longer than was previously reported (Weiss 1968). The study did not investigate whether or not the virus was associated with sperm cells or seminal plasma. However, the detection of virus in azoospermic semen samples suggested that the presence of virus was not dependent upon the presence of spermatozoa. The study was unable to determine the $TCID_{50}$ of infected bulls but isolation of virus at 1:1000 dilution was achieved, indicating the presence of a significant amount of virus in semen. Even though Carn and Kitching (1995) established that a viral inoculum

of 10^2 $TCID_{50}$ was sufficient to produce generalized infection following intradermal inoculation, and it would seem that the amount of virus present in semen found by Irons *et al.* (2005) was significant, it remains to be determined whether insemination with such semen would establish clinical LSD in susceptible cows.

There is currently no published information on the ability of semen infected with LSDV to establish clinical disease in cows inseminated with such semen, neither is there any data available as to the pathobiological mechanisms involved in the shedding of virus and viral particles into the ejaculate.

1.1.1.2 Bovine leukosis virus

Initial reports (Lucas *et al.* 1980; Moskalik 1987), identified bovine leukosis virus (BLV) as being shed in semen of seropositive bulls. Lucas *et al.* (1980) referred to trials done by Miller and van der Maaten to illustrate the fact that early attempts to produce evidence of virus shedding in semen of infected bulls was not consistently successful. Lucas and his coworkers inoculated sheep with the semen collected from a bull that had been identified as serologically positive for enzootic bovine leukosis (EBL) antibodies. Three of eleven sheep developed EBL antibodies as identified by AGID. The virus was also identified by electron microscopy. Monke (1986) reported on a Jersey dairy herd of 200 cows that failed to show seropositivity to EBL for a period of five years. This was despite

the fact that seropositive bulls provided 48.3% of all the semen used for artificial insemination (AI) in this herd over the period. It was therefore concluded that EBL was not transmitted in bull semen.

The role of leukocytes in the transmission of BLV was demonstrated by Kaja and Olson (1982). Semen and serum samples from 32 bulls were tested by AGID and 30 were found to have antibodies to BLV. The positive samples were then inoculated intraperitoneally into sheep. None of the sheep developed BLV antibodies. A later challenge with BLV infected lymphocytes produced antibodies in all the sheep. The authors therefore concluded that BLV cannot be transmitted via leukocyte free semen from BLV infected bulls.

Choi and co-workers (2002) studied BLV in 79 bulls, using PCR and agar gel immunodiffusion (AGID) and proved that some seropositive bulls in fact do not excrete virus or viral particles throughout the course of infection. They also managed to show the value of assuring that semen collection occurs via atraumatic techniques so that associated inflammation and leukocyte infiltration do not obscure the results of PCR and AGID on collected semen.

1.1.1.3 Bovine ephemeral fever virus

Bovine ephemeral fever virus (BEFV) is the causative agent of ephemeral fever in cattle. The disease is sometimes referred to as three-day stiff sickness. It

often presents as epizootics in Africa, Australia and Asia and impacts on reproduction via its principal effect on the locomotory system.

Bovine ephemeral fever virus has been reported (Burgess and Chenoweth 1975) to cause a consistent and dramatic increase in the percentage of midpiece abnormalities. The peak of this abnormality occurs about 35 days after peak fever. Simultaneously, a decrease in motility was associated with an increase in abnormal midpieces. The workers could not conclude whether this was the result of viral growth in testes or pyrexia. No work has been done on the effect that this reduced motility and poor morphology would have on conception figures.

Burgess (1973) failed to produce infection in nine heifers that were inseminated with two milliliters of BEFV suspension. In two heifers that were first inseminated with virus suspension and then inoculated intravenously (IV) two weeks later, antibodies were detected following the IV inoculation. The authors correctly stated that bulls suffering from ephemeral fever would be unlikely to be willing to serve and that results at that stage indicated that even if they were seropositive and shedding virus in semen, it was unlikely that this would produce disease in cows. The researchers point out that at that stage there was no concrete evidence pointing to shedding of BEFV in semen of infected bulls.

In studying the excretion of BEFV in bulls, Parsonson *et al.* (1974) showed that virus could be identified in one bull five days after IV inoculation of BEFV. This

finding suggested that in a small percentage of bulls virus could contaminate the semen sample if semen is collected during the period of viraemia. The research also stressed the importance of assuring that semen is not contaminated with blood during the collection of semen. Blood is a source of virus and injury to the penis and dissemination of the virus is then a further possible source of virus in the collected semen during this time. These workers demonstrated that it was not possible to induce infection in cows inseminated with semen infected with BEFV. There also appeared to be no effect on conception rates as 70% conceived with 2 services.

1.1.1.4 Bovine herpesvirus - 1

Bovine herpesvirus – 1 (BHV-1) is the causative agent of a respiratory syndrome, known as infectious bovine rhinotracheitis (IBR) as well as a genital disease known as infectious balanoposthitis/infectious pustular vulvovaginitis (IBP/IPV). It is widely recognized as the most commonly reported viral contaminant in semen (Kahrs *et al.* 1980; Afshar and Eaglesome 1990). Both clinical and subclinical infections can lead to establishment of persistent latent infections (Turin and Russo 2003), which are located in ganglion cells of the central nervous system (Kupferschmied *et al.* 1986). Under conditions of stress, the virus can become reactivated and migrates to the peripheral nerves where it is excreted. The infection is associated with reduced fertility and abnormal fetal development.

The distribution of BHV-1 in fractions of semen has been reported (Guérin 1993). The researchers used centrifugation for 30 minutes at 1500 g at 4 ºC to separate seminal plasma from the sperm cell fraction. Using titrations before, during and after different washing steps, it was concluded that a significant portion of BHV-1 found in semen of infected bulls was associated with the sperm cell membrane. It was shown that washing with trypsin yielded semen free from infective BHV-1. The authors also noted that no BHV-1 antigens have ever been found in bull testes, suggesting that viral shedding occurs in the epididymides or distal portions of the genital tract.

In a case report on a bull from an AI station (Kupferschmied *et al.* 1986), some of the frozen samples were retrospectively found to have been contaminated with BHV-1 even though the animal was seronegative upon arrival at the station, and showed no clinical signs whilst at the station. The authors reported that the use of this contaminated semen in the field resulted in seroconversion only, although slight IBR symptoms were observed in the experimental semen test. This article drew attention to the fact that at that stage laboratory methods for detecting IBR/IPV virus in semen were not sufficiently reliable. In a similar report from bulls at an AI station (Oirschot *et al.* 1993), Dutch workers found that seronegative bulls with no clinical signs of IBR or IBP shed BHV-1 virus intermittently for a period of several months. The virus was identified in the semen using complement fixation and neutralization tests. The researchers

concluded that the infection might have originated from a latently infected bull in which the virus was reactivated, shedding of vaccine virus or that the virulence of the causative virus was very low.

Numerous reports have been published in an effort to optimize laboratory methods available for detection of BHV-1 in semen samples. Pacciarini *et al* (1988) reported the use of a dot-blot hybridization assay with the ability to identify BHV-1 viral particles in a semen sample found to be negative by the cell culture inoculation technique. The test was reported to have very high sensitivity and specificity. Furthermore, the ability of the test to identify BHV-1 particles in preputial washes was also mentioned.

The polymerase chain reaction has been used for the detection of BHV-1 viral particles in semen samples. A nested PCR assay was reported to be able to detect BHV-1 for at least 1-10 days longer than virus isolation (Masri *et al.* 1996). The finding of Irons *et al.* (2005) that LSDV in semen is detected much longer by PCR than by VI has also been found by Van Engelenburg *et al.* (1995) with respect to excretion of BHV-1. The authors infected 11 Holstein bulls intrapreputially with BHV-1 and used virus isolation on both extended and fresh semen, as well as PCR to determine if virus was present in semen collected subsequently. They found reduced sensitivity of virus isolation on extended semen. Semen toxicity for cell culture necessitates that semen be diluted before use, and this can give false negative results when virus concentration in semen is

low (Rola *et al.* 2003). However, the workers concluded that not only does the dilution have an effect, but that the addition of extender and the freezing and thawing process also result in reduced sensitivity of virus isolation in detecting virus, and lower virus infectivity. Contrastingly, other reports (Kupferschmied *et al.* 1986; Drew *et al.* 1987) furthered the argument that cryopreservation and the use of extenders had a protective effect on the virus. Possible reasons for this could be the proteins in the extender and the cryobiology involved in semen preservation.

While it must be borne in mind that PCR only detects genetic material, and bears no significance on virus infectivity, it is possible that virus neutralizing antibodies could influence the sensitivity of PCR, as shown in this experiment. In contrast, virus isolation techniques depend upon intact, replicating virus. Bovine herpesvirus grows well on cell cultures, but is extremely fragile and easily disrupted or inactivated. This can also explain the observed better results with PCR than with virus isolation. Their results concurred with earlier reports (Snowdon 1965; Kőhler 1972) of varying patterns of excretion during acute phases of infection. The researchers later treated the experimental bulls with dexamethasone and from test results for PCR and VI, concluded that the virus could be spontaneously reactivated. It is unsure to what extent this occurs in LSDV shedding. They finally concluded that more than 90% of BHV-1 DNA can be detected in seminal fluid and almost no BHV-1 DNA is detectable in spermatozoa. This then also further underlines the necessity to determine

whether the virus in question is free or associated with cells, or even inside cells. Knowledge of this would enable scientists to better design effective methods of cleaning semen of virus.

1.1.1.5 Bluetongue virus

Bluetongue viruses (BTV) are capable of causing inapparent infection of cattle as well as a persistent infection of sheep in many countries of the world (Gibbs 1981). Bluetongue viruses produce prolonged seminal contamination, although researchers disagree on the regularity of shedding over time (Melville *et al.* 1993).

Gard *et al.* (Gard *et al.* 1989) found that semen from a bull naturally infected with BTV inoculated into sheep resulted in formation of neutralizing antibodies in the sheep, although the particular donor bull did not seroconvert. At the time of publishing, inoculation of BTV-infected semen into sheep was considered to be the method of choice to identify the presence of BTV in bull semen. More recently, Parsonson and McColl (1995) applied a well-characterized PCR to demonstrate the presence of BTV nucleic acid in formalin-fixed and frozen tissues from experimentally-infected sheep and cattle. Bowen *et al.* (1983) looked at the shedding of BTV in experimentally infected mature bulls and found that virus isolation from semen in no instance occurred without concurrent isolation from the blood. The seminal shedding of virus corresponded to the

peak of viraemia and virus was never isolated after the termination of viraemia. From earlier work by Irons *et al.* (2005) it is however clear that this is not the case with LSDV shedding. This could possibly be explained by the fact that BTV is a RBC-associated virus, and LSDV is not. In the study the authors noted that seminal shedding of BTV may be more common in older bulls due to the fact that all the bulls that shed virus in their semen were older than six years of age. It was not determined whether this is the result of occult inflammation with blood or inflammatory cells, which is known to be an age-associated occurrence in the genital tract of bulls. Another study that implicated age as a risk factor in seminal shedding of BTV in bulls (Kirkland *et al.* 2004) compared young (2-4 years old) and old (5-15 years old) bulls that were either experimentally or naturally challenged with different serotypes of BTV. No virus was detected in any of the semen samples collected from young bulls. Both 'wild-type' and laboratory-adapted serotypes of BTV were detected in semen samples of some of the older bulls, however detection in semen coincided or immediately succeeded the period of viraemia. This confirmed the findings of Bowen *et al.* (1983). At slaughter, virus was not detected in any tissue samples.

Spontaneous occurrence of BTV-induced abortion and fetal malformation has been reported (MacLachlan *et al.* 2000). Fetal anomalies characterized are meningoencephalitis and cavitating lesions in the subcortical white matter and cerebellum. The critical period for development of teratogenesis seems to be between days 70 to 130 of gestation. This knowledge has prompted

investigations into the ability of BTV-infected semen to cause teratogenesis if inseminated into susceptible cows. When 9 heifers were inseminated with semen infected from a bull experimentally infected with BTV, 6 became pregnant and 3 became viraemic and developed antibodies to BTV (Bowen and Howard 1984). No evidence of fetal infection could be found at 100 days of gestation in the heifer that became pregnant and infected, or in the five heifers that became pregnant but were not infected.

In a study involving 20 cows, the authors concluded that there was no evidence of congenital BTV-11 infection of fetuses (Parsonson *et al.* 1994) even though the cows developed BTV infection during pregnancy. Bluetongue virus antigen by immunofluorescence or viral particles by PCR could not be identified in any of the fetal tissue studied in this trial. The authors cautioned that the number of cows used was small and that further experiments might be needed.

In India, the effect of BTV infection on the semen quality of exotic rams has been studied (Mathur *et al.* 1990). The researchers found that within 4 days of artificial infection with BTV, semen from exotic rams were not suitable for use. Sperm motility declined and oligospermia was observed. The oligospermia progressed to aspermia. The major morphological defects observed were detached heads, swollen mid-pieces and coiled tails. The spermiogram gradually improved and had reached normal values within 67 days.

1.1.1.6 Bovine viral diarrhea virus

Few other infectious agents affecting cattle reproduction have received more publication than bovine viral diarrhea virus (BVDV). It is regarded by some workers to be economically the most significant infectious agent affecting cattle health and production (Campbell 2004). The clinical syndromes associated with bovine viral diarrhea virus (BVDV) infection are well known. They include effects on the gastrointestinal, respiratory, haematologic, immunologic, neurologic and reproductive systems (Baker 1995; Brock 2004). A recent review article summarizes the reproductive effects of BVD (Grooms 2004).

It is difficult to detect BVDV in unprocessed, raw semen due to the virucidal properties of seminal plasma, cell culture cytotoxicity and inhibition of reverse transcriptase enzyme (Schultz *et al.* 1982). Nevertheless, it is well-established that BVDV can be shed in the semen of acutely and persistently infected bulls. Kirkland *et al.* (1991) challenged five bulls with BVDV-infected serum in one nostril and BVDV-infected heparinized blood in the other nostril and observed them for 19 days. Semen samples were collected from each bull on four occasions between 7 and 14 days after challenge. Virus could be isolated from 9 of the 12 semen samples collected and from 3 of the 5 bulls challenged. Viraemia and concurrent isolation of virus from semen only occurred on the last day of viraemia, suggesting that the presence of virus in semen is not simply a consequence of passive transfer of the virus from the serum into reproductive

tissues. Shedding of virus continued beyond the period of viraemia, and the results of virus isolation and antigen detection suggested that viral replication took place in the reproductive tract. Results of virus isolation and antigen detection suggested that the seminal vesicles and prostate were the sites of most productive viral replication. The researchers found that semen quality was not affected in any of the bulls. They corroborated this finding in a concurrent study on a persistently infected bull. Contrastingly, Revell and co-workers (1988) found up to 45% abnormal nuclear defects in a persistently infected bull. It would appear that appreciable decline in semen quality is not a consistent finding in bulls either persistently or acutely infected with BVDV (Barlow 1986).

When 12 heifers that were confirmed not to have antibodies to BVDV were inseminated with semen from a persistently-infected bull, all 12 seroconverted 2 weeks later, thus providing evidence that BVDV may be transmitted in cattle by AI (Meyling and Jensen 1988). Four control heifers that were inseminated with virus-free semen, and were held in close contact with the heifers inseminated with infected semen did not seroconvert. Of the 12 heifers inseminated with infected semen, all became pregnant and gave birth to clinically normal calves. One of these calves was persistently infected with BVDV. The researchers concluded that infection of the foetus via placental circulation following artificial insemination does not occur commonly. In the light of this conclusion, the authors stated that the true epidemiological significance of semen-borne transmission of BVDV remains unclear. Although the mechanism by which fetal

infection occurs is not clear, there is some evidence that BVDV may cross the placenta by causing vasculitis on the maternal side of placentation, thus allowing access to the fetal circulation (Fredriksen *et al.* 1999).

Bovine viral diarrhea virus is known to be isolated occasionally from semen used for artificial insemination (Schultz *et al.* 1982). This is a significant finding due to the ability of the virus to survive cryopreservation (Meyling and Jensen 1988) and the potential widespread use of semen obtained from AI stations. Several authors have surveyed semen to establish the incidence of BVDV excretion. Howard and others (1990) surveyed four large AI centers and found infection in only 12 of 1,538 bulls. They used virus isolation on blood and serum neutralization tests to identify infected bulls and also performed virus isolation on semen samples according to published procedures. Whitmore *et al.* (1978) experimentally infected 9 bulls *per os*, intramuscularly and intraperitoneally with BVDV and collected semen from the bulls for 14 days. Bovine viral diarrhea virus was only found in 4 of 98 semen samples. Upon slaughtering of 6 bulls between 60 to 90 days after inoculation, necropsy failed to show any affects on the reproductive tracts, and virus was only found in one testis of one bull. The authors failed to show a decline in semen quality of any of these bulls and concluded that the probability of shedding of virus in semen is low in inoculated bulls.

The possibility that the virus may be localized in the testes of a bull was recognized by Voges *et al*. (1998) when they found that a postpubertal bull at an AI station was shedding BVDV for a period of 11 months, without showing viraemia. The bull had high BVDV circulating antibody titers, proving that the immune system was challenged repeatedly. At post mortem examination the virus was found to be localized in the testes of the bull. Interestingly in this case, the authors postulated that the bull may have become infected prepubertally before the blood-testis barrier became functional. The blood-testis barrier is formed by the tight junctions between adjacent Sertoli cells and effectively shields the haploid developing spermatids from the bull's immune system. The developing blood-testis barrier would then also protect the BVDV from the immune system. Infection before the onset of puberty is not essential for development of this prolonged shedding state as experimental production of persistent, localized testicular infection with BVDV has been achieved (Givens *et al.* 2003). In some of these experimentally infected bulls, BVDV persisted in testicular tissue for up to seven months as demonstrated by reverse transcriptase-nested polymerase chain reaction (RT-nPCR). Virus isolation failed to detect virus after day 21 post infection (p.i.). Despite the inability to isolate virus, BVDV in semen collected five months after challenge proved to be infectious when administered to a seronegative calf via the intravenous route. Niskanen *et al.* (2002) inseminated three susceptible heifers with BVDV-infected semen from a non-viraemic seropositive Holstein bull. Pregnancies were established in two of the three heifers. Conception failed in the third heifer that

became systemically infected with BVDV. Upon slaughter at 42 days after infection, virus could not be found in ovarian tissue, nor could histopathological changes be demonstrated. The authors cite other reports of the occasional birth of a persistently infected calf following insemination with BVDV-infected semen and caution against the use of semen from bulls in BVDV-free herds.

Due to the previously-mentioned difficulties with semen as a sample when attempting detection of BVDV, several diagnostic tools have been developed. Several reports have compared the analytical sensitivity of these tests. Afshar *et al.* (1991) compared the fluorescent antibody test (FAT) and microtiter immunoperoxidase assay (IP assay) with regards to their analytical sensitivity in detecting BVDV in bull semen. The IP assay was found to be a suitable alternative to the FAT, could be applied to a larger number of samples and did not necessitate the acquisition of expensive fluorescence microscopic equipment. Da Silva *et al*. (1995) described a reverse transcription-polymerase chain reaction (RT-PCR) for detection of BVDV in semen and cell culture supernatant. The Spanish workers refined the PCR-procedure available at that time by adding a chromatography step to remove seminal inhibitors prior to RNA extraction, enabling the effective detection of BVDV in raw and extended semen samples. Oligonucleotide primers were designed to amplify the p80 gene from BVDV. These primers managed to consistently amplify a 440-bp fragment from several non-cytopathic and cytopathic biotypes of BVDV. The RT-PCR was reported to be extremely sensitive - 0.4 $TCID_{50}$ can be directly detected. Givens *et al.* (2003)

compared the analytical sensitivity of RT-nPCR with virus isolation assays for detection of BVDV in the semen of an infected bull. It was found that viral detection was more sensitive in extended semen samples than in raw semen samples and more sensitive by RT-nPCR than by virus isolation. Application of both these diagnostic procedures to serum and semen samples collected from 558 post-pubertal bulls in southeastern United States revealed that the prevalence of BVDV-infection of semen in this region is less than 0.54%. The finding that viral detection in raw samples was less sensitive than in extended samples support the theory that virucidal properties and inhibitors of reverse transcriptase in semen are involved in the difficulties encountered with viral detection in semen samples, but argue against the contention of Kirkland (Kirkland *et al.* 1991) that low titers of virus in semen play a role. The authors cautioned that although the RT-nPCR assay was a rapid and sensitive method to detect BVDV in extended semen samples, it requires sample preparation before use and meticulous care to eliminate false-positive results. The test could be used to identify persistent testicular infection with BVDV, even though the prevalence of this phenomenon appears to be low.

The threat of introducing BVDV into embryo production systems has long been recognized (Givens and Waldrop 2004). The virus has been shown to be associated with oocytes, cumulus cells, follicular fluid, oviductal cells, uterine fluids and serum used in media for flushing the uterus and for maturation. It has even been associated with liquid nitrogen in which embryos are preserved. Fray

et al. (2000) examined sections of cryopreserved ovaries from persistently-infected heifers and found that there was no difference in the percentage of BVDV-infected oocytes between those in primordial, primary and secondary follicles. They failed to establish however, whether these infected oocytes are developmentally competent, and therefore what risk for transmission is posed by these oocytes. A need to screen raw materials used in in vitro fertilization (IVF) systems was identified by Trachte *et al.* (1998) when they showed that washing and trypsin treatment of IVF embryos or non-fertile or dead ova failed to remove all BVDV. Canadian workers (Bielanski and Loewen 1994) used semen from three bulls persistently infected with BVDV for the production of IVF embryos. They failed to demonstrate infective virus in any of the sonicated embryos, pointing to absence of virus transmission by spermatozoa during fertilization or to blocking of viral replication. The trial did however confirm that spermatozoa subjected to the swim-up procedure still contained virus and therefore it was concluded that either the amounts of virus associated with spermatozoa during ovum penetration was below the limits of the assay or the culture conditions affected virus viability adversely.

Interestingly, a congenital persistent infection with BVDV has been reported in pigs (Terpstra and Wensvoort 1997). Three piglets from a litter of 13 survived beyond three months of age. One of these three remained viraemic and immunotolerant until slaughter at 26 months of age and excreted virus in semen, urine and oropharyngeal fluid. Ejaculates did however, not contain spermatozoa.

While it was mentioned that pigs probably played a negligible role in the epidemiology of BVDV infection in cattle herds, that the presence of persistent carriers in pig populations could not be ignored if eradication schemes were to be successful.

1.1.1.7 Bovine immunodeficiency virus

Bovine immunodeficiency virus (BIV) is a lentivirus of debatable significance in bovine health (Gradil *et al.* 1999). Gradil and coworkers have identified the virus in the semen of artificially infected bulls using virus isolation and PCR, but failed to identify virus particles in frozen-thawed semen samples from 30 bulls in an AI station. The exact significance of these findings remains to be determined.

1.1.1.8 Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is a disease of exceptional economic significance and its presence in animal products is a major constraint to international trade in animal products, including gametes and embryos (Hasler 2003; Sutmoller and Olascoaga 2003).

It was established that FMDV is shed in the semen of experimentally infected bulls before the appearance of clinical signs, as soon as 12 hours after experimental infection (Anon 1963). The sperm quality of bulls experimentally

infected with FMDV showed a marked deterioration (Sellers *et al.* 1968), and abnormalities included teratozoospermia and oligozoospermia. Foot-and-mouth disease virus is shed in the semen of infected bulls for up to 60 days, although the role of semen in the transmission of FMD is unknown (Gajendragad *et al.* 2000). The workers in this study demonstrated that the oropharynx and semen were involved in the persistent shedding state. In studies done on African buffalo (*Syncerus caffer*), the maintenance host of FMDV in southern Africa, researchers failed to isolate FMDV from urogenital specimens collected from 20 animals (Bastos *et al.* 1999). The animals were found to be serologically positive to all three biotypes of FMDV known to occur in southern Africa. It should be noted however, that the diagnostic technique employed to detect virus in semen was virus isolation.

In pigs shedding FMDV in semen, it was found (Richmond 1978) that preadsorption of boar semen with kaolin removed the cytotoxicity, but not the antiviral properties, of semen and hence enhanced the detection of FMDV in boar semen samples. Further studies on boar semen have elucidated the use of diagnostic methods that were successfully used to identify FMDV in semen samples (Rijn *et al.* 2004; Guérin and Pozzi 2005). They include isolation on cell cultures, ELISA and PCR.

1.1.2 Shedding of virus in semen of other animal species

1.1.2.1 Equine arteritis virus

Equine arteritis virus (EAV) can infect horses and donkeys (Glaser *et al.* 1997). It can be transmitted by direct contact via nasal droplet or venereally (Paweska *et al.* 1995). Recently, vertical transmission through fomites has also been implicated as a mode of transmission (Guthrie *et al.* 2003). Although most animals are asymptomatic and develop a solid, long-term immunity to the disease, abortions and neonatal mortality can occur.

Following the epidemic in Kentucky in 1984, several trials focused on establishing the rate of persistence in stallions, the effect of the virus on semen quality and the anatomical site of viral persistence. Neu *et al.* (1992) observed marked changes in semen quality in stallions following experimental infection with EAV. The changes in motility, morphology and sperm concentration appeared to be temporary. These changes were attributed to the combined thermal effects of elevated body temperature and scrotal edema, rather than a virus-specific effect. Some 30-60% of acutely infected stallions will become persistently infected and shed the virus consistently in their semen (Timoney *et al.* 1986). This shedding state is in contrast to the intermittent shedding state observed with LSDV and BHV-1. The shedding of the virus in semen can persist indefinitely but some stallions can spontaneously clear themselves of the infection after a variable amount of time (Gilbert *et al.* 1997). The epidemiological

significance of asymptomatic, persistently infected stallions was illustrated in the first recorded outbreak in the British Isles (Higgins 1993). Here the source of introduction of virus could be traced back to a single stallion that shed the virus in his semen. The stallion was imported from Poland into England. Several reports have focused on the anatomical site of persistence of EAV. Neu *et al.* (1988) experimentally infected sixteen stallions intranasally with EAV. They isolated virus from genitourinary tissue samples in stallions that were still shedding virus at time of euthanasia. The areas where virus was detected most frequently were the ampulla of the vas deferens (73.3%), the vas deferens (68.8%) and the bulbourethral glands (62.5%). Wada *et al*. (1999) investigated the cell type associated with the genital tract in cases of stallions persistently infected with EAV and managed to localize the sites of persistence to the epithelium of the ductus deferens. In this experiment, stallions were artificially infected intranasally and tissue samples were examined histologically and immunohistochemically. The indirect immunoperoxidase technique was used and this allowed the researchers to identify antigens consistently in the ampullae of the ductus deferens and sporadically in the testis. They also noted that there was inflammatory cell infiltrate in the propria-submucosa of ampulla of the ductus deferens. They were however unsure whether this might have been attributable to a reaction to degenerated epithelial cells in which the viruses multiply. Interestingly, even though it was shown that asinine strain of EAV could be transmitted among horses and between donkeys and horses (Paweska *et al.* 1996), the strain seemed to be of very low transmissibility and pathogenicity. A

chronic infection of the reproductive tract of stallions could not be established with an asinine strain of EAV (Paweska 1997).

In a study to determine the complete genome sequence of EAV from a persistently infected stallion, it was found that the viruses detected in semen collected five years apart had only 85.6% and 85.7% nucleotide identity to the published sequence of EAV (Balasuriya *et al.* 2004). The variation that occurred in open reading frame (ORF) 5 resulted in the emergence of novel phenotypic viral variants. However, it is unlikely that this variation resulted in immune evasion and thus a persistent shedding state, due to the finding that all variants were neutralized by high-titre polyclonal equine antisera. Defective interfering particles were also unlikely to be the cause for maintenance or clearance of the persistent EAV status of the stallion as Nothern blot analyses of the viral variants failed to demonstrate any large genomic deletions. No evidence is currently available on viral variance and interfering particles in LSDV infections. Due to the stable nature of the virus it is unlikely that this could explain the persistent shedding of LSDV in semen of some bulls.

The testosterone-dependent nature of the persistent shedding state in the accessory sex glands is currently well-accepted. Stallions that are persistently infected and then castrated, cease shedding of virus (Glaser *et al.* 1997). In persistently infected stallions treated with a GnRH antagonist, no virus was detected in semen samples during the period of treatment (Fortier *et al.* 2002). It

could not be concluded that the treatment stopped the persistency, but that it inhibited seminal shedding of virus during the treatment period is clear. The researchers observed a concurrent decline in semen quality associated with the resultant decline of testosterone levels to basal. Establishment of persistence may involve a multifactorial process, with dependence on host and viral factors. Sexual rest following acute infection with EAV may reduce the likelihood of a stallion becoming a persistent carrier (Timoney and McCollum 1988).

Diagnostic methods to identify EAV in semen of stallions have been the subject of various papers. When a stallion tests seropositive, virus isolation on cell culture must be performed on a semen sample from the stallion. However, VI is laborious and time consuming, and the usefulness depends strongly on handling of samples (Huntington 1990). Starick (1998) compared the sensitivity of virus isolation with the molecular methods RT-PCR, nPCR and dot blot hybridization. The highest sensitivity was observed with the nPCR, and the sensitivity of RT-PCR could be improved by subsequently employing the dot blot hybridization test or the nPCR. The authors optimized the RNA extraction procedure by pretreatment with proteinase K and Chelex 100. The optimization achieved through this step is ascribed to removal of polyvalent cations which may disturb the enzymatic reaction. Contrastingly, Fukunaga and co-workers (1999) demonstrated that VI is more sensitive and specific at detecting EAV in semen of persistently infected stallions than PCR. This is the exact opposite to the situation in shedding of LSDV in semen of bulls (Tuppurainen *et al.* 2005).

However, the sensitivity of PCR was subsequently increased by using material which had been previously grown in cell culture. Dilution of the semen and the difference in sizes of LSDV and EAV may explain this difference. It is also known that EAV is much easier to culture and grows faster than LSDV. It would however seem that when properly optimized the PCR is a more sensitive technique than VI, yields results more rapidly and is less subject to the risk of cross-contamination (Larska and Rola 2003). The sensitivity and specificity of a nPCR for the detection of EAV were reported to be 100% and 97% respectively.

1.1.2.2 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the *Arterivirus* group of virus, similar to EAV and is associated with abortion, stillbirth, neonatal death and respiratory disease in piglets (Gradil *et al.* 1996). The presence and transmission of PRRSV in boar semen was initially shown by bioassay and then later detection was improved by development of a sensitive PCR to be used on boar semen (Christopher-Hennings *et al.* 1995).

In an experiment to establish the nature of excretion of PRRSV in semen of artificially infected boars, Swenson *et al.* (Swenson *et al.* 1994) infected four boars intranasally and then monitored them for a period of 56 days p.i. Virus could be detected in all four boars at first collection, 3 or 5 days p.i. and up to 13,

25, 27 and 43 days p.i. No difference between sperm morphology, motility or concentration between pre- and post-infection samples could be demonstrated, nor could any virus be isolated from reproductive tissues at the day of slaughter, 56 days p.i. No gross or microscopic lesions attributable to PRRSV could be demonstrated in the reproductive tract at slaughter. Christopher-Hennings *et al.* (1995) investigated the nature of the shedding of PRRSV in the semen of artificially infected boars and found that PRRSV RNA can be found in serum and semen of boars by PCR for variable amounts of time, thus pointing to a persistent shedding state in some boars. The researchers experimentally infected four boars intranasally with PRRSV isolate V-2332 and then monitored the boars by serum and semen collection 2-3 times weekly over a period of 100 days p.i. Blood samples were subjected to VI and PCR, as well as indirect fluorescent antibody (IFA) and virus neutralization (VN) tests, whilst semen was subjected to PCR only. Two of the boars shed virus in semen samples for a period of 56 and 92 days p.i. respectively. Virus could be isolated from the bulbourethral gland of the bull that shed virus in its semen on day 92 p.i. The recommendation was made that viraemia and serological status were inadequate indicators of when PRRSV shedding in the semen of artificially infected boars. In a subsequent investigation (Christopher-Hennings *et al.* 2001) the variation in duration of shedding of virus in semen between boars was attributed to host factors. It was found that Yorkshire boars appeared to be more resistant to shedding of virus in semen than Landrace boars, but the small sample size negated the possibility of showing a statistically significant difference. Interestingly, virus could be isolated

from the tonsil of a boar from which viral RNA was not detected in serum for a period of three weeks prior to slaughter. This finding pointed to lymphoid tissue as the site of viral replication in cases of persistent shedding. The findings of Wills *et al.* (1997) corroborates the finding that oropharyngeal tissue can be a site of persistence in cases of persistent virus shedding in semen. In this case, the virus could be detected up to 157 days p.i.

A further study into the site of persistence in boars shedding PRRSV in semen, involving vasectomized and non-vasectomized boars, concluded that PRRSV can enter semen independent of testicular or epididymal tissue (Christopher-Hennings *et al.* 1998). It was suggested that the source of PRRSV in semen was virus-infected macrophages/monocytes or non-cell-associated virus in semen and that the virus-infected macrophages/monocytes in semen originate from infection of local tissue macrophages or PRRSV-infected circulating macrophages. Shin and Molitor (2002) also investigated the site of persistence of PRRSV and found that there was a lack of agreement in the results of RT-nPCR on testis tissue samples obtained at days 14, 28 and 59 p.i. It was therefore suggested that PRRSV infection in the testes may be extremely restricted and thus would not constitute a major source of virus in semen during persistent shedding states.

1.1.2.3 Classical swine fever virus

Classical swine fever virus (CSFV) is recognized as a significant pathogen associated with boar semen (Rijn *et al.* 2004). In the epidemic in The Netherlands in 1998, it was recognized that AI could be the route through which the virus was introduced into the Dutch pig population (Smit *et al.* 1999). Subsequently, an animal model was established to assess the risk of further spread of CSFV. Three boars were experimentally infected with CSFV and between day 5 and day 18 p.i. semen was collected from these boars, extended, and used for artificial insemination of six sows on heat. Two of the six sows seroconverted following AI. The boars remained clinically healthy and seroconverted between 14 and 21 days p.i. In the fetuses of the two seropositive sows, CSFV was detected approximately 35 days p.i. This experiment established that adult boars infected with CSFV can shed the virus in their semen and infect sows and fetuses if the infected semen is used in AI programs.

In a subsequent study (Floegel *et al.* 2000) German workers confirmed that CSF could be transmitted via AI, and also showed that the virus could be detected in tissue samples of the epididymis but not from testicular tissue samples.

1.1.2.4 Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) is a lentivirus with many similarities to human immunodeficiency virus (HIV). It could therefore be a suitable animal model for studying AIDS.

Jordan *et al.* (1995) described the detection of replication-competent FIV in cell-free and cell-associated form in domestic cat semen. This report established the ability of FIV to produce infection of a feline lymphocyte cell line, even during the asymptomatic period of infection. Performance of PCR on cocultured FCD4E cells in seminal plasma samples detected virus in five of seven artificially infected cats. An interesting finding in this experiment was that the presence of virus in seminal fluid and seminal cells was not necessarily concordant. The workers explained this phenomenon by indicating that PCR positivity may represent quiescent, defective or incomplete virus, in addition to replication-competent virions. The other explanation offered was that cell-free virus may be shed by sources other than infected seminal cells, such as the epididymis, ductus deferens and accessory sex glands. Other tissues lining the reproductive tract can also be involved. Provirus could be detected in swim-up specimens, and suggested that virus may be associated with motile spermatozoa. It was also noted that virus expression varied among seropositive individuals and was not necessarily consistent within different fractions of semen from the same individual over time. More recently, the same workers

demonstrated that samples obtained before seroconversion may contain virus and that virus shedding differ among and within cats during acute infection (Jordan *et al.* 1999). The variability of shedding between individuals seems to be a common finding in studies of virus shedding in semen.

Whilst the most likely mode of transmission of FIV is through saliva in bite wounds, transmission through artificial insemination has been established (Jordan *et al.* 1996). The researchers synchronized folliculogenesis and ovulation and inseminated queens intrauterine via a laparoscopic approach. Three of six fresh inseminations performed produced infection, while none of the inseminations performed with frozen-thawed semen resulted in infection. Virus was not detected in any of the offspring of the queens that did become pregnant. In this study it was also found that FIV could be detected in vaginal washes from infected females.

1.1.3 Summary of literature with reference to LSDV

From the literature review on the mechanisms involved in the shedding of various viruses in the semen of domestic animals, the following deficiencies in the knowledge base of LSDV can be identified:

- There are no reports on specific sperm morphological abnormalities associated with LSDV infection.

- No work has been done on the outcome of the use of LSDV-infected semen for AI.
- The distribution of LSDV in fractions of semen or in genital tissue is not known.
- The impact of LSDV infection in embryo production systems is unknown.
- The effect of swim-up procedures on LSDV association with spermatozoa is unknown.
- The possibility of a persistent carrier state for LSDV in other species than cattle has not yet been investigated.
- The possible interaction of hormone levels and LSDV shedding has not been studied.

1.2 Hypothesis

Lumpy skin disease virus is associated with LSD lesions in the genital tract in cases of prolonged shedding of the virus. The virus is not associated with sperm cells, but located in seminal plasma and viral DNA is shed in semen as it is disseminated from lesions during the ejaculation process.

1.3 Objectives

1. To determine the site of persistence in bulls shedding the virus for periods longer than 28 days. For the purposes of this study, a prolonged shedder

is defined as a bull that tests positive for LSDV particles by PCR and/or virus isolation for a period longer than 28 days from the day of initial challenge.

2. To determine if the virus is present in all fractions of semen
3. To study the development of lesions in the genital tract and compare it to the presence of virus in different fractions of semen.

Chapter 2

Materials and Methods

2.1 Experimental animals

Six healthy postpubertal Dexter bulls were used, this being an early-maturing, thin-skinned European breed. The age and mass of the bulls at the time of acquisition are summarized in Table 2.1. These bulls were confirmed to be seronegative by serum neutralization test (SNT) and were acquired from herds where vaccination is not practiced. Furthermore, their ability to produce semen was confirmed prior to purchase. This was done by collecting semen via electrical stimulation and examining the sample under an Olympus light microscope using 200 x magnification to identify spermatozoal motility.

Table 2.1 Age and masses of experimental animals

Bull identification	Age (months)	Mass (kg)
Bull A	14	210
Bull B	16	348
Bull C	15	212
Bull D	13	255
Bull E	17	248
Bull F	15	206

2.2 Experimental procedures

2.2.1 Preparation of animals

Upon arrival, the body mass of each individual bull was determined and recorded. The recorded masses and ages are summarized in Table 2.1. The six bulls were housed in vector-protected stalls at the University of Pretoria's Biomedical Research Centre (UPBRC) for the duration of the experiment. They were fed on a diet of grass hay and lucerne and received water *ad libitum.* An acclimatization period of two weeks was allowed before challenge occurred. During the acclimatization period, the experimental animals were numbered with ear tags (A – F) and semen was collected four times to accustom the bulls to the procedure. Final clinical examination, semen collection and evaluation were performed five days before experimental challenge occurred.

2.2.2 Experimental infection

South African field isolate V248/93, a pathogenic isolate of known history and characterization was used to challenge the experimental animals by inoculating the bulls intravenously (IV) with 2 ml of the virus suspension at a titre of 4.5-6.5 log $TCID_{50.}$ The day of experimental challenge was recorded as day 0.

2.2.3 Clinical examination

A general clinical examination of every bull was done daily to ensure that clinical signs were only attributable to LSD and that general health was acceptable. During this general clinical examination the author determined rectal temperatures of the bulls, evaluated the skin for the presence of LSD lesions, palpated the prescapular and superficial inguinal lymph nodes, examined the faeces for consistency and colour and evaluated the general health of the bull. Rectal temperatures of the bulls were taken twice daily, once in the morning and once in the late afternoon.

A reproductive examination of each bull was performed every other day and scrotal circumference recorded. The testes and epididymides were palpated for size, consistency, elasticity and symmetry.

2.2.4 Sample collection

Before every semen collection, sheath washing was performed on each bull by infusing 50 ml of PBS into the preputial cavity, vigorously massaging the full length of the preputium through the skin and recollecting the fluid. This fluid sample was retained for diagnostic purposes. After sheath washing, another one liter of sterile saline was infused into and drained from the preputial cavity with the aim of eliminating preputial contamination of the other fractions during collection. Before the insertion of the probe of the electro-ejaculator, the

accessory sex glands of the bulls were gently massaged transrectally for one to two minutes and the fluid dripping from the preputial opening was collected. This fluid was regarded to be fluid mainly from the vesicular glands. Semen collection was done by electroejaculation with an El-Torro II electroejaculator*. Semen was collected into new graduated collection tubes to which latex collection cones were attached. To prevent cross-contamination, each bull was assigned a collection cone which was used for only that bull for the duration of the trial and the collection cones were scrubbed and rinsed with distilled water between uses. An effort was made to only collect semen from a protruded penis. The semen collected via electro-ejaculation was centrifuged at 250g for 10 minutes and separated into a cell-rich fraction and supernatant.

Semen collection and processing and preputial washes were performed on the day of experimental infection and every other day until day 28. On day 28, only semen was collected and submitted to identify bulls that were still shedding viral particles, based on the results of PCR.

A 0.1 ml aliquot from each semen sample collected was examined under light microscopy for the presence of red blood cells. Semen evaluation was done weekly throughout the trial. This included pH determination, evaluation of macroscopic appearance, mass motility, individual motility, sperm morphology evaluation on Eosin-Nigrosin smears and foreign cell Diff-Quick smears.

* Electronic Research Group, P.O. Box 536, Halfway House 1685, Republic of South Africa.

At the time of preputial wash and semen collection, blood samples were collected in heparin and serum tubes. All the abovementioned samples were immediately submitted to the Virology Section of The Department of Veterinary Tropical Diseases (DVTD) of The Faculty of Veterinary Science for VI and PCR. SNT was done on the serum sample collected on day 28 p.i.

2.2.5 Ultrasound examination

At the time of semen collection, ultrasound examination of the testes and accessory sex organs was performed and recorded. For this purpose an Aloka SSD-500 ultrasound machine with a 5MHz probe was used. A rigid 15 mm Perspex tube, cut longitudinally along its 40 cm length and fitted to the rectal probe, enabled transrectal ultrasonography to be performed on the bulls even at times when it was expected that they would be painful due to the effects of LSD. Ultrasonography could be performed by manipulation of the Perspex tube, obviating the need for introduction of an arm into the rectum of the animal. Ultrasonographic lesions were compared to the pattern of excretion of virus or viral particles in the fractions of the ejaculate collected.

2.2.6 Post mortem sampling and examination

Bulls found to be excreting virus in semen based on the results of the PCR tests performed on day 28 of semen sampling and testing, were removed from the

vector-free housing and slaughtered on day 30 p.i. The bulls that were not excreting virus at this time were sold at market price. Slaughter was performed at the Onderstepoort Veterinary Institute (OVI) abattoir and entire genital tracts were removed, while the rest of the carcasses were sold as Grade C meat. Post mortem examination of the harvested genital tracts was done in association with a pathologist from The Section of Pathology of The Department Paraclinical Sciences of The Faculty of Veterinary Science. Tissue samples were taken from the left and right sides from the upper, middle and lower level of each testis, the head and tail of the epididymis, the seminal vesicles, the prostate gland and *lamina interna* of the preputium. Samples from gross lesions, were also taken. Each sample was taken in duplicate. One of the pair of samples was preserved in 10% buffered formalin and the other sample was deep frozen at -80ºC.

2.3 Analyses performed on samples collected

2.3.1 Blood samples

Heparinized blood samples were subjected to VI from day 6 p.i. Virus isolation was performed by inoculation of 0.5 ml heparinized blood onto bovine dermis (BD) cells. The cell cultures were observed daily for cytopathic effects (CPE). After 14 days negative cultures were frozen briefly at -70ºC and thawed. A second passage was done and observed for 14 days. This was done by the Virology section of the DVTD.

A serum neutralization test (SNT) was performed according to standard operating procedure (SOP) of DVTD on serum samples collected on the last day of the trial to establish whether seroconversion had taken place and to determine the antibody titers to LSDV that had developed.

2.3.2 Sheath wash, vesicular gland fluid and semen samples

The semen samples collected on the last day of the trial were subjected to PCR first. Polymerase chain reaction was done on sheath washes, vesicular fluid, supernatant and cell-rich fractions of the semen from day 10 p.i. to day 26 p.i. Virus isolation was not done on semen samples.

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

2.3.3 Necropsy samples

Histopathology using haematoxylin and eosin staining (H&E) was used to evaluate tissues for evidence of virus-induced necrosis and vasculitis, as well as to look for cytoplasmic inclusion bodies. Immunohistochemistry using immunoperoxidase staining was performed on histopathology samples that showed evidence of viral damage in an attempt to study the distribution of viral antigen in relation to lesions observed. The results of immunoperoxidase staining were validated against samples from the testes of bulls known to be free of LSD. Transmission electron microscopy (TEM) was performed on negatively stained preparations of testis samples to identify LSDV. PCR and virus isolation were performed on all the tissue samples obtained at necropsy.

Virus isolation was performed by studying cytopathic effects on monolayers of BD cells according to published protocols and SOPs of the Department of Veterinary Tropical Diseases. Briefly, this method involved mincing of tissue samples using sterile scissors and forceps and then (using aseptic techniques), ground with a pestle in a mortar containing sterile sand. Ten milliliters PBS containing gentamycin[†] (0.1 mg/ml), ampicillin[‡] (0.05 mg/ml) and amphotericin B[§] (5 µg/ml) were added. The suspension was left to stand overnight at 4ºC. The samples were partially clarified by centrifugation at 2000 rpm for 3-5 min to remove gross particles and 0.5 ml of supernatant was then inoculated onto the

[†] Genta 50 Phenix, 50 mg/ml
[‡] Intramed Ampicillin Injectable 500 mg
[§] Fungizone injectable, Bristol-Myers Squibb

FC cells in 25 cm^3 culture flasks. Cells were maintained in minimum essential medium (MEM) containing 4% fetal calf serum (FCS) and 1 ml gentamycin and flasks were incubated at 37ºC in an atmosphere of 5 % CO_2. Cells were observed daily for evidence of toxicity or CPE. The growth medium was replaced if it appeared cloudy due to bacterial or fungal contamination. After 14 days negative cell cultures were blind-passaged onto a fresh cell culture monolayer and observed for another 14 days or until typical CPE changes occurred.

A negative control for VI consisted of a cell monolayer without any virus and a positive control consisted of a cell monolayer inoculated with 0.2-0.5 ml LSD virus (V248/93) suspension at a titre of 4 log $TCID_{50}$.

2.4 Data analysis

Purely categorical information was reported due to the small number of animals involved.

Chapter 3

Results

3.1 Clinical signs

Of the six bulls that were experimentally challenged, Bull D and Bull E developed severe generalized disease, Bull C and Bull F were moderately affected and Bull A and Bull B showed no obvious signs of disease. On this basis the bulls were divided into groups as indicated in Table 3.1.

Table 3.1 Grouping of bulls based on severity of clinical signs observed

Group	Bulls	Mass (kg)	Age (months)	Breed
Severely affected	D	255	13	Dexter
	E	248	17	Dexter
Mildly affected	C	212	15	Dexter
	F	206	15	Dexter
Inapparently affected	A	210	14	Dexter
	B	348	16	Dexter

3.1.1 Temperature reactions and general health

The group affected by severe generalized lumpy skin disease displayed the highest temperature reactions.

Bull D had an average morning rectal temperature of 39.2ºC and an average afternoon rectal temperature of 39.3ºC. It developed a fever reaction (i.e. >39.5ºC) on day 6 p.i. and continued to be febrile until day 21 p.i. The maximum rectal temperature recorded in Bull D was 40.5ºC on day 11 p.i. This bull maintained a rectal temperature in excess of 40ºC between days 9 to 15 p.i. (Fig. 3.1) Bull D showed lymphadenopathy of the prescapular lymph nodes on day 6 p.i. By day 8 p.i. the superficial inguinal lymph nodes were also involved and generalized lymphadenopathy persisted in Bull D throughout the duration of the trial.

Bull E had an average morning rectal temperature of 39.5ºC and an average afternoon rectal temperature of 39.7ºC. It developed a fever reaction on day 6 p.i. and continued to be febrile until day 28 p.i.; the end of the trial period. The maximum rectal temperature recorded in Bull E was 40.7ºC on days 10 and 11 p.i. and the bull maintained a rectal temperature in excess of 40ºC between days 7 to 20 p.i. (Fig. 3.1) Bull E showed enlargement of the prescapular lymph nodes on day 6 p.i. and enlargement of the superficial inguinal lymph nodes by day 8 p.i. Generalized lymphadenopathy persisted in this bull for the remainder of the trial.

Both Bull D and Bull E developed respiratory distress, inappetence and partial rumen stasis and were treated with 5ml of phenylbutazone[**] IV every second day and 30 ml of a combination of procaine benzylpenicillin and dihidrostreptomycin[††] IM daily. Treatment was initiated on day 18 p.i. and continued until clinical improvement was seen. Bull E was treated for 5 days and Bull D for 4 days.

Bulls in the group that was mildly affected (Bulls C and F) displayed lower rectal temperatures and the febrile periods lasted for shorter periods of time compared to the group with generalized disease. Bull C had an average morning and afternoon rectal temperature of 38.4ºC. Fever in Bull C started on day 6 p.i. and lasted until day 10 p.i. The maximum recorded rectal temperature in Bull C was 39.3ºC on day 7 p.i. (Fig. 3.1) Lymphadenopathy of the prescapular lymph nodes could be demonstrated for the first time on day 10 p.i. and by day 12 the superficial inguinal lymph nodes were also enlarged. From day 18 p.i. onward, the lymph nodes had returned to their normal sizes.

Bull F had an average morning and afternoon rectal temperature of 38.8ºC. Fever in Bull F started on day 4 p.i. and lasted until day 11 p.i. Bull F also had fever on day 14 p.i. The maximum recorded rectal temperature in Bull F was 39.9ºC on day 4 p.i. (Fig. 3.1) Bull F showed an enlarged left prescapular lymph node from day 4 p.i. and an enlarged left superficial inguinal lymph node from

[**] Phenylarthrite®, 200 mg/ml, Centaur, Bayer
[††] Depomycin®, 200 mg/ml, Intervet S.A. (Pty) Ltd.

day 12 p.i. but by day 18 p.i. the lymph nodes had all returned to their normal sizes.

The temperature reaction in the group that showed no obvious signs of disease was transient. Bull A showed fever on day 8 p.i. and again on day 14 p.i. The average morning rectal temperature of Bull A was 38.6ºC while its average afternoon rectal temperature was 38.4ºC. The highest rectal temperature recorded in Bull A was 39ºC on days 8 and 14 p.i. (Fig. 3.1) Enlarged prescapular lymph nodes could be demonstrated in Bull A from day 4 p.i. and enlarged superficial inguinal lymph nodes from day 16 p.i. By day 22 all lymph nodes were of normal size.

Bull B had an average morning rectal temperature of 38.5ºC and afternoon rectal temperature of 38.3ºC. Fever in Bull B started on day 5 p.i. and lasted until day 7 p.i. The highest recorded rectal temperature in Bull B was 39.3ºC on day 7 p.i. (Fig. 3.1) Bull B never showed lymphadenopathy.

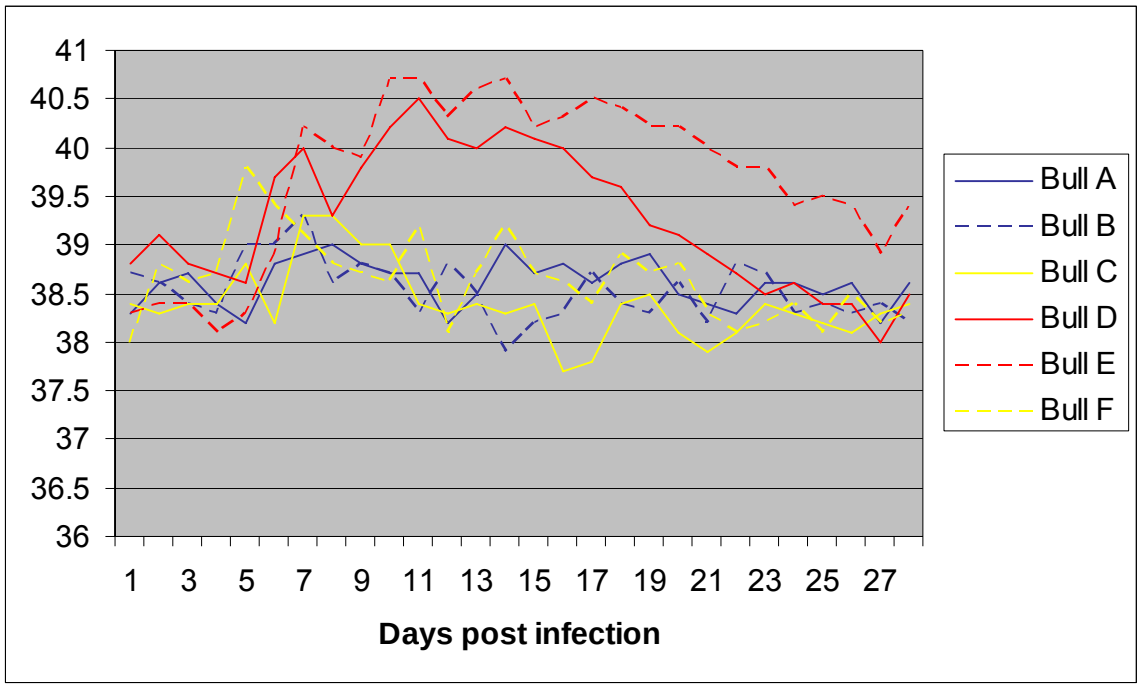

Fig. 3.1 Temperature reaction in bulls

Note: Severely affected bulls (D and E) indicated by red lines, moderately affected bulls in yellow, inapparently affected bulls in blue.

3.1.2 Development of lesions

In the group that showed severe generalized disease, lesions started appearing on day 7 p.i. Bull D had several skin nodules, averaging 2 cm in diameter, on the flanks and the paralumbar fossa on day 7 p.i. The lesions became more numerous and by day 14 p.i. extended to the hooves, including the ventrum and the scrotum. Over the course of the next 14 days the lesions became ulcerated and healed to leave areas of scarring. During the last week, Bull D displayed

corneal opacities and several ulcerative lesions could be seen on the muzzle and in the nares. The development of lesions followed a similar pattern in Bull E. The first lesions appeared on the flank on day 7 p.i., and then extended to include the paralumbar fossa, the perineum, ventrum, scrotum and the skin extending to the hooves. The entire skin was covered by lesions on day 16 p.i. The lesions began to ulcerate and suppurate by day 22 p.i. and by day 24 p.i Bull E had also developed corneal opacities and ulcerative lesions of the oral and nasal cavity. Fig. 3.2 is a photograph of Bull D on the day of slaughter. The distribution and nature of the typical lumpy skin lesions can be seen clearly.

Figure 3.2 Lesion distribution in Bull D (d28 p.i.)

In the group that showed mild clinical signs, lesions also started to appear on day 7 p.i. The first site of appearance was on the flanks. The lesions did not however, become more numerous and by day 14 p.i. no lesions could be observed in either Bull C or Bull F. The lesions in these bulls consisted of circumscribed round cutaneous swellings that did not ulcerate but simply disappeared.

Bull A and B showed no clinical signs of Lumpy skin disease and skin lesions could not be observed in either of these two bulls. Bull B showed some ulcers on its muzzle on day 14 p.i. which had cleared by day 18 p.i.

3.1.3 Scrotal circumference

Animals in the severely affected group showed outspoken changes in their SC over the course of the trial. Bull D had an average SC before experimental challenge of 31.75 cm. The SC for Bull D at the end of the trial period was 32.0 cm. On day 12 p.i. Bull D had a SC of 34.5 cm and the SC stayed above 34 cm until day 18 p.i. By day 26 p.i. Bull D had a SC of 30 cm (Table 3.2 & Fig. 3.3). Bull E had an average SC before experimental challenge of 30 cm. The SC for Bull E at the end of the trial period was 25.5 cm. On day 12 and 14 p.i. the SC for Bull E was 32.5 cm and by day 28 p.i. it had declined to 25.5 cm (Table 3.2 & Fig. 3.3). The SC of severely affected bulls therefore increased initially, followed by a decline below pre-infection values.

In the mildly affected group, Bull C had an average SC of 31 cm before challenge. The SC for Bull C at the end of the trial period was 32.5 cm. The highest SC for Bull C was measured at 32.5 cm on days 14, 24 and 28 p.i. and the lowest SC was measured at 30.5 cm on days 10 and 18 p.i. The average SC for Bull F was 26 cm before experimental challenge (Table 3.2 & Fig. 3.3). The SC measured for Bull F at the end of the trial was 26.5 cm. The highest SC measured for Bull F was 27.5 cm on day 24 p.i. and the lowest SC measured was 25.5 cm on day 10 p.i. (Table 3.2 & Fig. 3.3). In general the variations in SC measurements for bulls in the mildly affected group were far less than in the severely affected group and increased over the duration of the trial.

In the group of animals that were inapparently affected, Bull A had an average SC of 29.375 cm before challenge and the SC measured 29.5 cm at the end of the trial period. The highest SC measured for Bull A was 31 cm on day 18 p.i. and the lowest SC was measured at 28 cm on day 6 p.i. (Table 3.2 & Fig. 3.3). Bull B had an average SC of 27.7 cm before experimental challenge. The SC measured for Bull B was 29.5 cm at the end of the trial period. The highest SC recorded for Bull B was 30.5 cm on day 4 p.i. and the lowest SC recorded was 27.5 cm on day 8 p.i. (Table 3.2 & Fig. 3.3).

Table 3.2 Scrotal circumference measurements

Parameter	**Severely affected**		**Moderately affected**		**Inapparently affected**	
	Bull D	**Bull E**	**Bull C**	**Bull F**	**Bull A**	**Bull B**
SC before infection (mean)	31.75	30	31	26	29.375	27.375
SC at end of trial	32	25.5	32.5	32.5	29.5	29.5
Max SC measured	34.5	32.5	32.5	27.5	31	30.5
Days pi of Max SC	12	12,14	14,24,28	24	18	4
Minimum SC measured	30	25.5	30.5	25.5	28	27.5
Days pi of Min SC	26	28	10,18	10	6	8

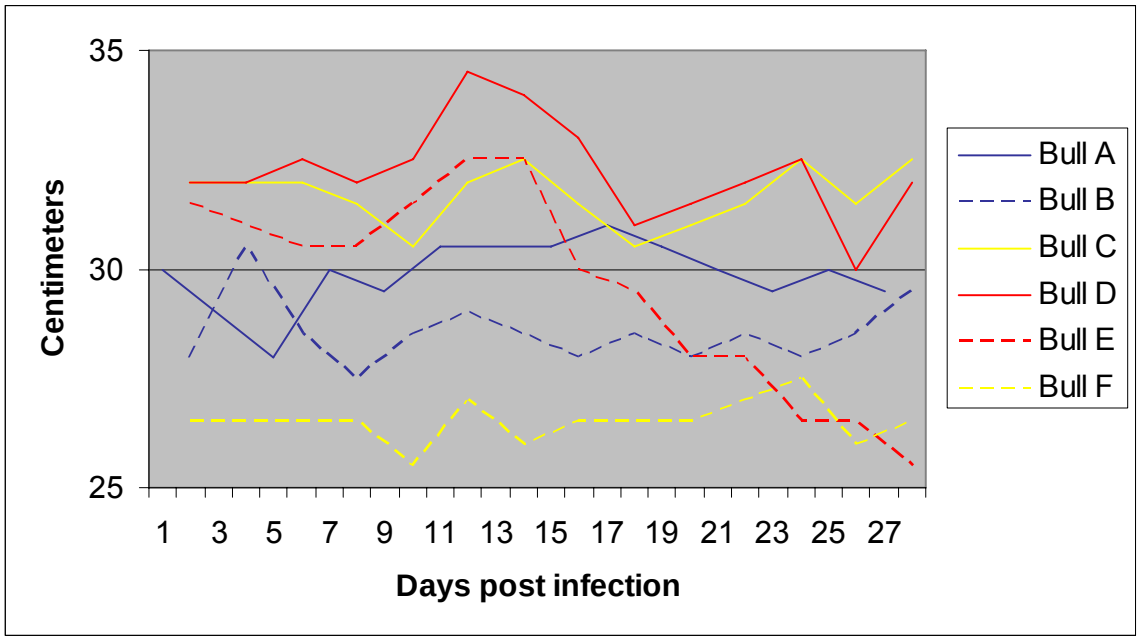

Fig. 3.3 Scrotal circumference value changes

Note: Severely affected bulls (D and E) indicated by red lines, moderately affected bulls in yellow, inapparently affected bulls in blue

3.1.4 Testes and epididymides

Abnormalities could only be detected in the epididymides and testes of Bull D and Bull E. They resort in the group that were severely affected and showed signs of generalized LSD.

LSD nodules could be observed on the scrotum of Bull D for the first time on day 10 p.i. By day 12 p.i. the testes were no longer mobile in the scrotum of Bull D and scrotal edema could be palpated. At this stage nodules could be palpated in

the epididymides; most notably in the *caput epididymidis.* The consistency of the testes of Bull D became markedly softer from day 16 p.i onwards and nodules could also be palpated in the *cauda epididymidis* at this stage.

The changes in the testes and epididymides of Bull E were similar to Bull D. Scrotal nodules could first be seen on day 10 p.i. and by day 12 p.i. the testes were no longer mobile within the scrotum. Epididymal nodules first appeared on day 14 p.i. in the *caput* and by day 16 p.i. the *cauda* was also involved. The testicular consistency at the end of the trial was very soft; even softer than in Bull D.

3.2 Ultrasonographic examination

Only Bull D and Bull E displayed ultrasonographic changes in their reproductive tracts. This was first noticed on day 10 p.i. and progressed in severity through the duration of the trial.

3.2.1 Ultrasonographic changes in accessory sex glands

Bull D showed decreased echogenicity of the parenchyma of the vesicular glands on day 10 p.i. Some oedema (4 mm) of the mucosa of the glands could be observed. The ultrasonographic appearance of the vesicular glands returned to normal by day 16 p.i. The *ampulla* of the *ductus deferens* showed a focal

hyperechoic area of 8 mm in diameter on day 22 p.i. that had not resolved by the day of slaughter. No other ultrasonographic changes could be demonstrated in the accessory sex glands of Bull D for the remainder of the trial.

Bull E showed a similar decreased echogenicity of the parenchyma of the vesicular glands that could first be appreciated on day 12 p.i. and that had subsided by day 18 p.i. Oedema of the mucosa of the glands was less distinct than in Bull E and measured between 2 -3 mm. No other ultrasonographic changes could be demonstrated for the remainder of the trial.

3.2.2 Ultrasonographic changes of the scrotum and scrotal contents

Similarly to the ultrasonographic findings in the accessory sex glands of Bull D and Bull E, the first changes were observed on day 16 p.i. and day 18 p.i. in Bull D and Bull E respectively. However, these changes became more outspoken through the duration of the trial when compared to the ultrasonographic changes demonstrated in the accessory sex glands.

In Bull D, the scrotal skin increased in thickness from 2 mm to 12 mm during the course of the trial. On day 16 p.i. a thin anechoic line (oedema) could be seen medial to the medial aspect of the scrotal dermis. This line could no longer be appreciated by day 20 p.i. At the same time, small hyperechoic foci, 6 – 8 mm in size, could be seen scattered through the testicular parenchyma (infarction).

Over time, these hyperechoic foci became associated with anechoic foci of similar size adjacent to them. On day 22 p.i. a 14 mm, round, hypoechoic focus could be seen in the proximal half of the left testis of Bull D. An 8 mm x 12 mm anechoic focus could be demonstrated between the *caput epididymis* and the *tunica albuginea* of the left testis on day 20 p.i. (epididymal granuloma). This lesion persisted until slaughter of Bull D. On day 18 p.i., a hyperechoic focus of 14 mm diameter could be demonstrated in the right epididymis of Bull D. This lesion persisted until slaughter of Bull D.

The scrotal skin of Bull E increased in thickness from 2 mm at the start of the trial to 14 mm upon completion of the trial. A thin, interrupted anechoic line could be seen adjacent to the medial aspect of the scrotal skin from day 18 p.i. This line persisted in Bull E until the day of slaughter. Scattered hyperechoic foci appeared in the testicular parenchyma of Bull E on day 20 p.i. and measured 6 – 8 mm in diameter. A large hyperechoic oval focus of 16 mm in diameter could be demonstrated in the distal half of the right testis of Bull E on day 22 p.i. (necrosis / infarction). The *mediastinum testis* of Bull E showed a gradual thickening over the course of the trial to reach a thickness of 10 mm by the end of the trial. The *mediastinum testis* was demarcated by a 2 mm anechoic line from day 24 p.i. onwards. On day 20 p.i. a hyperechoic focus of 11 mm in diameter could be demonstrated in the *caput epididymis* of the left testis of Bull E (hydrocoele). Fig. 3.4 shows the ultrasonogram of the left testis of Bull E on day 20 p.i.

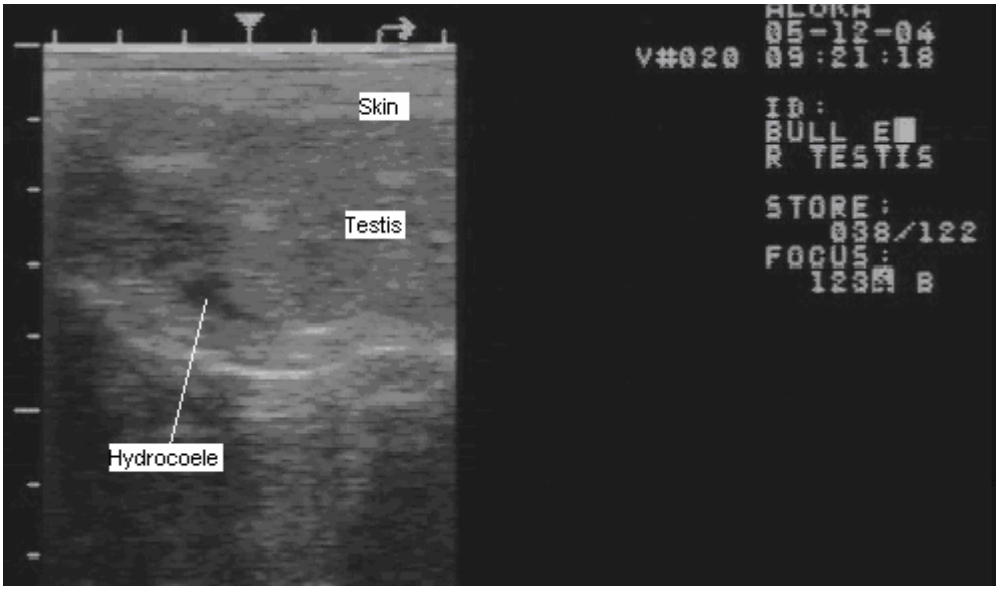

Fig. 3.4 Ultrasonogram of left testis of Bull E (d20 p.i.)

3.3 Necropsy results

3.3.1 Macroscopic pathology

The skin of the carcass of Bull D was in moderately poor condition with scattered ulcerated lesions. Some of the skin lesions showed serofibrinous exudates. Multifocal, roughly circular, necrotic areas could be identified on the muzzle and in the buccal cavity of Bull D. Moderate bilateral corneal opacities and ulceration could be seen. Mild to moderate generalized subcutaneous oedema was observed upon removal of the skin of the animal. Lymphadenopathy was present in all the superficial lymph nodes, but was especially pronounced in the superficial inguinal and prescapular lymph nodes. A mild, non-suppurative, chronic-active interstitial pneumonia was present in the lungs of Bull D. Severe, generalized, subacute dermal fibrosis of the scrotal skin was found. The testes

of Bull D were soft and did not bulge upon incision of the testicular parenchyma. On sagittal section of the testes of Bull D, multifocal areas of necrosis, fibrosis and infarction could be identified. These changes can be seen in fig. 3.5. The testes therefore showed severe, subacute to chronic testicular degeneration with multifocal infarction and necrosis. A single epididymal granuloma could be identified in the tail of the left epididymis of Bull D.

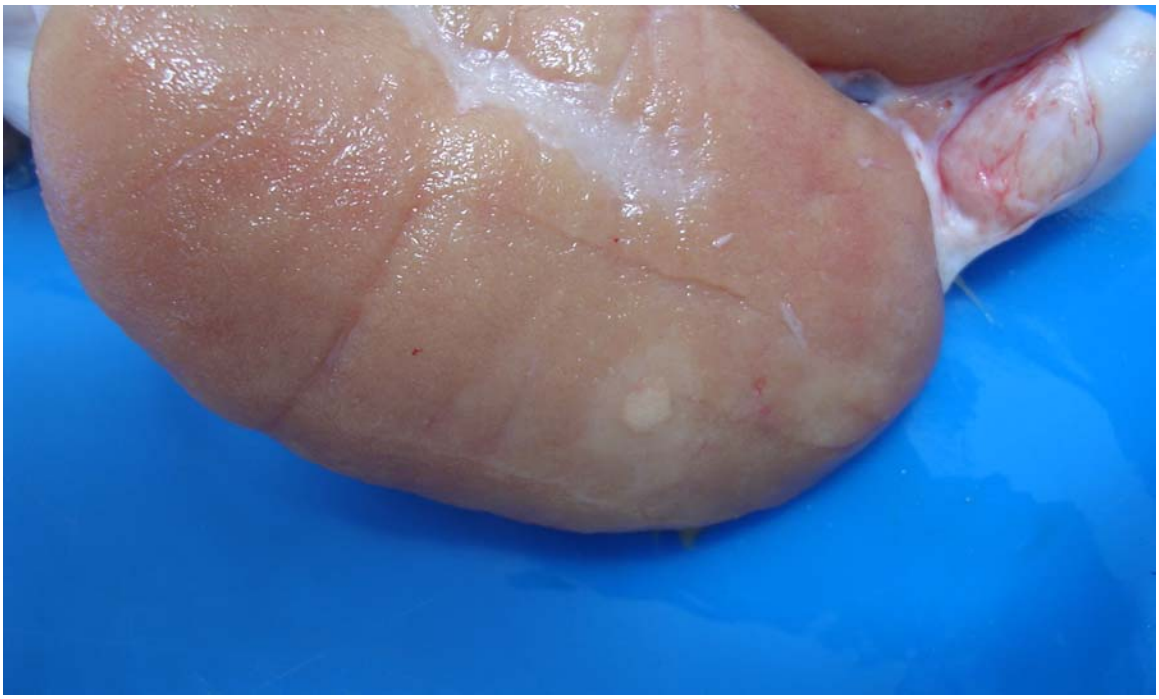

Fig. 3.5 Photograph of sagittal section of testis of Bull D at necropsy

The macroscopic lesions seen in Bull E were similar to those seen in Bull D except that no epididymal granulomas could be found in the epididymides of Bull E.

3.3.2 Microscopic pathology

In the accessory glands histopathology was restricted to the *ampulla ductus deferentes* in both bulls. There was mild chronic inflammation of the ampulla, which was characterized by fibrosis of the propria-submucosa and a multifocal perivascular and juxtaluminal infiltrate of lymphocytes and plasma cells with fewer neutrophils and macrophages. Some glandular lumina were obstructed (and secondarily dilated) by casts of necrotic spermatocytes, spermatids, spermatozoa and neutrophils.

There was moderate chronic diffuse interstitial orchitis in the testes of Bull D that was characterized by mild fibrosis and mild to moderate perivascular accumulation of lymphocytes, with fewer macrophages and plasma cells. In Bull D's testes there was quite prominent segmental necrosis of seminiferous tubules in all of the examined sections.

In one of Bull D's testes there was a wedge-shaped focus of coagulative necrosis (infarct) that extended from just beneath the *tunica albuginea,* a few millimeters into the parenchyma. At the periphery of the necrotic tissue there was a reaction zone of lymphocytes and plasma cells, with fewer macrophages and some fibrous connective tissue. The germinal compartment of the testicles revealed the presence of normal Sertoli cells, but there were very few spermatogonia and spermatocytes, with some tubules being totally devoid of spermatogenic cells. There was marked dissociation of primary spermatogonia from the basement

membrane in a number of the seminiferous tubules and many tubules contained necrotic spermatozoa and spermatogenic cells as well as a few degenerate neutrophils. These histopathological changes can be appreciated in fig. 3.6.

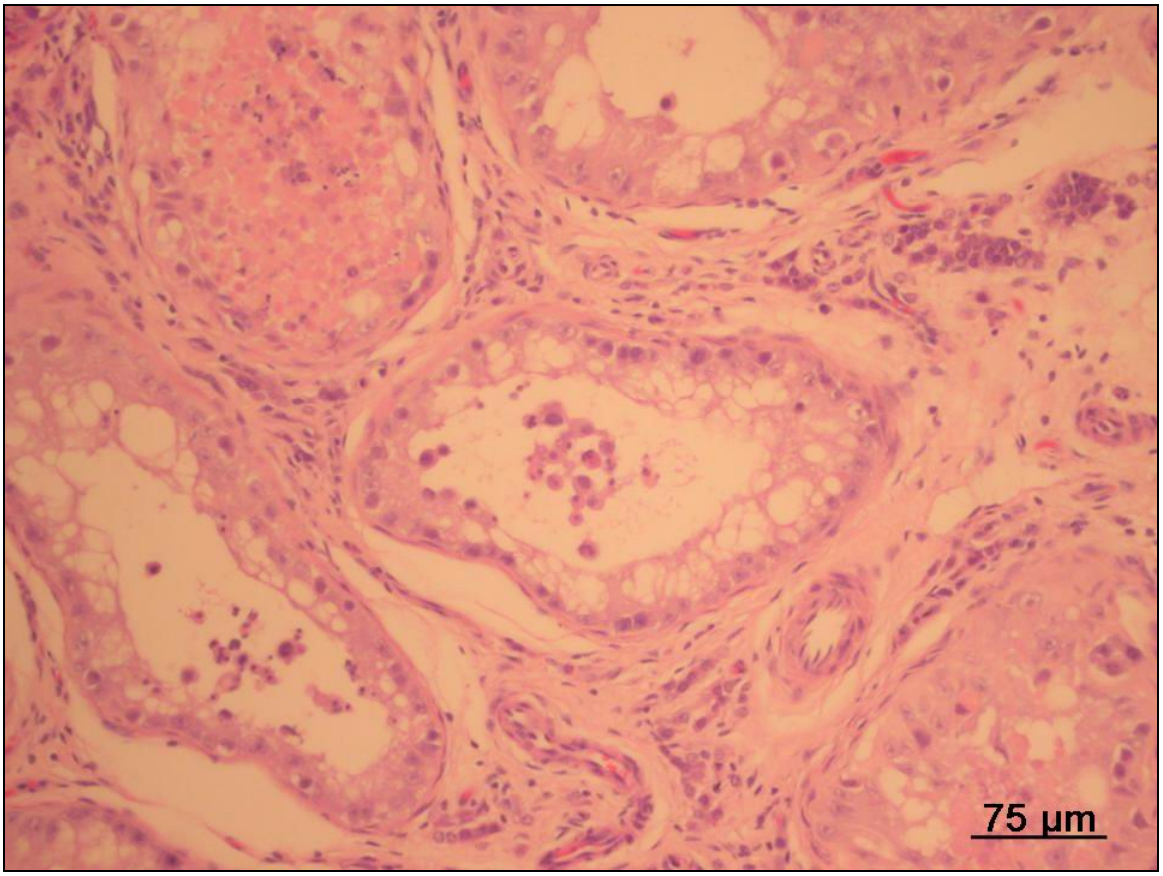

Fig. 3.6 Photomicrograph of section of testis of Bull D

There was dystrophic calcification of some of the aggregated necrotic cellular debris. The epididymis of Bull D revealed the presence of severe multifocal to coalescing necrogranulomata that were centred on ductular lumina. In the most severe lesions the ductules were completely obliterated by necrosis that was surrounded by lymphocytes, plasma cells and the occasional macrophage, as well as a thin rim of fibrous connective tissue. Fig. 3.7 is a photomicrograph of

the necrogranulomata observed in the epididymis of Bull D. The necrotic focus with the peripheral zone of round cells and fibrous connective tissue can be observed.

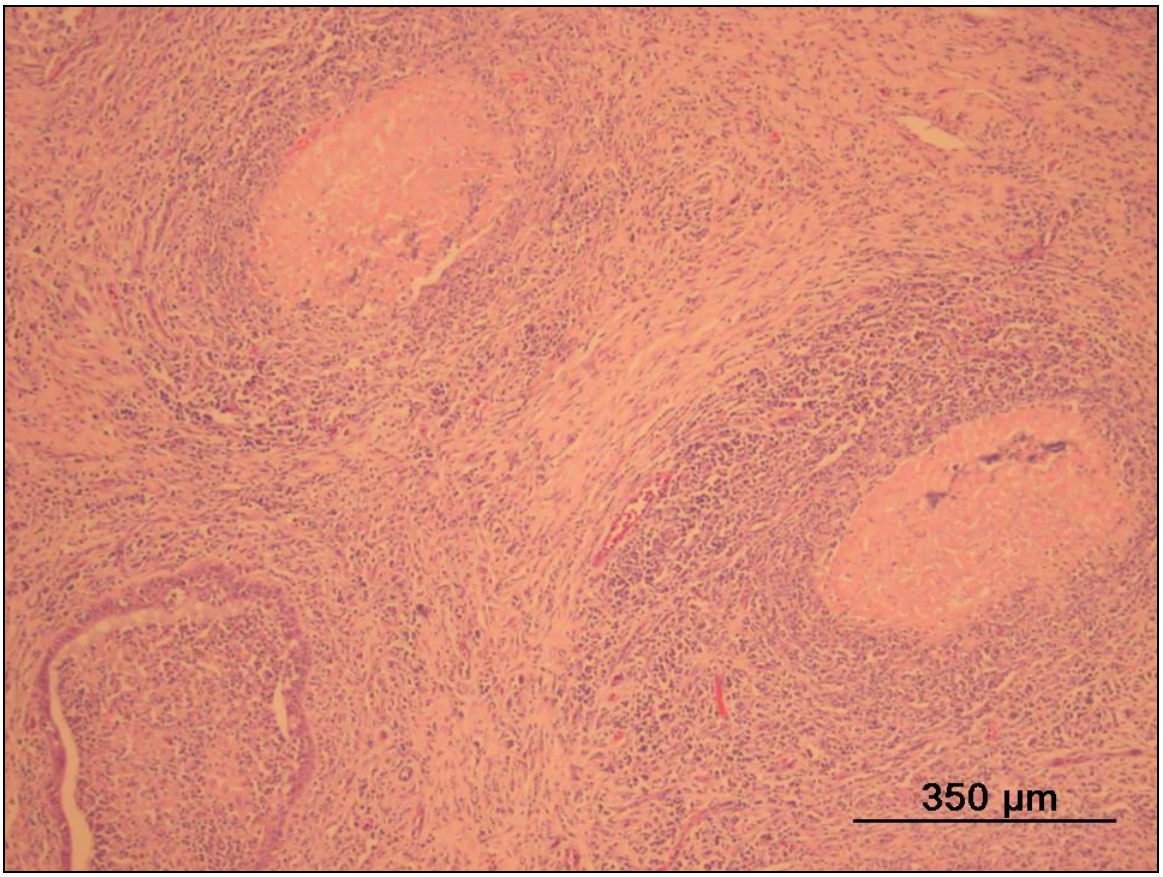

Fig. 3.7 Photomicrograph of necrogranulomata in epididymis of Bull D

Compared to Bull D there was only scant fibrosis in the interstitial compartment of Bull E's testes. In addition, there were relatively more seminiferous tubules that retained spermatogonia that were still in contact with the basement membrane. The necrosis within seminiferous tubules was far more subtle than in Bull D, affecting single cells or small clumps of cells within the occasional tubule. Bull E also had severe multifocal to coalescing granulomatous epididymitis, but in some

of the less affected ductules the intact lining epithelium was obviously metaplastic and hyperplastic (probably induced by the inflammation that was present).

3.3.3 Electron microscopy

Lumpy skin disease virus could be seen on positive staining electron microscopy of a section of the testis from Bull D. Fig. 3.7 represents a photograph of LSDV found on positive staining EM. The classic appearance of the virus can clearly be seen. No virus could be seen on a similar section of the testes of Bull E. Poxvirus particles could be demonstrated in wax-embedded pieces of tissue (from Bull D and E) that showed LSDV-positive staining on immunohistochemistry, as depicted in fig. 3.8.

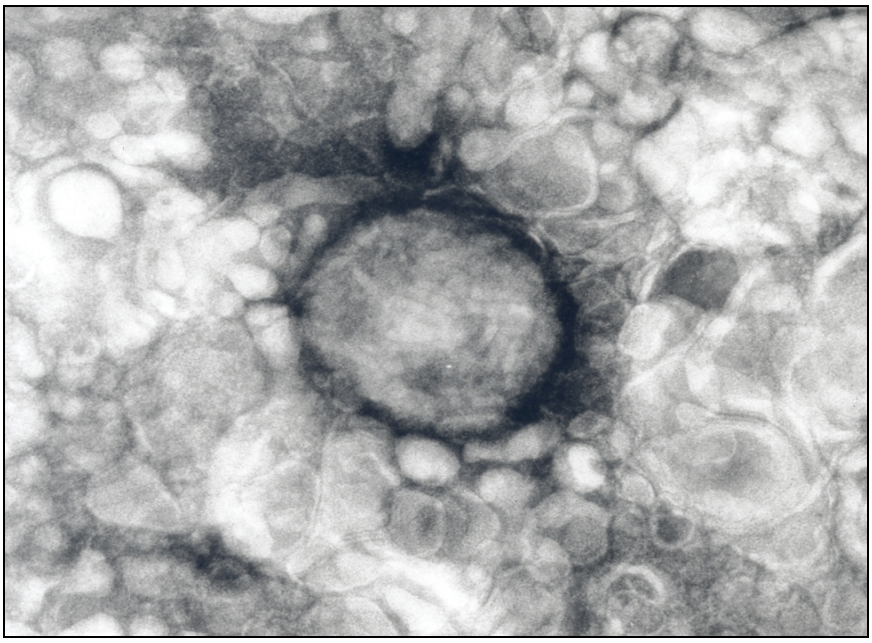

Fig. 3.8 Electron microscopic photograph of LSDV in testis of Bull D

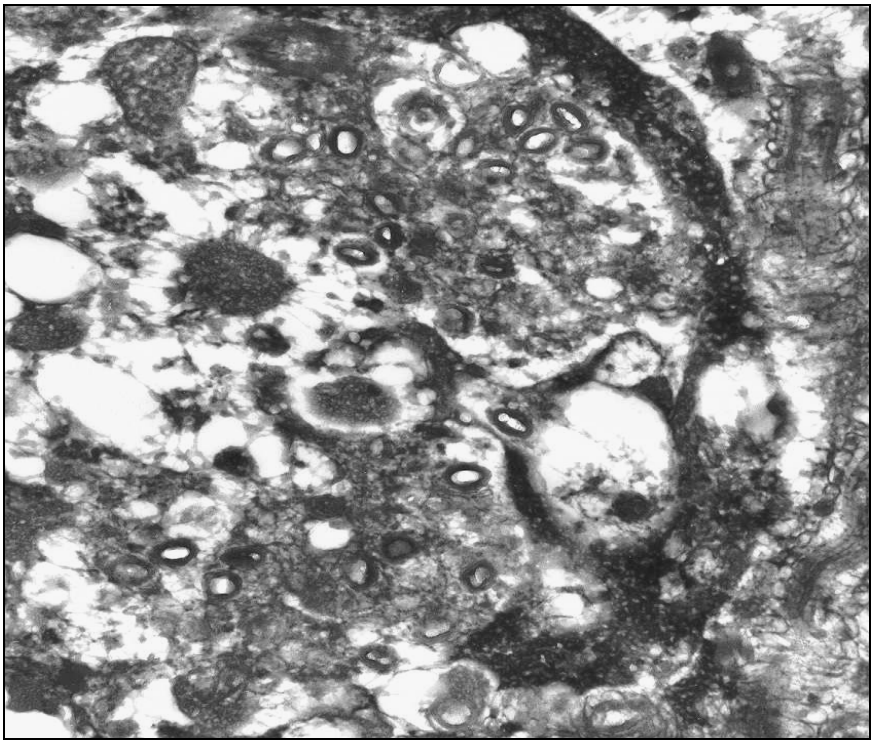

Fig. 3.9 EM photograph of poxvirus particles in affected section of testis of Bull E

3.3.4 Immunohistochemistry

Infarcted tissue in the testes of Bull D stained pale diffuse to granular dark orange indicating the presence of LSDV antigen. No positive staining could be observed in multiple sections of testes from both bulls with orchitis and segmental necrosis of seminiferous tubules. The necrogranulomata in the epididymis of both bulls showed similar positive staining. Some granules could clearly be seen in the cytoplasm of necrotic cells. Fig. 3.10 is a photomicrograph of the immunoperoxidase (IMP) staining observed in the epididymis of Bull D. The contrast with the surrounding tissue that does not contain viral antigen (and hence shows no staining) can clearly be appreciated.

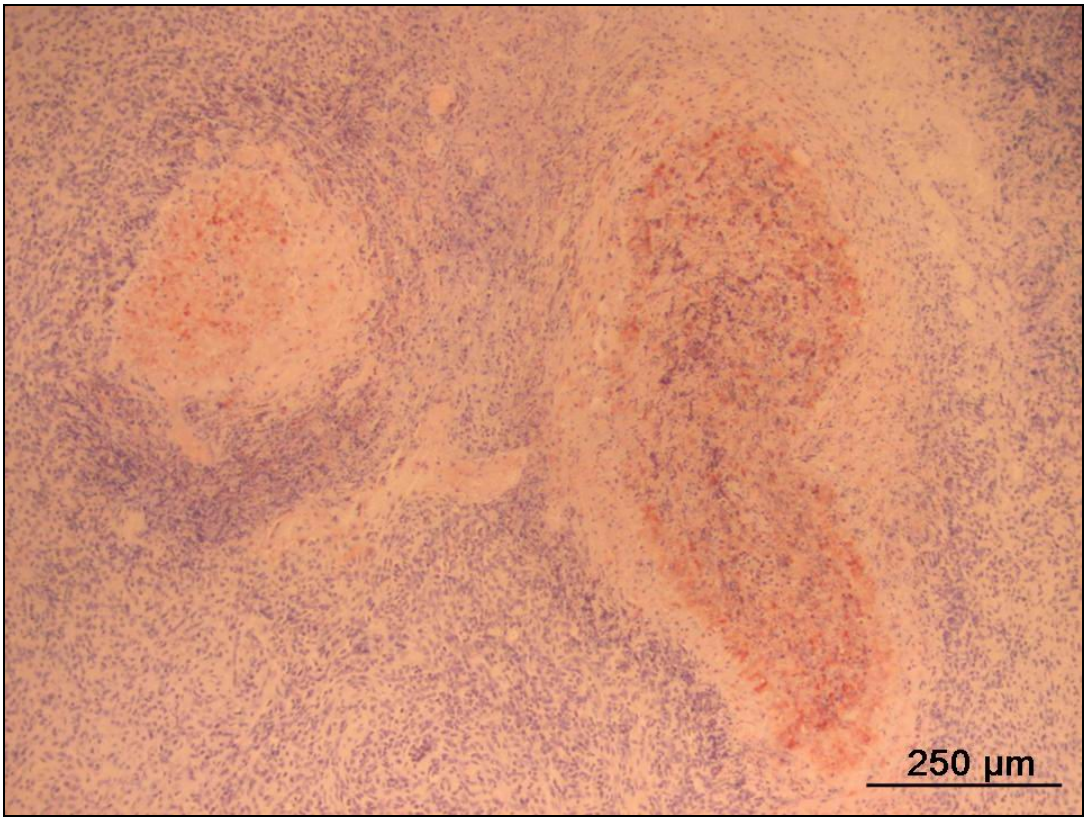

Fig. 3.10 Photomicrograph of IMP staining in epididymal necrogranulomata

3.4 Laboratory results on samples collected

3.4.1 Blood samples

In the severely affected group Bull D showed intermittent viraemia from 8 days to 20 days p.i. During this period viraemia could not be demonstrated on days 10 and 18 p.i. Bull E also showed intermittent viraemia from day 8 p.i. but continued to show viraemia until day 22 p.i. On days 10, 14 and 20 p.i. virus could not be isolated from the blood of Bull E. In both these bulls, no viraemia could be demonstrated for the remainder of the trial. The results of VI performed on heparinized blood samples are summarized in Table 3.3.

Fewer blood samples from the bulls belonging to the group that was moderately affected tested positive by virus isolation. Bull C showed viraemia only on day 6 p.i. Bull F showed viraemia on days 12, 16 and 24 p.i. All other blood samples from Bull C and Bull F tested yielded negative results for the isolation of LSDV (Table 3.3)

In the inapparently affected group, Bull A showed viraemia on days 14 and 18 p.i. Lumpy skin disease virus could not be isolated from any of the other blood samples collected from Bull A. No blood samples from Bull B tested positive via virus isolation (Table 3.3). SNT results show that on day 28 p.i. all bulls had seroconverted.

Table 3.3 Virus isolation results on heparinized blood samples

Days PI	Severely affected		Moderately affected		Inapparently affected	
	Bull D	Bull E	Bull C	Bull F	Bull A	Bull B
6	-	-	+	-	-	-
8	+	+	-	-	-	-
10	-	-	-	-	-	-
12	+	+	-	+	-	-
14	+	-	-	-	+	-
16	+	+	-	+	-	-
18	-	+	-	-	+	-
20	+	-	-	-	-	-
22	-	+	-	-	-	-
24	-	-	-	+	-	-
26	-	-	-	-	-	-
28	-	-	-	-	-	-

3.4.2 Sheath wash, vesicular fluid and semen samples

Sheath wash samples of Bull D tested positive by PCR on days 10, 12, 14, 20, 24 and 26 p.i. Vesicular fluid samples of Bull D tested positive by PCR on days 10, 12, 14, 16, 20, 22, 24 and 26 p.i. The supernatant of the semen samples of Bull D tested positive by PCR on days 14, 16, 20, and 22 p.i. The cell-rich fraction of semen of Bull D tested positive by PCR for the

first time on day 10 p.i. and tested positive on every day that samples were collected for the remainder of the trial (Table 3.4). Sheath wash samples of Bull E tested positive by PCR on days 10, 12, 16, 20 and 22 p.i. Vesicular fluid samples of Bull E tested positive by PCR on days 14, 16, 18, 20, 24 and 26 p.i. The supernatant of the semen samples of Bull E tested positive by PCR on days 12, 14, 20, 22 and 26 p.i. while the cell-rich fraction of the semen samples of Bull E tested positive on every day that samples were collected from day 10 p.i. Bull E was slaughtered on day 32 p.i. and the semen sample collected on that day tested negative by PCR. The PCR results for sheath wash, vesicular fluid and semen samples are summarized in Table 3.4.

The sheath wash samples of Bull C tested positive only on day 16 p.i., the vesicular fluid samples on days 14, 22 and 26 p.i. and the cell-rich fraction of the semen samples tested positive on days 12 and 14 p.i. Supernatant from the semen samples of Bull C tested negative throughout the duration of the trial (Table 3.4). The sheath wash samples from Bull F tested positive by PCR on days 12, 16 and 18 p.i. and the vesicular fluid sample on day 18 p.i. Neither the cell-rich fraction nor the supernatant of semen samples collected from Bull F tested positive by PCR on any of the days that samples were analyzed by PCR.

Table 3.4 PCR Results on sheath wash, vesicular fluid and semen samples

	Severely affected								**Moderately affected**								**Inapparently affecte**					
	Bull D				**Bull E**				**Bull C**				**Bull F**				**Bull A**				**Bull**	
Days PI	*Sheath wash*	*Vesicular fluid*	*Supernatant*	*Cell-rich*	*Sheath wash*	*Vesicular fluid*	*Supernatant*	*Cell-rich*	*Sheath wash*	*Vesicular fluid*	*Supernatant*	*Cell-rich*	*Sheath wash*	*Vesicular fluid*	*Supernatant*	*Cell-rich*	*Sheath wash*	*Vesicular fluid*	*Supernatant*	*Cell-rich*	*Sheath wash*	*Vesicular*
10	+	+	NA	+	+	-	NA	+	-	-	NA	-	-	-	NA	-	-	-	NA	-	-	
12	+	+	-	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	
14	-	+	+	+	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	
16	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	
18	-	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	
20	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
22	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	
24	+	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
26	+	+	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	
28	NA	NA	NA	+	NA	NA	NA	+	NA	NA	NA	-	NA	NA	NA	-	NA	NA	NA	-	NA	N
32					NA	NA	NA	-														

The cell-rich fraction of the semen sample collected from Bull A yielded positive PCR results only on day 18 p.i. The preputial wash sample collected from Bull B similarly yielded a positive PCR result on day 18 p.i. Additionally, the cell-rich fraction collected from Bull B on day 26 p.i. was positive by PCR (Table 3.4).

3.4.3 Necropsy samples

3.4.3.1 Virus isolation

Virus could not be recovered from tissue samples from the ampullae, vesicular glands and prostate glands of Bull D and Bull E. Virus was only recovered from the testes of Bull D and Bull E and the epididymis of Bull D, while no virus could be recovered from the epididymis of Bull E.

3.4.3.2 Polymerase chain reaction (PCR)

The PCR assay was consistently positive when performed on the testes of Bull D and Bull E and epididymis of Bull D, and gave a faint positive reaction after a second round was performed on dilutions of extracted DNA of the epididymis of Bull E. Samples from the ampullae, vesicular and prostate glands of Bull D and Bull E were negative by PCR.

Chapter 4

Discussion

The focus of this research was the identification of bulls that shed LSDV in their semen for protracted periods of time following artificial infection via the intravenous route and subsequent investigation of the mechanisms by which such protracted shedding occurs.

4.1 Clinical signs

The clinical signs observed in the bulls in this trial were remarkably similar to those produced in other trials and published in the literature (Hunter and Wallace 2001; Coetzer 2004; Tuppurainen *et al.* 2005). It remains unclear why only certain animals respond to the viral challenge. Genetic resistance determined by major histocompatibility complexes (MHC) found on the cell surfaces of individuals has been put forward as a possible explanation (Amills *et al.* 1998). Genetic variation in lumpy skin disease viruses is not involved in this phenomenon due to the very limited genetic variation associated with this virus (Kara *et al.* 2003). Contrastingly, genetic variation is a feature of viral persistence in semen of animals infected with equine viral arteritis virus (EVAV) (Hedges *et al.* 1999; Patton *et al.* 1999; Balasuriya *et al.* 2004) and porcine

respiratory and reproductive syndrome virus (PRRSV) (Christopher-Hennings *et al.* 2001). In a trial comparing the different routes of infection Carn and Kitching (1995) found that a greater proportion of animals showed generalized infection when challenged with a LSDV suspension intravenously. Contrastingly, Irons *et al.* (2005) found similar results to the results reported in the present study. It is possible that the smaller numbers of animals used in this trial and the trial of Irons, could have led to smaller numbers of animals showing generalized infection and hence skewed the results. Viral dose and virulence of the isolate may also explain variations in proportions of animals responding to challenge.

4.2 Scrotal circumference

The changes in the scrotal circumferences of the two bulls that showed signs of severe generalized lumpy skin disease were noteworthy. No report on changes in scrotal circumference associated with LSDV infection could be found in the literature. Jubb *et al.*(1985) states that testicular changes were associated with malignant catarrhal fever and bluetongue virus infections in cattle. The scrotal circumferences in the present study increased shortly after the onset of fever, reached a maximum two weeks post infection and then started to decline until the end of the trial. The values at the end of the trial were on average 15 to 20% lower than at the start of the trial. The softness of the testes and the decreased scrotal circumferences later suggest severe testicular degeneration. The extent of the testicular degeneration was confirmed at post mortem examination.

Histopathological examination of the tissue specimens from the two bulls in this trial suggested that necrosis was the main underlying pathomechanism at the time of slaughter. Although the authors did not specifically comment on testicular changes, Prozesky and Barnard (1982) concluded that vasculitis and thrombosis, leading to oedema and necrosis was central to the pathogenesis of the lesions in Lumpy skin disease. The increases in scrotal circumferences in the early part of the trial can therefore possibly be ascribed to inflammatory oedema.

4.3 Histopathology of testes

A study reported by Nagi (1990) detailed testicular and cutaneous lesions in tissue specimens from animals infected with LSDV during the first reported outbreak of the disease in Egypt. The author concluded that LSDV showed direct cytopathic effects on the vascular endothelium of both skin and testis and that this cytopathic effect resulted in luminal thrombosis and medial necrosis of affected vessels and subsequent alterations to the related tissues. The numerous seminiferous tubules that were totally devoid of primary spermatogonia as well as the extensive necrosis with obliteration of epididymal ducts on histological examination, suggested that these bulls would have suffered from reduced sperm production even if they did recover from the infection. The finding in the present study that some primary spermatogonia were also affected is contrary to the findings of Nagi (1990). A possible reason for the conflicting results could be the route of infection or the protracted illness that the bulls in the

present trial suffered from. The tissue specimens in the report by Nagi could have originated from animals with generalized infection as well as animals that showed only localized lesions. It has been shown that the first appearance and severity of local lesions was related to dose of virus (Carn and Kitching 1995). It is unsure whether the intravenous route of infection in the present study led to a higher viral load and hence a greater effect on the spermatogonia.

4.4 Ultrasonographic findings

Ultrasonography has become an invaluable diagnostic modality in medical and veterinary science. It can be applied to the genital tract to obtain images in a non-invasive manner. A 5 MHz probe is capable of providing images with a diameter of 3 to 5 mm in reasonable quality up to a depth of 10 cm, (Ginther 1998) making it a useful diagnostic tool, although not as sensitive as probes of higher frequencies.

Ultrasonographic changes in the accessory sex glands of the severely affected bulls were probably due to inflammation. The reason for the transient appearance of ultrasonographic changes in the ampullae and the vesicular glands is unknown. In Bull D the appearance of ultrasonographic lesions in the accessory sex glands and identification of viral particles in vesicular fluid samples by PCR coincided well. Only on one occasion could ultrasonographic lesions be observed without the vesicular fluid samples being positive by PCR. This

relationship was not well established in Bull E. In this bull viral particles could only be found in vesicular fluid samples on three occasions where ultrasonographic lesions were identified concurrently. The usefulness of ultrasonographic changes as a predictor of viral shedding in genital fluid is therefore questionable.

Furthermore, ultrasonographic changes in the testes and epididymides of the two severely affected bulls were evident later p.i. than PCR positive results on sheath wash, vesicular fluid and semen samples. It is an accepted clinical principle that ultrasonography of testes is not a reliable predictor of testicular pathology (Ginther 1998). However, in this study the gross post mortem findings did correspond well with the ultrasonographic lesions demonstrated in this study.

4.5 Viraemia related to clinical signs and seminal shedding

Virus isolation performed on heparinized blood samples yielded positive results at some point in time in all bulls except Bull B. Bull B also did not show any signs of lumpy skin disease. Similar to the findings of Tuppurainen *et al.* (2005), the duration of the viraemic period did not coincide with the severity of the disease, nor with the detection of viral antigen by PCR in the semen. This differed from the findings of Bowen *et al.* (1983) with respect to the shedding of bluetongue virus in the semen of artificially infected bulls. Only two semen samples collected during the present trial contained blood. The finding that viral nucleic acid was

also detected in other samples than those contaminated with blood, combined with the lack of coincidence with viraemia suggests that the virus does not enter the semen or fractions thereof due to contamination with blood. The mechanism of shedding of viral particles in semen of bulls artificially infected with LSDV is thus dissimilar to the situation with shedding of bovine leukosis virus (Choi *et al.* 2002) and bluetongue virus (Bowen *et al.* 1983).

4.6 Seminal shedding in relation to clinical signs

Virus was found most frequently from semen samples from the severely affected bulls. However, in agreement with Irons *et al.* (2005), detection of viral antigen was successful even at times when clinical signs of the disease were not apparent.

4.7 Viral presence in semen fractions

None of the different fractions of semen consistently produced a greater amount of positive PCR results. This seems to be a consistent finding for various viruses reported to be shed in the semen of various animal species for prolonged periods (Christopher-Hennings *et al.* 2001). The finding that sheath washes contained viral genetic material was noteworthy. Not only has this never been described before, but it also occurred in all bulls at some point during the experimental period. This finding is consistent with a predilection of LSDV for mucosal surfaces

as it is known that in bulls not showing generalized infection, ulcerated LSD lesions can occasionally be found in the oral cavity and omasum (Coetzer 2004). The intermittent nature of detection (via PCR) of viral antigen shedding precludes the routine use of sheath wash samples as an alternative to semen for diagnosing bulls shedding viral antigen in their semen. However, semen samples collected by routinely-used methods of electrostimulation and artificial vagina cannot be considered to be completely free of preputial fluid. The presence of virus in this component therefore contributes to the ability to detect viral particles in semen samples.

Immature spermatozoa released into the lumina of the seminiferous tubules reach the epididymis with the aid of fluid secretions from the Sertoli cells and rete testis, as well as contractile elements of the testicular capsule and cilia lining the efferent ducts. Ejaculation starts in the epididymis and travels along the ductus deferens. Simultaneously, there is contraction of the wall of the accessory sex glands, which forces their contents into the urethra and mixing of the contents occur. The urethra is then emptied by rhythmic contractions of various muscles (Roberts 1986; Garner and Hafez 1993). Viral particles can therefore be deposited into the ejaculate either from the Sertoli cells, from epithelial cells lining the rete testis and epididymis or from the accessory sex glands. It is also possible that the viral particles may come into contact with the ejaculate as it passes through the *vas deferens* and urethra during the ejaculatory process. The fraction of semen that yielded the most positive PCR results in this trial was

the cell-rich fraction of the severely affected bulls. Interestingly, this fraction was the only fraction from which viral DNA could be detected in one bull, and it could only be detected once. Numerous positive PCR results on supernatant fractions with a concurrent negative result on the corresponding cell-rich fraction adds impetus to the suggestion made earlier that the virus is not sperm-cell associated. Due to the large size of LSDV particles, they may have been located in the sperm-rich pellet after centrifugation despite not being bound to the spermatozoa in any way. PCR yielded no positive results on the vesicular fluid portion in the inapparently affected bulls but periodically in the moderately and severely affected bulls.

A possible explanation for these findings is that viral genetic material is swept into semen when the testicular fluid or accessory sex gland fluid comes into contact with LSD lesions. Contraction of the testicular capsule in the severely affected bulls would exert pressure on areas of necrosis and potentially liberate viral particles into the testicular fluid. An alternative explanation could be that epithelial cells in the epididymis are infected with virus that is liberated into the ejaculate. The immunohistochemistry at the time of slaughter does not support the latter explanation, but it is possible that it is the mechanism involved in the shedding at the early stages of LSD infection.

The finding that bulls were still shedding viral particles even though seroconversion had already taken place was also noteworthy. Contrastingly, it

has been reported that shedding of BVDV ceased with the production of detectable serum antibody (Kirkland *et al.* 1991). This provides further evidence that viraemia is not a mechanism by which shedding of LSDV particles takes place.

4.8 Effects of elevated scrotal temperature

It is of vital importance for the health of testicular tissue and normal spermatozoal morphology that sperm production occurs at temperatures of 4 – 6 ºC below core body temperature (Barth and Oko 1989). The effect of elevated scrotal temperature upon development of spermatozoal morphological defects (Kastelic *et al.* 1996) and testicular echotexture (Brito *et al.* 2003) has previously been established. In the latter study no changes in testicular echotexture was found up to 35 days after insulation of either the scrotal neck or the entire scrotum for a period of 4 days. The outspoken changes in testicular echotexture found in this trial would suggest that LSDV had an additional effect upon testicular and epididymal tissue other than only pyrexia. It should however be noted that the period of pyrexia in the two severely affected bulls in this trial was 16 and 24 days respectively; a period far longer than 4 days (Brito *et al.* 2003) or 8 days (Kastelic *et al.* 1996). It cannot be ruled out that the prolonged elevated scrotal temperatures were solely responsible for the testicular lesions in this trial. No reports could be found in literature on the effect of such prolonged elevated scrotal temperature upon testicular tissue as in this experiment.

4.9 Viral presence in tissue samples

Virus could be identified by PCR only in sections of epididymis of both bulls D and E and by virus isolation from the epididymis of only Bull D. Sections of the epididymides associated with outspoken necrosis showed positive staining on immunohistochemistry. Lumpy skin disease virus and viral particles could be demonstrated by positive-staining electron microscopy of sections of testes of both severely affected bulls. Virus could not be identified (either by PCR, virus isolation or immunohistochemistry) in tissue samples from the accessory sex glands and the testes of the two severely affected bulls. This is analogous to the findings of Givens (Givens *et al.* 2003) that BVDV antigen could not be found in testicular tissue samples from one of two postpubertal bulls acutely infected with BVDV. Contrastingly, Kirkland (Kirkland *et al.* 1991) concluded that BVDV replication predominantly took place in the seminal vesicles and prostate glands of acutely infected bulls and that a similar situation occurred in persistently infected bulls. Kirkland (Kirkland *et al.* 2004) failed to demonstrate bluetongue virus in tissues collected at necropsy from bulls that had been shedding virus in their semen. Neu and co-workers (1988) isolated EAV most frequently from the ampulla of persistently infected stallions and concluded that persistence could be attributed to a complex interaction between host and viral factors. It has to be remembered that shedding of EAV is a consistent finding over a long period of time and not sporadic over a protracted period of time, like shedding of LSDV and BHV-1 in semen of bulls. It has however been evident that protracted shedding of LSDV in semen of bulls is also dependent on a complex interaction

of host and viral factors. Most of these factors are still undetermined and require further investigation.

Immunohistochemistry is capable of showing viral predilection for a specific cell line and hence sheds light on the mechanisms involved in the protracted shedding state. Predilection for a specific cell line in the reproductive tract could not be shown in the present study. It has been established that sheeppox virus, (the prototype virus for the genus *Capripoxvirus*, to which LSDV belongs) spreads in macrophages around the body and becomes localized in various tissues, as well as in testes (Kitching 2004). It is possible that a similar mechanism is involved in the shedding of LSDV in semen and that at time of slaughter this mechanism has already resulted in the coagulative necrosis seen. Round cell infiltrates certainly did play a role in the development of the peripheral reactions to necrosis in the testes of the bulls.

4.10 Conclusions

This study confirms that the shedding of LSDV in the semen of artificially infected bulls is not associated with the presence of the virus in blood. Furthermore, the shedding of the viral genetic material is not consistently associated with a specific fraction of the ejaculate and protracted shedding is dependant upon the presence of LSD lesions. Such lesions can be detected by ultrasonography. The epididymides and testes are the primary sites of localization of the virus responsible for protracted shedding. Bulls developing severe generalized disease experience outspoken testicular degeneration. The extent of the testicular degeneration suggests that upon recovery the ability to produce sperm is diminished or lost. Collection of the cell-rich fraction of semen or the whole ejaculate is adequate for consistent identification of LSDV genetic material in bulls with lesions, but not in bulls that are subclinically infected. The epidemiological significance of shedding of LSDV particles in different fractions of semen of bulls not displaying signs of LSD warrants further investigation.

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