The bull as a source of trichomonosis and lumpy skin disease: An African perspective

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The bull as a source of trichomonosis and lumpy skin disease: An African perspective

De stier als bron van trichomonose en lumpy skin disease: Een Afrikaans perspectief

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Chapter 1

Introduction

The use of bulls in breeding herds is regarded as a practical, labour-efficient way of producing a calf crop. However, the risks of a poor crop due to unsatisfactory performance of bulls are significant. The causes of sub- or infertility of the bull are classified under the headings *Impotentia generandi*, referring to a lack of fertile semen, and *Impotentia coeundi*, or an inability to mate naturally due to physical causes or a lack of libido. Prior screening of bulls to reduce the risks is therefore prudent. Most of the causes of infertility can be detected in a systematic examination of the bull and a representative semen sample. The bull in which no causes of infertility are identified during such an examination is termed breeding sound, which is not a guarantee of fertility but implies a reduced risk of infertility.

The bull may also play a key role in causing reproductive wastage in the cow herd by acting as a source of infectious diseases. The costs associated with the introduction of an infectious disease into an uninfected herd are significant. *Brucella abortus* can cause dramatic abortion storms in susceptible herds, while trichomonosis and campylobacteriosis can cause pregnancy percentages of as low as 25% due to embryonal death and abortion.

Infectious diseases are amongst the most important causes of infertility in African natural service herds. Clearly testing of animals when they enter a herd is warranted. Bulls, being the animals most commonly introduced into herds, must be tested. However, standards need to be set for which diseases should be tested for, how samples should be collected, what tests should be used, and how many times an animal should be tested before being declared negative. Whatever tests are recommended have to be integrated into the complete set of tests and examinations done on a bull.

Selection of bulls for breeding purposes

Standardised procedures for performing breeding soundness examinations (BSE) and for interpretation of the findings and certification of the animals have been developed by the Society for Theriogenology in North America and the Australian Cattle Vets. Neither of these standards are deemed suitable for the African situation, one major deficiency of both being the lack of incorporation of testing for infectious diseases. The Australian standard states that routine testing for transmissible diseases is not conducted due to the lack of suitable technology(1). The Society for Theriogenology guidelines indicate that testing for infectious diseases may be indicated in specific instances but is not routinely applied(2).

Other deficiencies in the Society for Theriogenology and Australian standards which are addressed are the relative lack of emphasis on semen evaluation. The Australian model is relatively lenient on its requirements for sperm morphology evaluation, in that the client elects which procedures should be done, and this part of the evaluation is often omitted(1). The Society for Theriogenology model sets a low standard for motility. In this model, a semen sample may be evaluated either for mass motility or for individual motility(3). Individual motility of 30% is permissible for a satisfactory potential breeder. The fact that motility assessment is often underestimated due to negative effects which occur after ejaculation has confounded studies into the relationship between motility and fertility, most of which have shown that motility assessment has limited value in predicting fertility(4). However, the extrapolation that low motility values should be regarded as acceptable is questionable. In studies where extraneous influences have been controlled, where motility assessment is done by objective, repeatable means and where large numbers of breedings are included, motility has been shown to affect fertility, with combinations of motility parameters being highly correlated with fertility(5). The Australian standard requires a progressive motility of 60% or more for a bull to pass a semen evaluation(1). This corresponds more closely to the norm for bull semen. Our experience is that 70% is an achievable standard and the chapter on bull selection demonstrates how this is dealt with in practice.

For a breeding soundness examination protocol the practical implications of testing large numbers of animals at a time also have to be considered, as well as those of testing animals on farms in remote areas far from laboratory facilities.

The new certificate also meets legal requirements, resulting in the issuing of a document which is legally clear on sale of the animal.

It has become common practice to include tests for controlled diseases as well as for venereal diseases in the prepurchase examination of bulls. Testing for *Brucella abortus, Mycobacterium bovis, Tritrichomonas foetus* and *Campylobacter fetus* have therefore been part of the accepted breeding soundness standard in South Africa for many years. The recommendations are due for review in the light of new research. Of particular interest is the poor performance of testing methods for *T. foetus*. The availability of a new PCR test for this disease require a critical review of the requirements for bull breeding soundness certification, for which data concerning the field performance of the test is required.

This thesis also focuses on another disease which may be transmitted through semen - lumpy skin disease – and its implications for breeding soundness examination and international trade protocols.

Infectious diseases in bovine reproduction

Cattle suffer from an impressive array of infectious diseases which impact negatively on reproduction. There are obligate venereal pathogens such as *Tritrichomonas foetus* and *Campylobacter fetus* subsp. v*enerealis* which do not inhabit or otherwise affect any other organ system outside the reproductive tract. Then there are pathogens which are not spread venereally but have the reproductive system as their main target organs. *Brucella abortus* is one such pathogen, wreaking havoc in the pregnant uterus after entering the body by the oral route or via exposed mucous membranes in most instances(6). There are pathogens which affect reproduction as only one of a range of organ system effects. Bovine virus diarrhoea is one such organism, causing reduced semen quality and follicle development, endometritis, pregnancy loss and pregnancy loss and fetal malformations in addition to respiratory and enteric manifestations and profound effects on the immune system(7). Then there are the purely opportunistic pathogens, causing disease when conditions are just right, otherwise forming part of the natural flora of the animal. The bacterium *Arcanobacter pyogenes* is a good example, being a fairly ubiquitous organism which causes endometritis, vesiculitis and sporadic abortions under certain circumstances(8). Lastly there are those organisms which affect reproduction due to a general insult to the body. Bovine babesiosis is an example of this, abortion following in the wake of fever, anaemia and toxaemia(9).

Venereal transmission plays a role in the spread of many of the agents alluded to above, either due to transmission during coitus or their presence in semen which is used for artificial insemination or *in vitro* embryo production. Bulls are asymptomatic carriers of several venereal diseases. This combined with how bulls are used make the bull a highly strategic critical point for the control of these diseases(10). In natural mating production systems bulls are generally exposed to many female animals and are therefore an efficient transmitter for agents which require contact or close proximity for transmission. Bulls are also moved between herds more commonly than cows, with this being the only source of outside genetics in some instances. When artificial insemination is employed many thousands of females may be exposed to the semen from a bull. Should such semen be contaminated with an infectious agent which is able to infect the female when introduced into the uterus the impact could be vast, many orders of magnitude

greater than the worst scenario of infection emanating from a heifer or cow. The potential damage is similar when the semen is used for the *in vitro* production of embryos and the resulting embryos are infected. The focus of this work is therefore diseases which are harboured by the bull and pose a risk to susceptible female animals.

Two of this subset of diseases have special relevance to the South African situation, prompting this work. On the domestic front, trichomonosis has enjoyed a resurgence in prominence in the beef industry and is widely thought to be the commonest infectious cause of reduced calf crops(11). On the international level lumpy skin disease virus has attracted attention as it is an impediment to the export of bovine semen from South Africa(12).

Potential for spread of infectious diseases via frozen semen

Traditional husbandry methods limit the potential for spread of an infectious agent from an infected bull to the number of cows with which he has direct contact. Assisted reproductive techniques have multiplied this potential manifold both in terms of the numbers of animals which can be affected as well as the geographical area which can be spanned. It is therefore conceivable for a single infected semen donor to spread an infectious disease to many thousands of females in many countries. This is true both for diseases which have some effect on reproduction as well as for those which affect other organ systems but are shed in semen.

The OIE, recognising the potential role of semen in dissemination of disease, published a technical report on 'Diseases transmissible by semen and embryo transfer techniques' 22 years ago(13). Pathogens listed as being known to both be present in the semen of large ruminants and to transmit the disease through AI were foot-and-mouth disease virus, bluetongue virus, *Brucella abortus*, *Campylobacter* spp., *Mycobacterium* spp., bovine herpesvirus-1, *Tritrichomonas foetus*, *Leptospira*, bovine viral diarrhoea virus, *Haemphilus somnus*, and *Mycoplasma* spp.. Those where the organism was present in semen but where transmission had not been confirmed were rinderpest virus, *Mycoplama mycoides* (CBPP), lumpy skin disease virus, *Mycobacterium paratuberculosis*, *Coxiella burnettii*, bovine ephemeral fever virus, parainfluenza-3 virus, *Ureaplasma* spp., *Chlamydia* strains, enteroviruses, parapox virus, and transmissible genital papilloma virus. Several others were listed as being likely to be present in semen, and yet another group which could possibly be present(13). Much research has been done in this area since, as shown in several reviews(14,15,16,17).

Wrathall and coworkers evaluated the risks of viral transmission to recipients of bovine embryos arising from fertilisation with virus-infected semen(18). They considered viruses which could be found in semen from bulls with subclinical infections to be the most significant for international trade. The four which met these criteria were enzootic bovine leukosis virus, bovine herpesvirus-1, bovine viral diarrhoea virus and bluetongue virus. These authors noted the relative lack of success in removing pathogens from embryos produced *in vitro* using the standard processing method prescribed by the International Embryo Transfer Society compared to *in vivo* embryos. Interestingly, lumpy skin disease virus is not mentioned in this extensive review, most likely due to a total lack of publications on these aspects for this agent.

A range of biosecurity measures to prevent the spread of infections via semen have been employed. The most secure method is to ensure that the animal is not infected with the organism in question and is kept in an area or country where the disease does not occur before and during the period when semen is being collected. Where these measures are not attainable, alternative measures have been employed. These include keeping the animal in quarantine before and after semen collection for export, and testing the animal. Where a possibility remains of the semen being infected, batches of semen may be tested to detect the presence of the pathogen. Lastly, methods for removal of infection from semen while retaining the fertility have been devised. Processing of semen to remove pathogens is more feasible for *in vitro* fertilisation due to the small amount of semen which is required (18,19). It is important to ascertain the semen component involved in pathogen transmission, as this will influence the methods and success of removal of the pathogen(19). This has not been ascertained for lumpy skin disease virus.

While the preceding information is clear justification for great caution with regards to semen movements, there are diseases for which very stringent movement restrictions are regarded as being overzealous. Despite their proven presence in semen, the potential for the spread of some organisms is highly questionable. Enzootic bovine leukaemia virus (EBLV) was shown to infect susceptible females when infected leukocytes were placed in the uterus (20) and to be present in bovine semen(21), prompting the exclusion of seropositive bulls from semen donation. However, subsequent evidence has failed to demonstrate any risk and it is now accepted that transmission via semen is highly unlikely(22,23). A group of arthropod-transmitted, blood-borne infections have been shown to be transiently shed in semen at the time of viraemia of the donor. However, the potential for transmission at any other time is considered to be negligible, leading to calls for relaxation of international codes to permit the movement of semen from donors

shown not to be viraemic on the day of semen collection(16). This group includes the orbiviruses bluetongue, epizootic haemorrhagic disease and Ibaraki disease; the rhabdovirus bovine ephemeral fever; and bunyaviruses Aino, Akabane and Cache Valley virus.

Trichomonosis

Trichomonosis is a venereal disease of cattle caused by *Tritrichomonas foetus,* a parasitic protozoan inhabiting the bovine genital tract. Clinically it is characterised by aberrant returns to oestrus, infertility, some early abortions, and pyometra. The causative organism, *Tritrichomonas foetus*, is an obligatory inhabitant of the bovine reproductive tract. It is approximately 9–25 x 3–15 μm in size and is identified by three anterior flagellae and a characteristic undulating membrane visible under phase-contrast or dark-field microscopy(24). Its jerky, rolling motion are easily observed at 100x or 250x magnification using phase-contrast or dark-field microscopy.

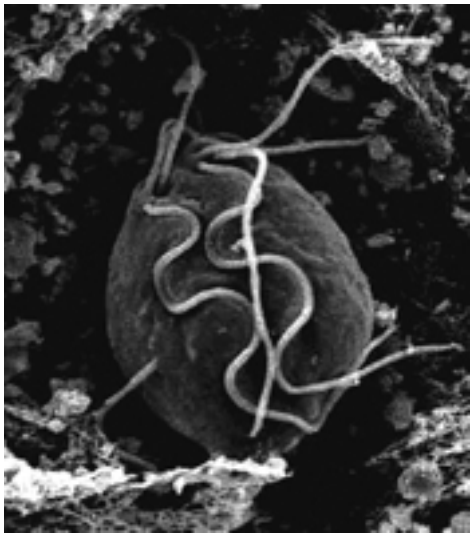

Figure 1. A *Tritrichomonas foetus* organism undergoing cell division

Since it's discovery, either in France in 1888 or in Italy in 1900 the disease has been diagnosed worldwide(25,26). It was first recorded in South Africa by Robinson in 1937 and was subsequently confirmed to be a major cause of infertility in beef cattle in the region(27). It is less problematic in dairy herds due to

the predominant use of artificial insemination in these herds(28). Considerable economic losses are associated with the disease due to increased days open, decreased calving percentage, decreased growing time of calves born later in the season, culling and replacement costs, and the costs of treatment(29,30,31). The losses increase exponentially as the prevalence of infection in a herd increases, with an expected loss of revenue of 35% for each cow exposed to an infected bull in a herd with a disease prevalence of 40%(32). Laboratories in various regions in southern Africa reported infection rates ranging from 0 to 25% of samples and 0 to 46% of herds(33,34,11,35,36,37). The population dynamics of herd bulls generally keeps the prevalence within a herd to below 50%(32).

Persistently infected bulls are the primary source of infection. Once infected, bulls generally remain life-long carriers, perpetuating the infection from one breeding season to the next(38). The main risk factor associated with an increased prevalence in bulls is increased age, with bulls up to three years of age being relatively resistant to infection(39,40,41,42). Mechanical transfer of infection by young bulls from infected to susceptible females has only been demonstrated in one case in which two services occurred within a short period of less than 30 minutes(41). Other risk factors are beef herds, sexual rest, commingled grazing, and exposure to a greater number of other herds(43). No clear breed susceptibility is seen, the reported variations in actual infection rates between breeds most likely reflecting differences in breed susceptibility, breeding activity, or management(42,39,34). No immunity has been recorded in bulls, although a weak circulating antibody response and secretion of antibodies into the preputial secretions is seen following challenge(44,45,46). Transmission during coitus is highly efficient, with 86-88% of susceptible females becoming infected following mating by an infected bull(47). *T. foetus* can also be disseminated by artificial insemination as it can survive the standard processing methods for semen(48). Transmission of *T. foetus* between bulls by means of homosexual activity may occur rarely but has not been proven conclusively(49). Iatrogenic transfer may occur during collection of preputial material for diagnostic purposes from bulls or when performing vaginal examinations on cows(31). Female-to-female transmission of the disease is unlikely(41).

Bulls are generally asymptomatic although a mild swelling of the prepuce with a mucopurulent exudate may be noted(50). The organism readily colonises the preputial and penile mucosa and, less commonly, the urethral orifice(51). Older bulls remain chronically infected although spontaneous recovery has been recorded(52). The increased susceptibility of older bulls is ascribed to the deeper epithelial crypts in the mucosa of the prepuce(24). The organisms do not invade the

mucosa(53) but have been isolated from other parts of the male genital tract, including the seminal vesicles(51,47). Females of all ages are equally susceptible. In females colonisation of the vagina is followed later by migration of organisms through the cervix to the uterus and oviducts. Cervicitis, and, in some cases, endometritis and salpingitis result, endometritis only developing from approximately 60 days after infection(54). Clinical evidence is very subtle and generally goes unnoticed. Conception is usually not interrupted although salpingitis may prevent fertilisation(54). Endometritis usually causes death of the embryo or foetus from 14 to 18 days up until five months of gestation due to interference with its nutrition or nidation(54). Where embryonal death occurs after recognition of pregnancy (Day 17), irregular oestrus cycles occur. Pyometra may develop due to retention of dead foetuses during the early stage of pregnancy(55). Occasional later abortions also occur(56). The majority of cyclic females spontaneously resolve *T. foetus* infections and regain normal fertility within three to seven months(31,54,57). Clearance from the vagina, cervix and uterus is simultaneous(57). Infection does, however, persist for longer in some females, resulting in a so-called carrier status which can perpetuate the infection in a herd despite commonly applied control measures(57,58). A partial immunity develops in infected females which enables the animal to eliminate the infection within three weeks if reinfected(57,59). The immunity lasts for 15 months, generally long enough to reduce pregnancy losses during the subsequent breeding season(59). Animals which develop pyometra or abort remain infected for protracted periods, with large numbers of organisms being shed following abortion. Rarely, females may carry the infection throughout pregnancy and calve at term(31,60). Two such cows remained infected for six and nine weeks post-partum, respectively(60). Permanent infertility resulting from chronic endometritis or pyometra is rarely seen(50). Antibodies to *T. foetus* have been demonstrated in some unexposed heifers in serum and genital tract secretions(57). These apparently occur naturally.

Calving percentages are typically very low following the infection of susceptible herds, whereas chronically infected herds often achieve calving rates of 70 and 85%(10,59). A marked staggering of gestational ages of pregnancies diagnosed during pregnancy examinations after a breeding season is often the first indication of the disease(38). Pyometra often presents in 5–10% of cows in a recently infected herd but has been recorded in 21% of exposed animals(38,55). Infection tends to be more insidious in dairy than in beef herds.

Trichomonosis: Limitations of current diagnostic tests

A positive diagnosis of trichomonosis is based on the demonstration of live *T. foetus* organisms or *T. foetus* DNA from preputial material of bulls, genital tract secretions of females, or aborted foetal and placental tissues. The most readily available laboratory methods are direct examination and culture. Direct examination has a low to moderate sensitivity of 20% and is therefore seldom used as the only diagnostic test(37). Culture of preputial samples has a sensitivity of 70–95%(61,62,63,64,65,66,67). Culture vials must be examined every other day for the presence of organisms until seven days before being declared negative. Culture of cervicovaginal mucus from clinically normal cows is even less sensitive than preputial material(31). In cases of abortion culture of foetal abomasal contents and foetal fluids is usually diagnostic.

A variety of other diagnostic tests have been used. Histopathological examination together with immunohistochemistry is suitable for demonstration of organisms in tissues from aborted foetuses(68,53). Vaginal mucus agglutination tests lack sufficient specificity for accurate diagnosis on an individual basis(69,50). Other diagnostic tests which have shown promise include an ELISA assay to the protective surface antigen TF1.17, a serological assay, a haemolytic test on blood samples and an intradermal test(38,46,70,71).

Various factors contribute to the moderate success of diagnostic methods. Firstly, the organism is labile, and can only be detected in samples which are placed in suitable transport media for a limited time after collection. Samples should be kept cool and transported to the laboratory as soon as possible, with same-day delivery being ideal. With transport in phosphate-buffered saline there is a reported reduction in sensitivity of culturing of approximately 10% after 24 hours(62,72). Enriched transport media inoculated immediately after collection prolong the viability of organisms. A transport medium suitable for both trichomonads and *Campylobacter fetus* is commercially available in South Africa (Steve's TM, Vrede Veterinary Laboratory). Products designed as both transport and in-practice culture systems are available, the most well-known consisting of a protease peptone medium in a clear pouch (In-Pouch TF, Biomed Diagnostics)(66). Another combined transport and culture system (Trichtube, Vrede Veterinary Laboratory) is commercially available in South Africa, but has yet to be extensively tested.

Another factor reducing the success of diagnostic sampling is the variation in the number of organisms in the preputial secretions of infected bulls caused by cyclical variation, breeding activity and treatment of infected bulls(73,59). Another very

common cause of false negative diagnoses is the overgrowth of culture media with contaminants, despite the fact that most media contain antibiotics. Care in collecting clean samples and filtration of samples before inoculation of media reduces but does not eliminate this problem.

The moderate sensitivity of the available diagnostic tests poses significant problems in effective control of the disease. Bulls are generally selected for testing due to their central role in the epidemiology of the disease and their frequent introductions to new herds. To reliably confirm a bull to be free of infection requires the collection of samples on three occasions a week or more apart(63). Due to logistical and cost implications, this is often not done, resulting in incomplete testing of animals entering herds. This has resulted in a failure to eradicate the disease.

Better sample collection methods and the use of more sensitive testing methods would facilitate control of the disease. Preputial material may be collected by washing or scraping. While studies have demonstrated the similarity in diagnostic accuracy of the two methods, many practitioners were unfamiliar with the technique and advantages of preputial scraping at the beginning of this work.

The use of PCR amplification technology enabled the development of highly sensitive and specific tests for diagnostic and research purposes(74,75,76). However, no publication is available demonstrating the optimal sample collection method, transport media, and effect of time delay on the accuracy of the PCR test for *T. foetus*.

Lumpy skin disease

Lumpy skin disease virus is a pox virus of the genus *Capripoxvirus* which causes acute, subacute or inapparent disease in cattle of all ages and breeds. It is endemic in Subsaharan Africa, Egypt and Madagascar with periodic increases in the prevalence of clinical disease. The disease is characterised by fever, skin nodules, necrotic plaques in mucosae, and lymphadenopathy. It causes considerable economic losses due to emaciation, damage to hides, infertility in males and females, mastitis, loss of milk production and mortality which is usually around 3% but may reach 40%(77). The morbidity in natural outbreaks may be 100%(77). For these reasons it was listed in the OIE's 'List A', which identified diseases with the potential for rapid spread and severe economic losses, until the inception of a single disease list in 2006.

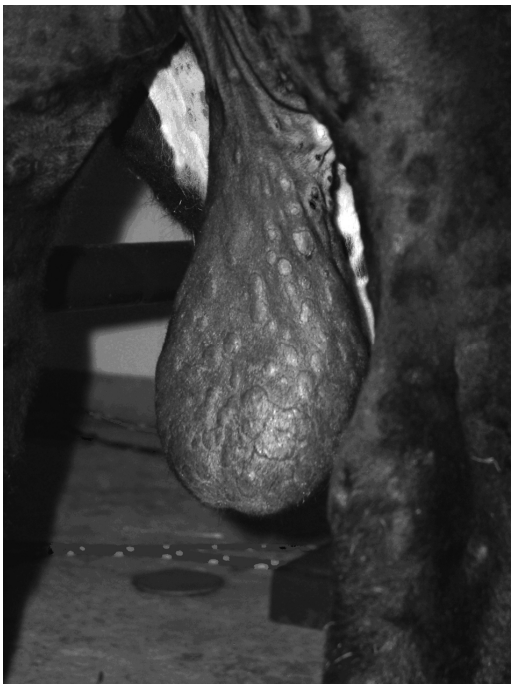

Figure 2. Cutaneous lesions on the scrotum and thigh of a bull with lumpy skin disease

Field observations and supporting evidence indicate that the disease is transmitted by biting flies(77). The mosquito *Aedes aegypti* has been shown to be a competent vector, transmitting the disease for up to 9 days following feeding on an infected animal(78). There are also indications of transmission in the absence of insect vectors, although transmission between animals by contact is extremely inefficient(79).

Lumpy skin disease impacts on male reproduction in various ways. Animals which develop clinical signs are febrile, depressed and painful, and unlikely to engage in mating activity. The disease also affects the semen quality of bulls which develop clinical disease, which has been described in experimentally-infected bulls(80). The effect on sperm output was related to the severity of clinical disease, with severely affected animals becoming azoospermic or severely oligospermic. Semen consistency, an indicator of sperm production, took six weeks to return to pre-infection levels in mildly affected animals whereas the severely affected animals

never quite recovered their pre-infection consistency. Sperm motility declined drastically within eight days of challenge in all animals, returning to preinfection levels at days 21 to 132 post-challenge. Percent morphologically normal sperm cells was unchanged until day 14 after challenge, when it showed a distinct decline in most animals lasting up to 10 weeks. The authors speculated that the duration of the poor semen quality in the two severely affected bulls exceeded what would have been expected due to the febrile response alone, and that other mechanisms were likely to be present.

The effect on fertility of bulls is permanent in some cases. Testicular lesions include thrombosis, medial necrosis and periarteritis of testicular blood vessels, diffuse degenerative changes in the seminiferous tubules, subacute focal interstitial orchitis, and a chronic fibrous interstitial reaction(81). Eventual atrophy of the testes may occur(82).

Vaccination against lumpy skin disease using attenuated virus vaccines is highly effective. The Neethling strain of lumpy skin disease virus has been used successfully in a vaccine for the control of lumpy skin disease in southern Africa(83,84). However, the effect of the vaccine itself on semen quality remains to be elucidated. Being a live vaccine, it is conceivable that there may be an effect.

Lumpy skin disease: Excretion in semen

Weiss found that the virus was excreted in semen for 22 days after the fever reaction following experimental infection, and that viral shedding in semen occurred in the absence of clinical evidence of infection in some cases(84). Although no further data exists as to the risk posed by semen in the transmission of the disease, the above information was sufficient to classify semen from countries where lumpy skin disease occurs as a potential hazard(85,86). Office Internationale des Epizooties recommendations for the importation of semen from an infected country where lumpy skin disease occurs is as follows: An international veterinary certificate attesting that the donor animals showed no clinical sign of lumpy skin disease on the day of collection of the semen and for the following 28 days; were kept in an establishment or artificial insemination centre in the exporting country where no case of lumpy skin disease was officially reported for the 28 days prior to collection, and that the establishment was not situated in a lumpy skin disease infected zone(87).

Since no additional information regarding the excretion of lumpy skin virus in semen was available, further studies were required to characterise the phenomenon.

Factors such as prevalence, associated signs, and ability to detect virus using modern detection methods were required. The origin of lumpy skin disease virus in semen was not known, nor in which fraction of the semen it is found. This information would be of value in assessing the likely course and duration of viral shedding, as well as the significance thereof and possible preventive measures or methods of reducing or removing the virus from infected semen.

Semen may be used for the insemination of live animals for normal calf production or *in vivo* embryo production, or it may be used for *in vitro* fertilisation and embryo production (IVF and IVP). *In vitro* embryo production is a powerful tool in genetic improvement, enabling the shortening of generation intervals by up to a year and four to five times the number of calves to be produced per unit time as with conventional embryo transfer(88). However, the use of semen containing pathogens is a concern in IVP. A recent review concluded that the use of virus-infected semen often results in the production of contaminated embryos, and that the removal of viruses from these embryos is more difficult than with *in vivo*-derived embryos(18). On the other hand, the absence of direct contact with recipient animals, the small amount of semen used and the processing of semen prior to use for IVF, which includes rigorous washing and the removal of seminal plasma, all reduce the potential for disease transmission via infected semen(89). Whether the use of semen containing lumpy skin disease virus in IVP will result in infected embryos has not been studied.

The risk of disease transmission by IVF embryos increases if the infectious agent is non-cytopathogenic for the oocytes or embryos(89). It is therefore important to establish whether lumpy skin disease affects embryo development in an IVP system, which has not been studied previously. No studies examining the ability of swim-up or any other methods to render semen safe from lumpy skin disease virus have been published. Since the fraction of the semen in which the virus occurs is not known, the likelihood of successful removal using semen sanitising methods cannot be rationally predicted.

Live viral vaccines have the potential to result in shedding of the virus in the semen of vaccinated animals. In bovines, recent work has demonstrated the shedding of virus-strain BVDV following vaccination of both pre- and postpubertal bulls with an attenuated live virus vaccine(90). Shedding of vaccine-strain lumpy skin disease virus would influence the inclusion of vaccination in protocols designed to allow for the export of bovine semen to countries where lumpy skin disease does not occur. No studies have been done on whether vaccine virus is shed in semen.

Objectives

The objectives of the work reported in the following chapters were therefore as follows:

- To formulate a new standard for the certification of bulls for breeding soundness in southern Africa (Chapter 2);
- To compare the relative diagnostic accuracy and practical use of preputial washing and preputial scraping in the diagnosis of Trichomonosis in bulls (Chapter 3);
- To determine the relative sensitivity and specificity of a PCR test for *Tritrichomonas foetus* from preputial samples collected by washing versus scraping, stored for 6 hours, 30 hours and 5 days, and preserved with guanidinium thiocyanate (GuSCN) (Chapter 4);
- To determine the incidence and duration of shedding of lumpy skin disease virus in the semen of experimentally infected bulls using PCR testing and virus isolation (Chapter 5);
- To determine the fraction of semen in which lumpy skin disease virus occurs, the tissues in which it is localised in bulls which become persistent seminal shedders of the virus, and the associated pathological changes (Chapter 6);
- To determine whether lumpy skin disease virus is removed by swim-up processing of bull semen, and whether the use of semen spiked with lumpy skin disease virus for the *in vitro* fertilisation of oocytes affects the embryo yield and results in the production of infected embryos after *in vitro* culture (Chapter 7);
- To determine whether bulls vaccinated with the Neethling strain modified live lumpy skin disease virus vaccine shed the virus vaccine in their semen or field strain virus following experimental challenge (Chapter 8).

In conclusion, the results of these studies and their possible implications for future research and for application in veterinary practice are summarised and discussed in Chapter 9.

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Chapter 2

Bull breeding soundness evaluation in Southern Africa

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ABSTRACT

The motivation for and process leading up to the publication of a new bull breeding soundness certification standard endorsed by the South African Veterinary Association is described. The Veterinary Certificate of Bull Breeding Soundness and Explanatory Notes and Minimum Standards are shown. The first component of the certificate is a declaration by the veterinarian that the bull complies with the minimum standards set for examinations for the selected purpose, these being for use as a natural service sire, as a donor of semen for distribution, and for insurance purposes. This is followed by the details of the bull and owner, and a list of the recommended examinations and tests for the bull with provision for which were performed. Certificates are available in book form with the Explanatory Notes and Minimum Standards on the reverse, and a carbon copy which remains in the book.

The clarity and ease of completion of the document are regarded as being positive features. Bulls are either classified as breeding sound or not, with no actual parameters indicated on the document and no certificate issued for those which do not meet the set criteria. Contact details of the parties involved are shown on the certificate to allow for communication as a means of avoiding disputes.

Key words: Bull breeding soundness, certification, South Africa

INTRODUCTION

Evaluating the fertility of bulls prior to use as breeding animals would make obvious economic sense if it were possible. Practically it is not possible to predict fertility by means of a breeding soundness evaluation. Applying minimum standards to a set of accepted procedures, however, enables one to make a decision with regard to the breeding potential of the bull. An animal found to satisfy these criteria is deemed to be breeding sound, and the procedure of performing such a set of tests is a breeding soundness evaluation(1).

The cattle breeder using natural service is reliant on the fertility of the bulls for the productivity of his operation. He also seeks to minimise the risk of the bulls infecting the cow herd with a disease with potentially serious consequences. The veterinary practitioner is ideally placed to evaluate the bull with the aim of detecting common causes of infertility, and to do the necessary testing and take appropriate samples to confirm freedom of disease.

The change of ownership of a bull introduces another set of considerations which must be combined with the basic biological and managerial factors affecting use of the bull. A change of herd holds the risk of introduction of infectious diseases to the destination herd. In addition, there is the expectation of a certain level of performance from the animal and the ability to rectify the situation should this expectation not be met.

The logical end point where animals are subjected to breeding soundness evaluations is the issuing of a certificate by the practitioner for those animals which fulfil the set criteria. Besides the selection of the tests and the methods to be used for each, the legal implications of certification add another layer of complexity to the procedure. Certification has grown into an important role of the veterinary profession. As the financial value of products and the responsibility inherent with this important task have gained increased recognition so the legal ramifications of certification have become increasingly important. The principles of certification are as applicable to a bull as they are to any other product, despite the unique aspects inherent to the use of breeding bulls.

A breeding soundness certificate does not guarantee fertility or the absence of risk of transmitting ill-effects to herds the bull is used on, but it suggests a reduced risk due to certain causes. The benefits of examining bulls for breeding soundness are well-documented. Bulls classified as satisfactory for breeding soundness achieved a 9% higher pregnancy rate in a breeding period in single-sire breeding herds than

bulls of questionable breeding potential(2). Bulls which are unsatisfactory could be completely infertile with total loss of the potential calf crop in the single-sire situation. Examples of conditions which cause infertility and which are easily detected are lameness(3), persistent frenulum(4), poor sperm morphology(5), and Trichomonosis(6). For this reason the evaluation of breeding soundness of bulls prior to use is recognised as an integral part of sound herd management in many countries. A cost-benefit analysis of breeding soundness evaluation suggested a direct, short-term financial benefit to be gained by producers under South African conditions(7).

As the practice of the evaluation of bulls evolved, so did a range of services. While the main components of the examination are dictated by the main causes of infertility, the methods used to evaluate these components, depth of the examination, and additional aspects included in the examination vary greatly between practitioners. Advances in veterinary research have led to the introduction of new approaches in this field, and the challenging of some earlier beliefs. Such changes are not adopted in a uniform fashion by all individuals, increasing the tendency towards variation in the application of field examinations. Such variation is a source of dissatisfaction to both clients and practitioners, both groups experiencing inconsistency in the costs and outcomes of the examinations. This situation resulted in some loss of credibility of breeding soundness evaluation in South Africa, and erosion of the perceived value of the procedure.

The motivators mentioned in the preceding paragraph led to production of the first standardised set of guidelines for bull breeding soundness certification by the South African Veterinary Association in 1989. This parallels the situation in other countries, where similar requirements for standardisation resulted in the development of guidelines for bull breeding soundness certification by the Society for Theriogenology in 1983(8), with an updated set of guidelines being published in 1993(9). A recent article describes the process of devising an industry standard for examination of bulls in Australia(10). In this context the revised South African standard of 2000, which has been in use for six years and has been recently updated, is presented here.

MATERIALS AND METHODS

The Livestock Health and Production Group of the South African Veterinary Association convened a working group to revise the existing guidelines. Specific requirements for the revisions were to clearly define what tests should be included in the standard breeding soundness evaluation, and to review recent literature to provide evidence-based standards for each parameter where possible. The brief was for a document which was simple enough to gain wide acceptance in the industry, yet clear enough to eliminate doubt as to what the standards were. In particular, the guidelines should aim to reduce the potential for disputes arising out of the transfer of a bull to a new owner and subsequent unsatisfactory performance of such a bull.

Clinicians of the Faculty of Veterinary Science took the lead in formulating a draft certification document, which was then circulated within the veterinary profession, to breeders groups, and to the main livestock insurance underwriter for comment. Once a set of guidelines had been agreed on, this was presented to veterinarians in a series of workshops in different regions of South Africa and in Namibia and, after having considered their input, the certificate was finalised and made available in print form by the South African Veterinary Association.

When the need arose for a second print run of certificates input was solicited from practitioners via the branch structure of the veterinary association and via an electronic discussion forum to which a large proportion of practitioners subscribed. Minor changes to aspects which had proven to be problematic during field application of the standards were made.

RESULTS

The standard Veterinary Certificate of Bull Breeding Soundness is shown in Fig. 1, and the accompanying Explanatory Notes and Minimum Standards in Fig. 2.

The first paragraph of the document consists of a declaration by the veterinarian that the bull was examined for the selected use class and complies with the minimum standards for this use class. The person or entity who commissioned the certification is also specified in this paragraph, indicating the legal ownership of the information.

Veterinary Certificate of Bull Breeding Soundness

I the undersigned, being a veterinarian registered with the South African Veterinary Council, hereby certify that on this _____ day of _____ 20___, at _____, on the request of _____, I performed the examinations and tests as indicated hereunder on the bull identified below. In my opinion the bull is breeding sound in terms of the standards listed on the reverse side of this certificate for the purposes of (encircle the letter):

A:	Sale or own use	B:	Semen donation for the purposes of sale	C:	Insurance

Identification of the bull

Breed: _____ Age: _____ Colour: _____

Tattoos: _____ Brand: _____

Ear notches: (drawn in) R  L

Ear tags: _____

Name: _____

Name of owner: _____

Residential address: _____

Telephone: _____ Fax: _____

Residential address of the animal *(at the time of examination)*: _____

Examinations and tests: (Those tests for which the "Done" blocks are circled were performed whereas those for which the "Not done" blocks are circled were not performed).

	Circle the options that apply to the bull	**Use classes for which tests are recommended**	**Reason not done:** (not necessary to complete if test not indicated for this bull)

General clinical examination			A	B	C	Test must be done
Detailed clinical examination	Done	Not done		B	C	
Examination of the genital system			A	B	C	Test must be done
Semen collection and evaluation			A	B	C	Test must be done
Contagious abortion (blood test)	Done	Not done	A	B	C	
Tuberculosis (skin test)	Done	Not done	A	B	C	
Trichomonosis (sheath wash or scraping)	Done	Not done	A	B	C	
Number of tests for trichomonosis	0	1 2 3				
Campylobacteriosis (sheath wash or scraping)	Done	Not done	A	B	C	
Number of tests for Campylobacteriosis	0	1 2 3				
Bovine virus diarrhoea (antigen test)	Done	Not done	A	B	C	
Serving ability	Done	Not done		B	C	

Other tests (specify): _____

Further comments: _____

_____

_____

Signed _____ Address or stamp of practice:

Name _____

Qualification _____ SAVC no. _____

Date _____ day of _____ 20 _____

See reverse side for minimum standards and explanatory notes

Explanatory Notes and Minimum StandardsDefinition

A bull is certified as breeding sound if it passes a specified battery of tests relating to reproductive well-being. The extent of the list of tests and, hence, the scope of a breeding soundness certificate may vary, depending on the need. A breeding soundness certificate does not guarantee fertility or the absence of risk of transmitting ill-effects to herds the bull is used on, but it suggests a reduced risk due to certain causes.

Bulls for which a breeding soundness certificate is not issued are not necessarily infertile, of low fertility or pose a risk of transmitting ill-effects to herds they are used on. It is not essential to have a breeding soundness certificate in order to use, buy, sell or insure a bull but the associated risk is carried by the user and (or) seller and (or) buyer and (or) insurer.

Clinical examination

A **general clinical examination m**ust be done in all cases. It is accepted to consist of visual inspection of general health status, condition score (2-3½ out of 5 acceptable), eyes, bite, legs, gait, and conformational defects. A **detailed clinical examination** includes assessment of the digestive, respiratory, circulatory, and locomotory systems and integument in addition to the above.

Examination of genital system

Must be done in all cases.

Sheath: Inspect and palpate. Should not hang lower than the line from the knee to the hock

Penis: Palpate the whole penis from the S-bend distally. Attempt to extrude and visualise during semen collection. If the penis was not seen with a full, normal erection this should be indicated under "Comments".

Scrotal circumference:
Minimum standards according to breed and age (months)

Breed	18 m.	21 m.	24 m.
Bos taurus	32cm	33cm	34cm
Zebu (Brahman)		32cm	32cm
Synthetic Bos taurus x Bos indicus		32cm	33cm
Indigenous			32cm

Alternatively, breed minima may be applied where available, providing they exceed these guidelines.

Testes: Mobile, symmetrical, firm, elastic, with no palpable irregularities. Rotation and slightly uneven height acceptable unless specified by breed society.

Epididymides: Symmetrical, even consistency, normal size throughout.

Scrotum: Neck and scrotal skin normal, testes mobile, ventral cleft ≤3cm.

Internal genital organs: No palpable abnormalities of ampullae, prostate, vesicular glands and pelvic urethra.

Semen evaluation

All of the following evaluations should be done in all cases:

Volume: Only reliable if collected by artificial vagina, but should be recorded in all cases

Colour: White, ivory or yellow

Consistency: Classified as follows:

Description	Approximate sperm concentration
Thick creamy	2,5 x10^9/ml
Creamy	1,75 x10^9/ml
Thin creamy	1,0 x10^9/ml
Milky	0,5 x10^9/ml
Watery	0,2 x10^9/ml

Minimum standard: Milky or thicker

pH: 6,2 – 7,4

Mass motility: Scored as follows:

0 No motility
1 Few motile sperm, <10% alive
2 Many motile but no wave motion
3 Slow waves visible
4 Well-defined, strong waves with rounded turns, reaching periphery
5 Waves as for 4, but with a whiplash effect

Minimum standard: ≥2 out of 5

Individual sperm motility: ≥70% linear motile

Foreign cells: High numbers of spermatogenic cells are unacceptable. Leukocytes and bacteria are unacceptable if there are indications that they do not originate from the preputial cavity.

Morphology: ≥75% normal sperm with no indication of a progressive disorder of the reproductive tract or known heritable morphological defect.

Disease tests

In cases where the herd is known by the practitioner to be negative for contagious abortion and tuberculosis, this status has been confirmed at a recent herd test, and the herd is closed, this is acceptable justification for not retesting each animal.

Currently available tests for trichomonosis and campylobacteriosis are only 75-90% sensitive. Three tests are therefore necessary to certify an animal free of these diseases with a high degree of certainty. Practical implications may preclude complete testing of all animals.

BVD testing must confirm that an animal is not a persistently infected carrier.

Serving ability

Required for use classes B and C and recommended for A. A healthy female in oestrus or restrained in service stocks is required. The bull's ability to mount, achieve intromission and to thrust are assessed. Covering should be achieved within 5 and 10 mins in experienced and young bulls respectively. Longer periods may be allowed for *Bos indicus* bulls.

Other examinations or tests

Optional additional tests include a serving capacity test, tests for other infectious diseases such as leptospirosis, tests for genetic defects and additional tests that an insurer may require.

General recommendations

This certificate indicates the status of the animal at the time of examination. However, arrangements for examinations should be made a month before the certificate is required to allow for the complete evaluation of all bulls.

The user must observe the bull for breeding activity and serving ability at the beginning of the breeding season.

In the case of purchase of a bull, show this certificate to the local veterinarian and consult him/her regarding further actions which should be taken prior to use of the bull.

Copies of certificates, records and smears are retained by the veterinarian for a minimum of three years.

The second, third and fourth paragraphs make provision for description and identification of the bull, the name and contact details of the owner, as well as location where the animal is kept, the latter being a requirement for certification by the South African Veterinary Council. Provision is made for all of the commonly-used identification methods. Where unequivocal identification methods are not already present on the animal, practitioners are encouraged to apply tamper-proof ear tags during the course of their examination.

The fifth paragraph is a list of the examinations and tests, indicating which are recommended and which were performed on each bull. Certain of these are regarded as integral to the evaluation, without which the bull cannot be evaluated. For the remainder, provision is made for circumstances under which the practitioner assesses the risk of omitting that test as acceptably low to indicate this on the certificate as a motivation. Examples of this are where a recent whole-herd test has indicated freedom of certain diseases and the herd has been closed since the test. Failing to achieve the set cut-off points on any one of the mandatory or recommended examinations or tests renders the bull not certifiable for breeding soundness. So, for example, if the bull is found to have a scrotal circumference below the accepted norms, or be positive for Trichomonosis, the practitioner gives the client a report stating reasons for unsoundness and a prognosis and possible remedies. This may still permit sale of the bull should the defect or defects causing him to be unsound not be of a nature that the bull will be infertile. Such a sale is termed a conditional sale.

Books of certificates in English and Afrikaans were printed and made available to veterinarians. The books contain 50 certificates, each with the Explanatory Notes and Minimum Standards on the reverse, and with a carbon copy which is left in the book after removal of the original. Each certificate bears the logo of the South African Veterinary Association.

Qualitatively assessed, uptake of the standards has been good. Assessment of acceptance is facilitated by the small size of the rural practitioner fraternity in South Africa and the good communication channels. Sale of certificate books continues, with a second print run required. Only minor changes were necessitated for the second print run as a result of comments received from users of the certificate.

DISCUSSION

The first breeding soundness certificate published by the South African Veterinary Association in 1989 was a one-page certificate allowing for identification of the animal and giving blank spaces for the practitioner to list the tests and examination procedures performed. No guidelines or explanatory notes were issued with the certificate, and no standard manual was published. This led to a variation in the manner is which the certificate was used.

During consultation with different parties it became apparent that bulls used in different scenarios were subjected to different expectations and that the risk profile depended on the use of the bull. Provision was therefore made for the application of different standards depending on the purpose for which the bull is to be used. The three main purposes for which bulls are examined by South African practitioners are for use as natural service sires, as donors of semen for distribution, and for insurance purposes. These were therefore defined in the guidelines as three different 'use classes' with differing requirements.

The ease of completion of the certificate is considered to be a major feature of the document. Each relevant piece of information is filled in manually in the spaces provided.

Due to the requirement for a document which could accompany an animal at the change of ownership, each bull is dealt with as an individual. Group reporting is commonly practiced by practitioners in South Africa, but a standard is not set for the format for such reporting.

The view of the breeding soundness certificate as a quality assurance tool is integral to the final product. Thus, for the sake of clarity, a bull is classified as either being breeding sound or not. Bulls found to be breeding sound are eligible to be certified as such using the standard document. Bulls deemed not to be breeding sound are dealt with by means of the communication of findings and recommendations to the producer in verbal or written reporting according to the preference of the practitioner. This differs from the Society for Theriogenology and the Australian standards, both of which include intermediate categories. The Society for Theriogenology standard makes provision for classification to be deferred pending further evaluations in bulls which do not fully satisfy the criteria for a Satisfactory Potential Breeder(9). The Australian standard classifies bulls as fertile, subfertile or infertile depending on the parameters for different traits(10).

Due to the inability to demonstrate a clear advantage in fertility of bulls with differing values for parameters above the threshold value, there is no provision for specifying the value for each of these parameters on the certificate which emanates from the evaluation. For example, a bull with semen with a mass motility of 3 need not be any less fertile than one with semen with a mass motility of 4 or 5, with some variability being due to differing environmental conditions at the examination site.

The unambiguous nature of the information contained in the document contributes to the ease of interpretation. This is further facilitated by the page of explanatory notes and minimum standards, which specifies the standards applied for the sake of clarity to all parties.

Disputes arising out of bull sales are often characterised by a lack of communication between the different parties involved in the transaction. It was therefore deemed important to encourage communication between parties by ensuring that the identity and contact details of both the seller and the certifying practitioner are indicated on the certificate. Continuity between practitioners is also addressed by a general recommendation that the certificate be shown to the buyer's veterinarian on arrival of the bull. This places them in a position to assess what further measures need to be taken before the bull is used.

Another principle embodied in the certificate is that of transferring some responsibility to the buyer to ensure certain aspects of the performance of the bull, particularly those that are not tested for in the practice setting and for which the lay observer can gather useful information. Thus it is specified in the explanatory notes that the user observe the bull for satisfactory libido and serving ability when he is used. Care must be taken in assessing the serving ability of bulls to minimize the stress imposed on restrained animals and the potential for disease transmission.

While use of the certificate and the principles embodied therein has been good, further marketing of the concept of bull breeding soundness certification and of this standard in particular is required to improve penetration into the market. Other future prospects include the possibility of distributing the certificate in electronic template form and to automate the completion of the form from hospital records.

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Collection of preputial material by scraping and aspiration for the diagnosis of *Tritrichomonas foetus* in bulls

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ABSTRACT

Two trials were done to assess the diagnostic sensitivity and practicality of preputial scraping as a method of collecting preputial material from bulls infected with *Tritrichomonas foetus*. In the first, preputial material was collected by simultaneous scraping and aspiration from three infected and one uninfected bull ten times over a five week period. In the second trial, samples from five infected bulls were collected by both sheath washing and scraping on six occasions, while eight uninfected animals were sampled three times. Samples were cultured using a modified Trichomonas culture medium (Oxoid).

In the first trial, 29 out of 30 samples from infected bulls were found to be positive. In the second trial, 83 per cent of samples collected by both methods tested positive. In neither trial were any samples from the control bulls found to be positive.

Scraping was found to be quick, safe, and offered the advantages over preputial washing that urine contamination was easily avoided, samples were smaller and more concentrated and contamination by environmental contaminants was reduced. It may be subject to greater operator variability than sheath washing.

It is concluded that preputial scraping is equal in sensitivity to washing and represents a suitable alternative to preputial washing for the collection of material for direct examination and culture of *Tritrichomonas foetus*.

Key words: *Tritrichomonas foetus*, venereal disease, bull, diagnosis, preputial scraping, preputial wash

INTRODUCTION

Tritrichomonosis is a major source of economic losses for the beef industry in South Africa. The disease is characterised by embryonal and foetal death, resulting in lowered calving percentages, prolonged intercalving periods, heifers failing to conceive, sporadic abortions, aberrant oestrus cycles, and the presence of post-coital pyometra in some animals. Prevalence of the disease for various regions in southern Africa range from 0 to 46 per cent of herds[6,13,17,22] (Bloemfontein Veterinary Laboratory, pers. comm., 1999)(Louis Trichardt Veterinary Laboratory, pers. comm., 1999)(S M Pefanis, Vrede Veterinary Laboratory, pers. comm., 1999).

Despite awareness of the disease and proven control programmes based on well-researched epidemiological principles, the disease remains problematic. One factor contributing to this situation is the lack of a highly sensitive and specific test for carrier animals. Culture of preputial material from bulls is the most commonly-used test. While this technique yields sensitivities of almost 100 per cent in some hands**,** diagnostic rates in the 70 to 90 per cent range are more commonly reported[1,3,5,9,12,14,18,19,20,21,23,25,27,28]. Factors contributing to reduced sensitivities include remoteness of farms, fractious animals**,** sample and animal identification errors, collection of large numbers of samples at once, sample contamination and overgrowth, and inconsistent laboratory techniques[7,20]. It is therefore necessary to test a bull repeatedly to obtain a reliable result, a requirement which is not universally adhered to due to the associated cost and inconvenience.

Sheath washing and scraping are the two most widely utilised methods for the collection of preputial material. Material may also be collected by rinsing the liner of an artificial vagina after semen collection[11]. Scraping is most commonly performed with a dry Perspex artificial insemination (AI) pipette attached to a rubber bulb or syringe, which enables aspiration of preputial smegma as the preputial lining is scraped. Custom-made instruments for the collection of preputial scrapings have not shown any advantage[15]. Despite the fact that both sheath washing and scraping have been well described [2,3,24,27] and do not differ in sensitivity[23], sheath washing remains the predominant technique used by veterinary practitioners and animal health technicians in South Africa.

The aim of this trial was to show that scraping and aspiration is a practical method of collecting preputial material for testing bulls for the presence of *Tritrichomonas foetus* infection, and that samples collected by this method achieve a high diagnostic sensitivity when subjected to culture.

MATERIALS AND METHODS

In Trial 1, three adult Bonsmara bulls, which were known to be *Tritrichomonas foetus* carriers and one uninfected two-year-old Jersey bull were used for the study. Preputial material was collected on ten occasions over a five-week period, with a mean interval of 3,2 days between collections. Collection was by means of scraping and simultaneous aspiration using a dry perspex AI pipette (AI pipettes, Kyron Laboratories) connected to a sterile disposable 20 ml hypodermic syringe by means of a silicon-rubber tube. For collection bulls were restrained in a sturdy crush with a neck clamp and by means of an assistant applying a tail-grip. Additional restraint consisted of tying one back leg or application of low level electrical stimulation delivered via an electro-ejaculator probe placed in the rectum**.** This was only necessary in a minority of collections where reaction of the bull was such that safety of the operator was deemed to be at risk.

The technique of collection was as follows: The collection apparatus was held in one hand by grasping the syringe. The tip of the pipette was guided into the caudal reaches of the preputial cavity and manipulated vigorously with an in-and-out movement while suction was applied with the syringe. The tip of the pipette was guided to different areas of the preputial membrane and glans penis using the other hand (Fig. 1). After an average of approximately twenty strokes the pipette was withdrawn and the contents inspected. If insufficient cloudy material was present the procedure was repeated for a longer period.

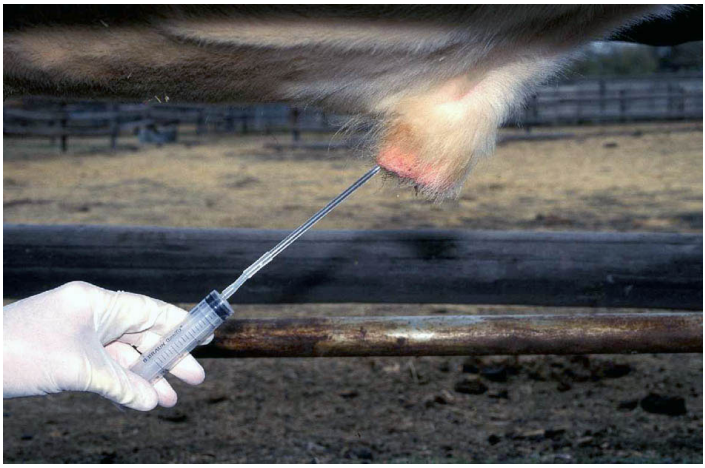

Figure 1. Collection apparatus in the preputial cavity of a bull.

The material was then transferred to a plastic tube containing approximately 4ml of phosphate-buffered saline (PBS Dulbeco, Onderstepoort Biological Products). The material in the pipette and syringe was flushed into the PBS by aspirating the medium up into the pipette repeatedly (Fig. 2).

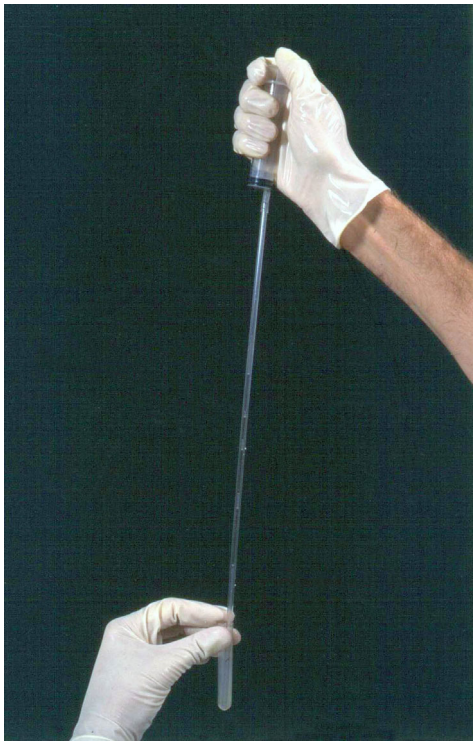

Figure 2. Transferring the content of the pipette and syringe to a tube containing buffered saline.

The collection apparatus and disposable latex gloves were changed for each bull.

Tubes were marked with a sample number only, placed in a polystyrene cool box with a frozen ice pack and transported to the Agricultural Research Council - Onderstepoort Veterinary Institute within two hours of collection. Laboratory staff were unaware of the origin of each sample. Samples were cultured in modified Oxoid's consisting of Trichomonas medium (CM161, Oxoid Limited), horse serum, distilled water and antibiotics. Cultures were examined by direct microscopy.

In Trial 2, five known positive bulls on two extensive commercial farms were sampled by sheath washing and scraping on six occasions over a period of 18 days. The washing was done first by instilling 50 ml PBS into the preputial cavity through a sterile latex tube, massaging the preputium vigorously for approximately 100 strokes, and then siphoning the fluid back into the sample bottle through the tube. This was followed by sheath scraping samples, which were collected as described in Trial 1. Eight uninfected bulls were sampled three or more times each. Four of the control bulls were on one of the farms described above, while four were housed semi-intensively in a university teaching animal unit. Samples were collected by one of two operators on each occasion. Samples were chilled and transported to the laboratory within 6 or 24 hours. Only a small fraction of each sample was utilised for culture, the remainder being used for other purposes.

The two-tailed Chi squared test was used to test for differences between the samples collected by the two sampling methods.

RESULTS

Besides some mild discomfort during collection no bull showed any adverse reaction to the repeated collection of material at short intervals.

In all cases sufficient opaque whitish or bloody, mucoid material could be obtained using scraping. Collection of the sample could generally be accomplished on the first attempt without additional restraint of the bull. Good samples could usually be obtained without the use of the free hand to guide the tip of the pipette, which enhanced operator safety. As the trial progressed the time required for the collection of sheath scrapings reduced.

In Trial 1, 29 of 30 samples collected from the infected bulls were found to be positive on laboratory examination giving a sensitivity of 0,96 with a 95 per cent confidence interval of 0,83 to 0,99. The one negative result was obtained on the eighth collection. None of the 10 samples from the uninfected control bull were positive.

The results of tests on infected bulls in Trial 2 are summarised in Table 1. Twenty four of 29 samples collected by both methods tested positive (0,83). There was agreement on 21 of the 29 samples. One bull was unavailable for testing on one occasion. No positive results were obtained from the control animals.

There was a significant difference in sensitivity between operators in the sheath scraping samples in Trial 2 (9/14 compared to 15/15, $P<0.05$).

Table 1: Culture results from five infected bulls collected by sheath washing and scraping on six successive occasions.

Bull	Sample Operator Transport time	1 A 6 hours	2 A 24 hours	3 B 6 hours	4 A 24 hours	5 B 6 hours	6 B 24 hours
9763	Wash Scrape	- +	- +	+ +	- +	+ -	- -
9924	Wash Scrape	+ +	+ +	+ -	+ +	+ +	+ +
9968	Wash Scrape	- +	+ +	n/a n/a	+ +	+ +	+ -
99001	Wash Scrape	+ +	+ +	+ +	+ +	+ +	+ -
BIG	Wash Scrape	+ +	+ +	+ +	+ +	+ +	+ +

n/a: Not available for sampling

DISCUSSION

The collection of preputial material by scraping with simultaneous aspiration has several practical advantages over preputial washing. Speed of collection, the ability to collect the sample without an assistant, and the fact that contamination of the sample by urine is easily avoided are significant advantages. Although *Tritrichomonas foetus* organisms do survive in urine[16], dilution of the cellular content of the sample is undesirable. The use of disposable collection apparatus eliminates the possibility of cross-contamination of samples or of the transmission of organisms between successive animals. Mechanical transmission of *Tritrichomonas foetus* is a potential hazard whenever infected animals are examined[10]. Special receptacles containing the larger volume of PBS do not have to be ordered beforehand, and the smaller volume of the sample obtained by scraping facilitates sample transport and laboratory processing. Lastly, as the sample is

collected primarily from the caudal reaches of the preputial cavity there is less likelihood of contamination from the environment, particularly in bulls which have gross contamination of the cranial portion of the preputial cavity caused by habitual eversion of the lamina interna. This is in agreement with findings of other workers sampling for *Campylobacter fetus*[26].

The presence of blood in the sample did not constitute a problem, which confirms the findings of other authors[2]. Roughening the tip of the pipette, as is commonly advocated, was found to be unnecessary to obtain a satisfactory sample. The fact that scraping was done after washing on all occasions may have biased the results in Trial 2 by reducing the number of organisms in the preputial cavity**.**

The high diagnostic sensitivity attained in Trial 1 is ascribed to animal factors, sampling technique, proximity to the laboratory facilitating rapid delivery of samples, optimal handling facilities, experienced staff and the small number of bulls sampled on any one occasion.

The lower diagnostic sensitivity attained in Trial 2 for both methods is ascribed to the less optimal collection conditions and to the fact that only a small volume of each sample was available for direct examination and culture. While some authors have found a decreased sensitivity with a 24 hour delay in processing of preputial samples[15,25], we could not demonstrate any effect.

There was a tendency for more false negative tests towards the end of the sampling period in Trial 2 but not in Trial 1. This phenomenon has been seen by other authors, who ascribed it to an increase in bacterial contamination in the sheath after repeated sampling[8]. It is known that more false negative cultures are obtained from bulls during periods of active breeding, presumably due to a reduction in numbers of organisms in the preputial cavity[4]. A similar reduction in the number of organisms due to frequent sheath washing is one plausible explanation for our observation. Alternative explanations include the increased exposure of organisms to blood containing antibodies and other serum factors by virtue of repeated scrapings, and an increase in bacterial contamination of the preputial cavity.

The difference between operators in the sensitivity obtained on sheath scraping suggests that this technique is more prone to operator variability than sheath washing, although larger sample sizes may have demonstrated differences in the latter method as well. This warrants further investigation. If this is the case, thorough training of operators would be a necessity to attain consistent diagnostic accuracy.

Whether sheath scraping or washing are equally suited to the collection of samples for molecular diagnostic techniques requires further work.

It is concluded that preputial scraping is a suitable alternative to preputial washing for the collection of material for culture of *Tritrichomonas foetus*, offering several important practical advantages over that method. Routine use of this technique by competent operators can be expected to render at least equal diagnostic rates to preputial washing.

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Chapter 4

Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: Effects of sample collection method, storage and transport medium on the test.

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ABSTRACT

The objectives of this study were to determine the detection limit of a PCR test for *Tritrichomonas foetus*, to investigate the effect of sampling method, guanidinium thiocyanate (GuSCN), and sample storage, and to confirm the accuracy of the test on field samples. Serial tenfold dilutions of culture material were used to determine the detection limit. For the sample handling trial, 5 positive bulls were sampled by sheath washing and scraping on 6 occasions over a period of 18 days (n= 29 samples) and 8 control bulls were sampled 3, 4 or 6 times (n=28 samples). Samples were cultured, while portions with and without GuSCN were subjected to DNA extraction within six hours, after 30 hours and after 5 days at 4 °C. PCR and agarose gel electrophoresis was performed. A two-tailed Chi square test was used to test for differences between treatments. The PCR assay showed a specificity of 98%. Its sensitivity declined with storage time, from 90% at six hours to 31% at 5 days. Sampling method and GuSCN had no effect on test sensitivity. The detection limit of the assay was 100 organisms. Parallel testing of 193 field samples gave complete agreement between culture and PCR results.

Key words: *Tritrichomonas foetus*, diagnosis, PCR, sample handling

INTRODUCTION

Bovine trichomonosis is a venereal disease of cattle causing reproductive failure, characterized by early embryonic death with irregular estrus cycles and infertility(1). The causative organism is the protozoan *Tritrichomonas foetus*, a trichomonad characterized by three anterior and one posterior flagellae and an undulating membrane. The disease is transmitted from infected, asymptomatic bulls to heifers or cows during coitus (2).

Despite increased awareness and proven control strategies, trichomonosis continues to be a major source of economic losses in Southern Africa, North, Central and South America, Australia, and Asia (3-9). One factor contributing to this situation is the lack of a highly sensitive and specific test for carrier animals.

Culture of preputial material from bulls is the most commonly used diagnostic test. Sensitivities of over 90 % are attainable under optimal conditions (10-15). However, sensitivities of 70 - 90 per cent are more representative of samples collected or processed under sub optimal conditions as is often the case in the field (13,14,16-19). Specificity of diagnosis by culture methods may also be problematic, as other motile protozoa which are found in preputial samples may also survive in culture media(20-23).

Amplification of DNA material by polymerase chain reaction (PCR) is potentially a highly sensitive and specific diagnostic test. Minute quantities of genomic material may be successfully amplified, and primers can be designed to detect subtle genetic differences(24,25). PCR technology has been shown to be effective in the diagnosis of *Trichomonas vaginalis* in humans, with a sensitivity of 95 per cent and a specificity of 98 per cent (26). It has also been applied to the detection of *Tritrichomonas foetus* (27,28) and to the study of genetic relationships between trichomonad species and strains (29-31). While much emphasis has fallen on primer selection and the optimization of laboratory protocols to render satisfactory results, little attention has been given to sample collection and handling procedures. Factors relating to sample collection and handling which may be expected to affect test outcome are sample selection, collection method, transport medium, holding temperature, and time delay until processing can take place (25).

As the bull is the most important maintenance host (32), and the organisms are found almost exclusively in the superficial layers of the preputial mucosa (33-35), preputial specimens are the sample of choice. Preputial material is generally collected by sheath washing or sheath scraping combined with aspiration, which

are both well described (36-38) and have similar sensitivity(13). Scraping is the more traumatic of the two procedures, samples often containing blood and other products of superficial epithelial damage in addition to thick, mucoid smegma. Components of blood inhibit the polymerase reaction(39). Smegma originating from sheath scraping has been shown to reduce the sensitivity of PCR procedures(28). On the other hand, sheath washes often contain substantial amounts of urine, which also has inhibitory properties (39,40). Scraping also renders a more concentrated sample of smaller volume than washing. The effects of these factors on PCR diagnosis of *Tritrichomonas foetus* have not been reported.

The decline in the numbers of viable organisms in samples stored prior to culture is well documented (14,16,41-43). While immediate culture is the ideal, a delay of 24 hours may result in a loss of approximately 10 per cent in diagnostic sensitivity, with longer delays causing more drastic declines. This has lead to the general recommendation amongst South African laboratories of overnight shipping of samples to the laboratory, and the utilization of enriched transport media where longer delays are unavoidable.

Similar work has not been reported for PCR-based diagnostic tests for *Tritrichomonas foetus*. Time delay may lead to a decline in sensitivity of PCR testing due to degradation of the nucleic acids(24). *Tritrichomonas foetus* is known to secrete a range of hydrolytic enzymes (44,45). These enzymes cause rapid DNA breakdown following cell lysis(46,47). Other compounds either from the organisms themselves, from other organisms which are present in the preputial cavity, from the preputial membrane or the upper urogenital tract may also affect the integrity of nucleic acids over time. The addition of preservatives to prevent degradation of the nucleic acid component of stored samples may improve the sensitivity of PCR testing. The chaotropic agent guanidinium thiocyanate (GuSCN) causes cell lysis, inactivates nucleases, binds DNA and preserves its helical structure, enabling its purification and detection (46,48). These activities render it a potentially suitable transport medium for samples collected for the purposes of PCR detection of *Tritrichomonas foetus*. This has not been investigated.

The objectives of this study were to investigate the sensitivity of the PCR assay for diagnosis of *Tritrichomonas foetus* and the effect of sampling method, the addition of GuSCN, and sample storage on the diagnostic sensitivity and specificity of the test. The detection limit and the routine applicability of the assay were also investigated.

MATERIALS AND METHODS

Determination of the detection limit of the PCR assay was done using a field *Tritrichomonas foetus* strain isolated at the Onderstepoort Veterinary Institute. Organisms were cultured in a liquid medium consisting of a commercial medium (Onderstepoort Biological Products, Onderstepoort, RSA) supplemented with 10% heat-inactivated horse serum and antibiotics as described by Ribeiro (21). Cultures were read by a highly experienced technician throughout this work, limiting the potential for misdiagnoses. The concentration of organisms in the medium was determined using a hemocytometer. Serial 10 fold dilutions ranging from 10^5 to 10^{-2} organisms in 10 ml were made by addition of the applicable number of organisms in culture medium to PBS and to a sheath wash sample which tested negative for *Tritrichomonas foetus* by both culture and PCR. The 10 ml aliquots were centrifuged at 800 x g, the supernatants discarded and the pellets resuspended in 200 µl of sterile PBS. DNA was extracted from the resuspended samples using the GuSCN and silica method(47). The final elution of DNA from the silica-nucleic acid pellet was done using 30 µl TE buffer. Five µl of the extracted DNA was used as template for PCR.

PCR was performed using the method described by Felleisen et al. with minor modifications(27). Primer TFR3 and TFR4 were used with an annealing temperature of 60 °C. Takara Taq DNA polymerase (Takara Biomedicals, Separations, Cape Town, RSA) was used. The primers amplify a 347-bp segment of the 5.8S rRNA and the flanking internal transcribed spacer regions ITS1 and ITS2(27). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and visualized under ultraviolet light.

Detection limit determinations were done three times on three different cultures. The detection limit was taken as the number of organisms in the last dilution to give a positive test, and the average detection limit of the three determinations was calculated. The detection limit was expressed as number of organisms processed.

The effects of sample collection and handling procedures were investigated using samples collected from 5 bulls infected with *Tritrichomonas foetus* and 8 uninfected bulls. The infected bulls were housed in groups under extensive management systems on two commercial beef farms. Four of the control bulls were also pastured together on one of these farms, while 4 were housed semi-intensively in a university teaching animal unit. None of the bulls were used for breeding shortly before or during the test period.

Sample collection and handling procedures were as follows: Samples were collected by sheath washing and sheath scraping, as described by Irons et. al.(49). Sheath washes were collected in 50 ml PBS and sheath scrapes were suspended in 5 ml PBS. Washing was done first on all occasions. Sampling was carried out by one of two operators on each occasion. The positive bulls were sampled once on each of 6 occasions over a period of 18 days. Control bulls were sampled 3 times (n = 6), 4 times (n = 1) or 6 times (n=1). A total of 29 samples were collected by each method from the infected bulls, and a total of 28 samples were collected from the control bulls. A resting period of 2 to 4 days was allowed between samplings.

Once all samples had been collected for the day half of each sample was decanted into a second vial, to which guanidinium thiocyanate (GuSCN) was added to achieve a final concentration of 200 mMol/L. Samples were chilled to 4 °C and transported to the laboratory within three to six hours of collection.

A portion of the GuSCN-free sample was cultured on arrival at the laboratory or the following morning. Aliquots of both the GuSCN-treated and GuSCN-free samples were subjected to DNA extraction within 6 h, after 30 h and after 5 days of storage at 4 °C. An 8 ml aliquot from the sheath wash samples was centrifuged as described above and the pellet was resuspended in 400 µl of sterile PBS. Half of the resuspended sample (200 µl) was used for DNA extraction as described above. 200 µl of the 5 ml sheath scrape samples were processed directly as described above without prior concentration by centrifugation. The extracted DNA was used as template for the PCR as described above. The sensitivity was calculated as a percentage of known infected animals that gave a positive test result. A two-tailed Chi square test was used to test for differences between treatments.

The accuracy of serial sampling was determined as follows: Test results for samples from the positive bulls without GuSCN were subdivided into two series of three by considering the first and last three samples as two series of three each. If any one or more of the three in each series was positive, the series was considered to be positive. The sensitivity was calculated as above.

Field evaluation of the PCR method was done using 193 sheath wash samples submitted to the Onderstepoort Veterinary Institute for diagnostic purposes. Samples were submitted to the laboratory within six hours of collection. Samples were tested by both culture and PCR as described above.

RESULTS

The average of the detection limits of the PCR assay in the three experiments was 10^2 organisms. A sample gel is shown in Figure 1. There was no difference between dilutions of organisms in PBS or in preputial material. Consistent results were obtained in all three repetitions.

Results for the sample collection and handling trial on samples from the infected bulls are shown in Table 1. Twenty-four of the 29 samples (83%) collected by each of the sampling methods tested positive on culture. When results from the sheath washing and scraping were considered together, all of the infected bulls tested positive on culture on each sampling occasion throughout the sampling period with the exception of one bull, which tested negative on the last day of testing. This bull tested positive on PCR on this day, the only occasion for which the overall culture and PCR results for the day differed for any animal. No positive results were obtained for samples collected from the eight control animals when tested by both culture and PCR.

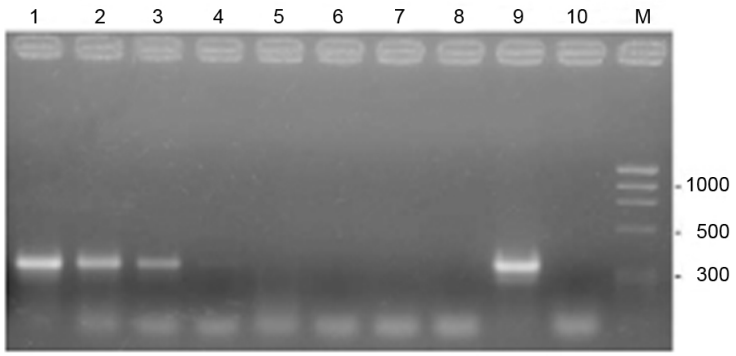

Figure 1. Detection limit of the PCR assay. *Tritrichomonas foetus* PCR product resolved on an agarose gel containing ethidium bromide. Lanes 1 to 9 have 2.4 x 10^5, 2.4 x 10^4, 2.4 x 10^3, 2.4 x 10^2, 2.4 x 10^1, 2.4 x 10^0, 2.4 x 10^{-1}, 2.4 x 10^{-2} *Tritrichomonas foetus* organisms/ml respectively as a template source. Lane M represents molecular weight markers.

There was no difference between the sensitivity of culture of fresh samples and PCR testing at any interval, with the exception of the sheath scraping samples extracted after a delay of 5 days, where culture of the fresh sample was more sensitive than PCR testing. Holding time reduced sensitivity of the PCR test for samples collected by both methods, the difference becoming significant at 5 days.

The addition of GuSCN did not improve the sensitivity of the test. Sampling method had no effect with the exception of samples held for 5 days with GuSCN, where sheath washing gave higher sensitivity than sheath scraping. A combination of sampling method and holding time did render significant differences, with samples collected by sheath washing and tested after 6 hours giving significantly better sensitivity than those collected by scraping and held for 30 hours.

Table 1. Sensitivity (and 95% confidence interval) of culture and PCR testing of 29 preputial samples collected from five infected bulls showing the effect of storage time, collection method and addition of guanidinium thiocyanate (GuSCN).

Collection method	Addition of GuSCN	Culture	PCR		
			Time delay before DNA extraction		
			6h	30h	5d
Wash	No	0.83 (0.64-0.94)	0.9[a] (0.73-0.98)	0.69 (0.49-0.85)	0.62[b] (0.42-0.79)
	Yes	N/A	0.9[a] (0.73-0.98)	0.72 (0.53-0.87)	0.62[b] (0.42-0.79)
Scrape	No	0.83[a] (0.64-0.94)	0.83[a] (0.64-0.94)	0.62 (0.42-0.79)	0.41[b] (0.24-0.61)
	Yes	N/A	0.72[a] (0.72-0.53)	0.62 (0.42-0.79)	0.31[b] (0.15-0.51)

Within lines, treatments with differing superscripts differ in sensitivity $P < 0.05$

Performing serial sampling on three consecutive occasions for each bull increased the sensitivity of the PCR assay at 30h and 5 days (Table 2).

Of the 193 diagnostic samples tested by PCR and culture, ten (5.18 %) were found to be positive on culture and PCR. There was complete agreement between the culture and PCR results, with no samples being found positive with one test and not the other.

Table 2. Effect of serial sampling on sensitivity of the PCR assay after storage time of 30 h and 5 days

	30h		5day	
Sampling Method	Wash	Scrape	Wash	Scrape
Number of bulls	5	5	5	5
Number of sampling occasions per bull	5.8	5.8	5.8	5.8
Sensitivity (%) without serial sampling	67	77	70	53
Number of series of 3 samples	10	10	10	10
Number of series of 3 testing positive	10	10	10	9
Sensitivity (%) with Serial Sampling	1	1	1	0.9

DISCUSSION

The detection limit of the PCR assay for the diagnosis of *Tritrichomonas foetus* was found to be 100 organisms per sample. This is equivalent to two organisms/mL of a 50 ml sheath wash sample, assuming that the concentrating procedure is efficient. Hammond and Bartlett (1943) have reported that the average number of *Tritrichomonas foetus* organisms in a sheath scrape collected from experimentally infected bulls is 50 organisms/ml (33). In the same report, it is stated that the average number of organisms from a naturally infected bull is 141 organisms/mL, with the least organisms being found in the region of the galea glandis, where 20 organisms/mL were recovered. The PCR assay is therefore sensitive enough to detect the numbers of organisms that are typically found in naturally infected bulls.

In the sample collection and handling work, sheath washing was always done before sheath scraping in order to avoid contamination of the sheath wash samples with blood or other products of mild tissue trauma associated with sheath scraping. This may have led to some reduction in sensitivity of the sheath scrapings.

For the purposes of calculating the sensitivity and specificity of the PCR test, the disease status of each bull was determined by culture, using the combined result of the sheath wash and sheath scrape samples collected on the same day such that the bull was considered positive if either of the samples were positive. The one animal which tested negative on culture on the last day of sampling could not be assumed

still to be infected. Spontaneous cure in older, chronically infected bulls has been documented (12). Samples collected from all animals on that day contained a lower number of viable organisms as evidenced by weaker positive results on culture and PCR. This could either be due to poor sampling or sample handling or a true reduction in numbers of viable organisms in the animals on that day. The estimate of the specificity of the PCR test in this part of the trial is therefore considered conservative, as this bull may still have been infected, in which case the discrepancy between culture and PCR results would be ascribed to a superior sensitivity of the PCR test.

Our results showed that the PCR has a specificity of at least 98% as only one possible false positive test result was observed out of 57 samplings from known or possibly negative animals in the sample handling trial. No false positives were seen in the field evaluation trial. Sample collection and handling had no effect on test specificity. We did not see the nonspecific amplification products leading to difficulty in interpretation of results, as has been described elsewhere(22,27). The high specificity makes the PCR assay suitable for confirming the presence of an infection with a very high probability. However, these results should be interpreted with caution. As this trial did not constitute a diagnostic test validation protocol test sensitivity and specificity based on this limited data are likely to be overestimated (50).

The main feature of our findings was the decline in sensitivity of PCR testing of stored samples with time. This effect had not reached significance at 30 hours, possibly due to sample size, but was significant or highly significant in all treatments after 5 days of storage. The loss of sensitivity with time was not prevented by the addition of GuSCN at this concentration. The Silica-GuSCN DNA extraction method which has been shown to eliminate inhibitors of DNA processing enzymes (48) was used to isolate DNA from the sheath wash and sheath scrape samples. GuSCN was chosen as a preservative during transport due to its compatibility with the extraction buffers. Preliminary tests showed that concentration of GuSCN higher than 200 mM lysed *Tritrichomonas foetus* organisms in sheath washes. This would have led to a loss of DNA during the first step of processing, which is centrifugation to concentrate the organisms before lysis. Based on this, a concentration of 200 mM was chosen.

Besides DNA degradation, other causes for a decline in sensitivity with storage time are accumulation of inhibitory compounds or contamination of the sample(25). We cannot exclude either of these possibilities on the basis of the available data.

Serial testing could compensate for the loss of test sensitivity if a time delay between sampling and assay is unavoidable. Serial sampling on three consecutive occasions improved the sensitivity of the PCR assay to 100% at 30 h and 90-100% at 5 days. Serial sampling is accepted practice in South Africa for *Tritrichomonas foetus* diagnosis by culture and direct microscopic examination, due to the long distances which make rapid transport to the laboratory impractical. Our results show that serial testing with the PCR assay would adequately compensate for the loss in sensitivity associated with time delay.

The present results did not demonstrate any difference between the two sampling methods when PCR was used for the diagnosis of *Tritrichomonas foetus* in bulls. Evidence of the superiority of sheath washing over sheath scraping only approaches significance after sample storage for 5 days. Such a difference may be caused by less DNA being present in the sheath scraping samples initially, more inhibitory compounds in the sheath scraping samples, more rapid DNA deterioration in the sheath scrape sample, or a combination of these factors.

The previously mentioned low numbers of organisms on the last day of sampling may emulate the published effect of mating activity, where the numbers of organisms in the preputial cavity decline with breeding activity(51). This effect has not previously been noted after intensive sampling. This supports the commonly applied practice of spacing sample collections at weekly intervals for diagnostic purposes.

The routine applicability of the PCR test was demonstrated by the 100% agreement between culture and PCR results for 193 diagnostic sheath wash samples submitted to the Onderstepoort Veterinary Institute's Bacteriology Laboratory for screening. However, the PCR assay gave results within 24 h of the samples being submitted while it took up to 6 days in some cases for culture to field a positive diagnosis. This emphasizes the advantage of the PCR in terms of a rapid diagnosis and sample turnover time.

We conclude that preputial samples for PCR testing for *Tritrichomonas foetus* may be collected by sheath washing or scraping and should be submitted to the laboratory as soon as possible. The use of the DNA-preserving agent GuSCN did not prove advantageous, but its inclusion at different concentrations should be investigated further.

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Chapter 5

Excretion of lumpy skin disease virus in bull semen

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ABSTRACT

This work was done to establish the incidence and duration of excretion of lumpy skin disease virus (LSDV) in semen of experimentally infected susceptible bulls. Six serologically negative bulls 11-20 months of age were experimentally infected with a virulent field isolate (strain V248/93) of LSDV. Animals were observed for the development of clinical signs, blood was collected until day 90 after infection, and semen was collected every second day until day 18, then twice a week till day 63 and twice a month until three consecutive samples were negative when tested for LSDV by polymerase chain reaction (PCR). An aliquot of each sample which tested positive using PCR was inoculated onto cell monolayers for the recovery of virus.

Two bulls developed severe lumpy skin disease (LSD), two bulls showed mild clinical signs and two bulls showed a transient fever only. Multiple samples were positive on PCR from both of the severely affected bulls and one of the mildly affected bulls; between days 10 and 159, days 8 and 132, and days 10 and 21 respectively. Only one sample from each of the other three bulls was positive on PCR. Virus was only isolated from one sample from one of the severely affected bulls and from five semen samples from the other.

This study confirmed the excretion of LSDV in bovine semen for prolonged periods, even when obvious clinical signs of the disease were no longer apparent.

Key words: Lumpy skin disease virus; Capripoxvirus; bull; semen

INTRODUCTION

Lumpy skin disease virus (LSDV) is a pox virus of the genus Capripoxvirus which causes acute, subacute or inapparent disease in cattle of all ages and breeds. It is endemic in Subsaharan Africa and Egypt with a marked increase in the prevalence of clinical disease in southern Africa from 1990 to 1999(1). The disease is characterised by fever, skin nodules, necrotic plaques in mucosae and lymphadenopathy. It causes considerable economic losses due to emaciation, damage to hides, infertility in males and females, mastitis, loss of milk production and mortality of up to 40%, although mortality rarely exceeds 3%(2). The morbidity in natural outbreaks may be 100%(2). For these reasons it is listed in the Office International des Epizooties' 'List A', which identifies diseases with the potential for rapid spread and severe economic losses.

Field observations and supporting evidence indicate that the disease is transmitted by biting flies, although this has not been conclusively proven(2). There are also indications of transmission in the absence of insect vectors, although transmission between animals by contact is extremely inefficient(3). Weiss, citing unpublished observations, stated that the virus was excreted in semen for 22 days after the fever reaction following experimental infection(4). Although no further data exists as to the risk posed by semen in the transmission of the disease, the movement of semen from countries where LSDV occurs represents a potential hazard(5,6). No standard procedure to detect the presence of LSDV in semen, or to render semen safe by biosecurity procedures or laboratory processing methods have been published.

The objectives of this work were to confirm the excretion of LSDV in semen of experimentally infected susceptible bulls and establish the duration and infectivity in relation to clinical signs, serological status and the presence of virus in blood.

MATERIALS AND METHODS

Animals and housing

Six unvaccinated bulls with no detectable neutralising antibody titers were identified. Two were Holstein / Friesians and four were Dexters or Dexter crosses. All bulls has been reared in groups under semi-extensive conditions within 80km of Pretoria. All were between 11 and 20 months of age, and semen containing motile spermatozoa was collected from all animals by electrical stimulation. Bulls

were housed in vector-free housing for the duration of the experiment, which was performed in early summer, before the peak vector season.

Experimental infection, observations and sampling

After an acclimatisation period of 11 days, the animals were artificially infected by the intravenous injection of a virus suspension. Virus was prepared by culture of a virulent field isolate (strain V248/93) on bovine dermis cell monolayers, harvesting, establishing the infectivity by titration and adjusting the infective dose to a titre of 10^5 $TCID_{50}$ /ml. Two millilitres of this suspension was injected intravenously into each bull on day 0. Animals were then observed for the development of clinical signs. Rectal temperature was measured twice daily until the end of the fever response, after which it was checked twice a week. Blood was collected daily until day 30, then every 3 days until day 90 and virus isolation and serum neutralisation tests were performed.

Semen was collected by electroejaculation prior to infection, then every second day until day 18, then twice a week until day 63 and twice a month after that only in those bulls still giving positive samples when tested by PCR. Semen collection was terminated when three consecutive samples were found to be negative for LSDV on PCR. Semen was collected into new graduated sample tubes using collection cones which were scrubbed and rinsed thoroughly between uses to prevent cross-contamination. Semen was delivered immediately to the laboratory where it was frozen at –20°C for later testing by PCR and at –70°C for virus isolation. Whole semen was used for the PCR test and, where applicable, virus isolation.

PCR test

DNA extractions for polymerase chain reaction (PCR) were performed using a modification of the methods of Gubbels et al. and Schwarts et al. (7,8). Semen was tested by PCR using primers developed from the gene for the viral attachment protein(9). The forward and reverse primers had the sequences 5'TTTCCTGATTTTTCTTACTAT3' and 5'AAATTATATACGTAAATAAC3' respectively, rendering an amplicon of 192bp. A negative control consisting of water, a negative semen control consisting of bovine semen, and a positive control of bovine semen with LSDV added, were included with every PCR sample run.

Virus isolation

Those semen samples which tested positive with PCR were subsequently tested by virus isolation to ascertain the presence of infectious virus. Samples were thawed and diluted by 1:10 to reduce toxicity of semen to the tissue cultures. Minimum essential medium (MEM) containing 5% foetal calf serum and higher than normal concentrations of antibiotics was used to control bacterial growth. Antibiotics in the medium were 0.2 mg/ml amoxicillin, 200 IU/ml penicillin, 200 mg/ml streptomycin, 0.2 mg/ml gentamycin and 0.5 µg/ml amphotericin B. Diluted samples were left for 24-48 hours at +4°C before inoculation onto a confluent bovine dermis cell monolayer. Cultures were examined 1.5 hours after inoculation and if any indications of toxicity were seen, the culture was washed with Dulbecco's phosphate buffered saline (PBS+) containing 0.05 mg/ml amoxicillin and the growth medium was replaced. All cultures were washed with PBS+ with amoxicillin and the media was replaced 6 hours after inoculation with a growth medium consisting of MEM with 5% foetal calf serum and 0.05 mg/ml amoxicillin, 50 IU/ml penicillin, 50 mg/ml streptomycin, 0.1 mg/ml gentamycin and 0.5 µg/ml amphotericin B. Cultures were examined daily for 14 days for cytopathic effects. A negative control consisting of a bovine dermis cell monolayer and a positive control of a bovine dermis cell monolayer with 0.3-0.5 ml of a LSD virus suspension added into it was used on each occasion that virus isolations were performed. In a pilot study this method was able to detect virus in spiked semen samples at 10^{-3} dilution.

Infectivity of semen

Titrations in tissue cultures to determine the infectivity of virus particles was performed on one positive semen sample taken from a bull showing overt clinical signs of lumpy skin disease(LSD) 10 days after experimental infection, using the method of Reed and Muench (10).

RESULTS

Clinical signs and serology

Two of the six bulls, numbers 1 and 2, showed severe clinical signs of lumpy skin disease (LSD), both becoming inappetant and depressed and remaining febrile for 12 and 9 days, respectively.

Table 1: Time of shedding of LSDV antigen in semen from six bulls infected on day 0, as determined by PCR and virus isolation (VI)

Bull		Days																												
		3	4	6	8	10	12	14	16	18	21	25	28	31	35	38	42	45	49	52	56	63	70	97	117	132	145	159	179	
1	PCR		-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	-	+	-	
	VI				+	+	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-			-		-		
2	PCR	-	-	-	+	+	-	-	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	-	-	-	
	VI				-	+		+	+	+	-	-	-	-	-		+	-	-	-		-	-	-		-				
3	PCR	-	-	+	-	-	-	-	-	-		-		-	-		-													
	VI			-		-																								
4	PCR	-	-	+	-	-	-	-	-	-		-		-	-		-													
	VI			-		-																								
5	PCR	-	-	-	-	-	-	-	-	+	-	-	-	-	-		-													
	VI					-				-																				
6	PCR	-	-	-	-	+	+	+	+	+	+	-	-	-	-		-													
	VI					-	-	-	-	-	-																			

+: Positive PCR or virus isolation
- : Negative PCR or virus isolation

Multiple skin lesions consisting of 0.5-2 cm diameter firm raised nodules developed primarily on the head and neck, sides, ventral trunk, scrotum and limbs. Antibiotic and anti-inflammatory treatment was given to these two animals for respiratory symptoms secondary to LSD. Two other animals showed mild symptoms consisting of fever for 4-6 days, few skin lesions and mild lymph node enlargement, while symptoms in the other two animals were limited to a transient fever. Fever appeared on day 4 or 5 in all animals. Skin lesions appeared on days 5, 6, 7 and 9 in those animals which developed lesions. All animals became serologically positive using the serum neutralisation test by days 12 to 18, and all remained serologically positive for the duration of the trial.

Virus in semen

The semen of all bulls tested positive for LSDV on one or more occasions using PCR. The time of shedding as determined by PCR is depicted in Table 1. Bull 1 tested positive on days 10 – 159 post infection with four negative samples out of 23 taken in that period, Bull 2 tested positive on days 8 – 132 with seven negative samples out of 22, Bulls 3 and 4 tested positive on day 6 post infection, Bull 5 tested positive on day 18, and Bull 6 tested positive on days 10 – 21. No false positives were encountered in the negative control samples on PCR.

Virus was only isolated in samples from the two severely affected bulls. Semen from Bull 1 tested positive on day 8 and 10 post infection. Virus was isolated from samples from Bull 2 taken on days 10, 14, 16 and 42 post infection. Virus could not be isolated from any other samples.

Clinical signs were present throughout the time that virus was recovered from semen samples in all animals. Fever preceded the first positive semen samples by 1 to 13 days. Once the fever had subsided, skin lesions persisted beyond the time that virus was present in semen samples. However, these lesions were inconspicuous, consisting of scars from active skin lesions or a limited number of skin nodules.

Virus concentration in semen

Virus was isolated from the positive semen sample diluted 1:1000. The $TCID_{50}$ could not be determined due to toxicity to tissue cultures at lower dilutions.

DISCUSSION

This work provides evidence of shedding of LSDV in semen of infected bulls for considerably longer than was previously shown. Shedding of virus in two of the six bulls persisted despite termination of the viraemic phase, seroconversion and clinical recovery from the disease. Previous to this study, the longest period for which virus could be found in any tissues of infected animals was 33 days in skin nodules(4). Persistent shedding of virus in the semen of immunocompetent bulls has been reported for other viral agents, namely bovine herpesvirus type 1(11), bovine virus diarrhoea virus (BVDV)(12,13), enzootic bovine leucosis(14), and possibly bluetongue virus(15,16). Whether the virus is associated with the sperm cells or the seminal plasma was not investigated. However, as several ejaculates from both of the severely affected bulls tested positive on PCR at a time that they were severely oligospermic it is evident that presence of the virus is not dependant on the presence of spermatozoa.

Whether the infectivity of the semen as determined in this study is likely to cause infection following mating or insemination of susceptible female animals cannot be determined. Carn and Kitching (1995) found that a viral inoculum of 10^2 $TCID_{50}$ was sufficient to induce generalised infection following intradermal inoculation(17). The exact $TCID_{50}$ in the semen of an infected bull could not be determined in this study, but the fact that the virus could be isolated at 1:1000 dilution indicates that a significant amount of virus was present in the semen. The effect of introduction of this amount of virus into the female tract requires investigation.

The inability to isolate the virus from large number of samples which were positive on PCR was noteworthy. One possible explanation for this discrepancy is that non-infectious virus or fragments of viral nucleic acid were present. However, the possibility that infective virus is present even when isolation on tissue cultures is negative cannot be ruled out. This was demonstrated by Givens et al. with BVDV. Using reverse transcription nested polymerase chain reaction (RT-nPCR), virus was found in semen for 209 days following experimental infection but could not be isolated from semen from day 17 post infection(13). By using a bioassay consisting of the inoculation of susceptible calves with raw semen, these authors demonstrated that infective virus was present in the semen despite the inability to isolate it.

The low sensitivity of virus isolation may be due to the cytotoxicity of semen to tissue cultures, which has been reported for bovine semen(18). Cytotoxicity was

observed in some samples in the present study. These cytotoxic effects necessitated dilution of samples, thus lowering virus titers and potentially causing false negative results. Observation of the cell cultures for toxic effects early after inoculation and washing where necessary was also employed to compensate for the toxicity of samples. Repeated passage is another way of compensating for cytotoxic effects(19). These samples were blind passaged another two times if no cytopathic effects were seen after 10 days on the primary tissue culture. However, it is possible that viable virus was still present in these samples at a titer lower than the detection threshold of the virus isolation system.

With the exception of one sample, we were unable to isolate the virus from the semen beyond the time of seroconversion. This is in agreement with the observations of Givens et al. with BVDV(13).

The site of persistence of virus in the genital tract of these bulls was not identified. Persistence of viruses in the testes may be facilitated by the immunologically privileged status which the seminiferous tubules enjoy, sheltered by the tight junctions between Sertoli cells and the basement membrane which together form the blood-testis barrier. The shedding via semen of BVDV for prolonged periods by seropositive immunocompetent bulls has been reported(12,13). While Voges et al. postulated that this situation arose as a result of infection before the formation of the blood-testis barrier and subsequent protection of the virus by this barrier, Givens et al. demonstrated that BVDV may persist in seminiferous tubules of bulls infected after puberty when the barrier is fully formed(13).

The lower temperature within the scrotum compared to the remainder of the body may also play a role in enhancing survival of the LSDV in this environment. Poxviruses may be temperature sensitive in culture, and the severity of some diseases caused by mammalian pox viruses is enhanced by low environmental temperatures(17).

This work demonstrated the excretion of LSDV in bovine semen for much longer than was previously thought, and beyond the time when obvious clinical signs of the disease were present. Further research is needed to investigate the nature of this persistent shedding and the ability to prevent it by vaccination and other biosecurity measures. Until further data regarding the effect of infected semen in the female genital tract become available, bulls which recover from clinical lumpy skin disease should be regarded as potential sources of the virus to susceptible animals for up to six months after the disease.

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Chapter 6

Sites of persistence of lumpy skin disease virus in the genital tract of experimentally infected bulls

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ABSTRACT

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

Two of the bulls developed severe lumpy skin disease (LSD) and were found to be shedding viral DNA in their semen on Day 28 p.i. Viral DNA was identified in all semen fractions from all bulls, but mostly from the cell-rich fraction and from the severely affected bulls. The PCR assay was positive on post mortem samples of testes and epididymides from the two severely affected bulls. Virus could be recovered from the testes of these two bulls and from the epididymis of one of them. Immunoperoxidase staining was observed in sections of testes and epididymides exhibiting necrosis.
This study suggests that the testis and epididymis are the sites of persistence of LSDV in bulls shedding virus in semen for prolonged periods and revealed that viral DNA is present in all fractions of the ejaculate.

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

INTRODUCTION

Lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*, causes an acute, subacute or chronic disease of cattle, characterized by fever and the formation of multiple firm, circumscribed nodules in the skin of affected animals and necrotic plaques in the mucous membranes, as well as generalized lymphadenopathy [1]. The disease has significant economic implications not only in subSaharan Africa and Egypt, where it is endemic, but also internationally [2,3]. It is therefore classified as a disease that is notifiable to the OIE [3]. Economic losses are incurred due to damage to hides, reduced feed intake, pneumonia, mastitis and infertility. Although mortality rarely exceeds 3%, it can be as high as 40% [1]. The deleterious effect that the disease has in affected bulls on their ability to mate and to produce fertile sperm is often not appreciated, but has important economic implications, especially in the context of subsistence farming and in farming systems where single sires are used. A recent review article [4] has identified the need for research into the epidemiology and transmission of lumpy skin disease (LSD) in South Africa due to its resurgence over the last decade of the 20th century.

The exact method of transmission of LSDV remains unknown but circumstantial evidence suggests that biting flies play a role as vectors [5]. Direct or fomite-mediated transmission of LSDV between animals is inefficient [6]. The potential for transmission via semen has serious implications, particularly for the international movement of bovine semen [11]. The presence of LSDV in semen can be detected by virus isolation (VI) and by polymerase chain reaction (PCR), and recently several methods of improving the diagnostic sensitivity of these methods have been published [7,8]. Although Weiss [9] stated that LSDV is shed in semen for 22 days after the fever reaction, Irons [10] isolated viable virus in semen 42 days after infection and identified viral DNA up to 5 months after infection. The risk of the presence of LSDV in semen poses a potential threat to movement of semen from countries where LSD is endemic [11]. There is currently no information on the ability of semen infected with LSDV to establish clinical disease in cows inseminated with it, nor is there any data available as to the pathobiological mechanisms involved in the shedding of virus and viral particles into the ejaculate.

The objectives of this work were to determine the site of virus persistence in bulls shedding virus in semen for a period longer than 28 days, to determine if the virus is present in all fractions of the semen, to study the development of lesions in the

genital tract and to compare such lesions with the presence of virus in different fractions of the semen.

MATERIALS AND METHODS

Animals and housing
Six healthy Dexter bulls, which had not been vaccinated for LSD, and with no detectable neutralizing serum antibodies to LSDV were selected. All bulls were reared in semi-extensive conditions on a commercial farm and were between 13 and 17 months of age. Semen containing motile spermatozoa was collected from them by electrical stimulation prior to their selection for the trial. They were housed in vector-protected stalls at the University of Pretoria's Biomedical Research Centre (UPBRC) for the duration of the experiment.

Experimental infection, observations and sampling
After an acclimatization period of 14 days, the bulls were artificially infected by intravenous injection of 2 ml of virus suspension. Virus was prepared by culture of a pathogenic field isolate of LSDV of known history (strain V248/93) on bovine dermis cell monolayers. After harvesting it, its infectivity was established by titration and adjusting the infective dose to a titre of 10^5 $TCID_{50}$/ml. From the day of infection (Day 0) the animals were observed daily for the development of clinical signs. Rectal temperatures were measured twice daily. A reproductive examination was performed every other day, which comprised measuring the scrotal circumference, palpation of the testes and epididymides, and an ultrasound examination of the testes and accessory sex glands using an Aloka SSD-500 ultrasound machine[*] and 5 MHz linear probe. A rigid 15 mm Perspex tube, cut longitudinally along its 40 cm length, was attached to the ultrasound probe and enabled evaluation of the accessory sex glands without introduction of the arm of the operator into the rectum of the animal. Blood samples were collected every other day for the duration of the trial and were subjected to virus isolation from Day 6 p.i. On Day 28, serum samples were collected and subjected to a serum neutralization test to confirm whether or not seroconversion had occurred.

After infection of the bulls preputial washes were performed every other day for the duration of the trial by infusing 50 ml phosphate buffered saline (PBS) into the preputial cavity and then massaging the preputium vigorously for one minute before collecting the samples. The vesicular glands were massaged per rectum

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prior to insertion of the probe of the electro-ejaculator and the fluid emanating from the preputial opening was collected. Semen was collected by electrical stimulation into new graduated collection tubes to which latex cones were attached. It was then centrifuged at 250*g* for 10 min to separate it into a cell-rich fraction and supernatant. Each bull was assigned a collection cone that was used only for that bull for the duration of the trial and the collection cones were washed, scrubbed and rinsed with distilled water between uses to prevent cross-contamination of samples. Unfractionated semen samples were collected on the last day of the trial and subjected to PCR to identify the bulls that were still shedding viral DNA in their semen at that stage. A PCR was done on the sheath washes, vesicular fluid, supernatant and cell-rich fractions of the semen from Day 10 p.i. to Day 26 p.i. Virus isolation was not performed on semen samples as it has previously been determined that this method has a low sensitivity for the detection of LSDV in semen [10].

Bulls that were positive by PCR on the whole semen sample collected on Day 28, were slaughtered and a diagnostic post mortem was performed. The genital tracts and tissue samples from them were taken and treated accordingly for histopathological evaluation, immunoperoxidase staining, transmission electron microscopy, virus isolation and PCR.

Polymerase chain reaction test

The PCR was performed using a QIAmp® extraction kit with commercially available primers for LSDV. The forward and reverse primers had the sequences 5'-TTTCCTGATTTTTCTTACTAT-3' and 5'-AAATTATATACGTAAATAAC-3' respectively, rendering an amplicon of 192 bp [12]. A positive control consisting of bovine semen spiked with LSDV and a negative bovine semen control as well as a water control were included in the PCR. Amplified products were analysed using a 100 bp DNA ladder as a molecular marker on 1.5% agarose gels. Amplicons were visualized using an UV transilluminator at a wavelength of 590 nm and positive reactions were confirmed according to size.

Virus isolation

Virus isolation was performed on heparinized blood samples from Day 6 p.i. and on tissue samples collected at necropsy. The cell cultures used comprised bovine dermis (BD) cells, maintained in minimum essential media (MEM) containing 4% foetal calf serum (FCS) and 1 ml gentamycin (0.1 mg/ml). Flasks were incubated at 37ºC in an atmosphere of 5% CO_2. Virus isolation from the blood samples was performed by inoculation of 0.5 ml from each sample into the cell cultures. The cell cultures were observed daily for cytopathic effects (CPE). After 14 days,

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

Histopathology and electron microscopy
Tissue sections for histopathology were prepared using routine procedures and were stained with haematoxylin and eosin (H&E). Immunohistochemistry, using immunoperoxidase staining, was performed on tissue sections prepared from parts of the genital tract that, on microscopic examination of H&E stained sections, revealed evidence of viral-induced damage. The 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.

RESULTS

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

inflammatory drugs and antimicrobials for pneumonia. Semen from these bulls was PCR positive for LSDV viral DNA on Day 28 and they were subsequently slaughtered.

Bull M1 and Bull M2 showed mild clinical signs of LSD consisting of fever for 4-7 days, a small number of skin lesions and mild enlargement of superficial lymph nodes, while infection in the remaining two bulls (I1 and I2) was inapparent with the exception of a transient fever. All the bulls were serologically positive at the end of the experiment as determined by the serum neutralization test.

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

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

Post mortem examination and histopathology
The macroscopic pathology of Bulls S1 and S2 was typical of that of severe LSD and included multiple nodular skin lesions, corneal opacities, generalized

lymphadenopathy and chronic-active interstitial pneumonia. Areas of fibrosis, necrosis and infarction could be visualized in the testes and epdidymides.

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

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

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

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

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

PCR Assay
The PCR amplified viral DNA in all of the fractions of the ejaculate from all the bulls at some stage p.i.. The cell-rich fractions of Bull S1 and Bull S2 were PCR

positive for all samples analyzed from day 10 p.i. Viral DNA could be identified in 66.7% (6/9) of sheath wash samples; 88.9% (8/9) of vesicular fluid samples; and 44.4% (4/9) of supernatant samples collected from Bull S1. This is compared with 55.6% (5/9) for all these samples collected from Bull S2 during the same time. The PCR assay was consistently positive when performed on the testicular tissue from Bulls S1 and S2 and epididymis of Bull S1, and gave a faint positive reaction after a second PCR was performed on dilutions of extracted DNA of the epididymis of Bull S2. Samples from the ampullae and vesicular and prostate glands of Bulls S1 and S2 were negative by PCR. Virus could not be recovered from tissue samples of the ampullae, vesicular glands and prostate glands of Bull S1 and Bull S2. Virus was only recovered from the testes of Bulls S1 and S2 and the epididymis of Bull S1, but not from the epididymis of Bull S2.

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

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

Note: * VI blood: Virus isolation on blood; SW Sheath wash; VF vesicular fluid; SN supernatant; CR cell rich; US ultrasound; A Ampullae; T Testes

[#] Semen collected on Day 28 p.i. was not fractionated

DISCUSSION

This work further confirms the phenomenon of persistent shedding of LSDV in the semen of infected bulls as described by Irons *et. al* [10]. Lumpy skin disease viral DNA was found in all fractions of semen and in sheath wash samples from the bulls that were shedding virus in the semen for protracted periods of time.

Evidence of the presence of viral DNA was found in tissue sections of the testes and epididymides. The scrotal circumference of all the bulls increased shortly after the onset of fever, reached a maximum 2 weeks p.i. and then declined progressively until the end of the trial. The scrotal circumference of Bull S1 was the same value as it was at the commencement of the experiment, while that of Bull S2 was 15% lower than at the start. The softness of the testes and the decreased scrotal circumference suggested severe testicular degeneration, which was confirmed at post mortem examination. Histopathological examination of the tissue specimens of the testes of Bulls S1 and S2 in this experiment revealed that necrosis was the main underlying pathological change. Although not commenting specifically on testicular changes, Prozesky and Barnard [13] concluded that vasculitis and thrombosis, leading to oedema and necrosis was central to the pathogenesis of the lesions in LSD. The initial increase in scrotal circumference can thus possibly be ascribed to an inflammatory oedema. The extent of testicular degeneration in Bull S2 was more severe than in Bull S1. The reason for this is unknown. It has been postulated that a possible explanation for different animal responses to challenge with LSDV is genetic resistance as determined by major histocompatibility complexes (MHC) found on cell surfaces of individuals [14].

The testicular and epididymal pathology reported here is more severe than that described by Nagi [15] as numerous seminiferous tubules were totally devoid of primary spermatogonia and extensive necrosis with obliteration of epididymal ducts could be demonstrated. Possible reasons for these differences could be the route of infection and/or the protracted illness that the bulls in the present study suffered from. The tissue specimens in the report by Nagi may have originated from animals with a generalized infection as well as from those that showed only localized skin and mucous membrane lesions. It has been shown experimentally that the first appearance and severity of such localized lesions is related to dose of virus that the animal received [16]. It is possible that the intravenous route of infection in the present study led to a higher generalized viral load, which had a greater effect on the spermatogonia. Spermatogenesis is determined by the amount of functional seminiferous tissue [17]. Severe pathology of the epididymal ducts as was present in these two bulls may also have resulted in permanent occlusion of

many of them, which would also have resulted in a decline in fertility. It is therefore likely that these bulls would have suffered from reduced sperm production if they had recovered from the infection, and not been slaughtered.

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

The results obtained in this experiment indicate that LSDV found in the semen of affected animals is not due to contamination of the semen with blood. The semen was positive by PCR after the time when virus was no longer detectable by virus isolation in the blood. Furthermore, semen samples were tested for the presence of blood and found to be free.

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

The demonstration of viral antigen by immunoperoxidase staining in necrotic testicular tissue is significant. A general characteristic of members of the *Poxviridae* is that they are relatively resistant to unfavourable conditions, and are not dependant on the presence of live cells for their survival; LSDV has, for example, been shown to survive in skin scabs for up to 33 days [8]. If virus can persist for this length of time in necrotic skin, it is likely that it survives for at least as long in necrotic testicular tissue. The voiding of such necrotic tissue into patent

seminiferous tubules, would explain the presence of LSDV into semen for prolonged periods and as no evidence was encountered of viral activity in other parts of the genital tract, this is deemed to be the most likely explanation for this phenomenon. Further studies are required to confirm or refute this possible phenomenon.

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Effect of lumpy skin disease virus in bull semen on *in vitro* embryo production

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ABSTRACT

This study investigated the risks associated with the use of semen infected with lumpy skin disease virus (LSDV) in *in vitro* fertilisation.

Six straws of frozen-thawed semen from uninfected bulls were spiked with LSDV and two straws from experimentally infected bulls shedding LSDV in their semen were subjected to swim-up and then tested for virus by PCR and virus isolation. The samples from infected bulls were negative, but the spiked samples all tested positive by PCR but not virus isolation after swim-up. Virions could be demonstrated by electron microscopy, of which 9% were attached to sperm cells.

Semen was spiked with virulent LSDV and used for the fertilisation of oocytes from abattoir ovaries. Control and treated groups consisted of 497 and 488 oocytes respectively. LSDV was detected by PCR at all steps of the embryo production system in the treated group, and in three control batches of 100 presumptive zygotes each at day two and five following fertilisation, but not day seven or ten. Virus was isolated from two batches of day ten embryos. There was no difference in cleavage rate at day five, day seven embryo development rate was higher and day ten hatching rate was lower in the treatment than the control group.

If oocytes are fertilised using infected semen, viable virus does persist and infected embryos are produced, but viral titers are low implying a low risk of infection of susceptible embryo recipients. Embryo development is negatively affected by the presence of virus once hatched from the zona pellucida.

Key words: Lumpy skin disease virus, *Capripoxvirus*, bull, semen

INTRODUCTION

Lumpy skin disease virus (LSDV) is a poxvirus of the genus *Capripoxvirus* which causes acute, subacute or inapparent disease in cattle of all ages and breeds. It is endemic in sub-Saharan Africa, Egypt and Madagascar(1). The disease is characterised by fever, skin nodules, necrotic plaques in mucosae and lymphadenopathy. It causes considerable economic losses due to emaciation, damage to hides, infertility in males and females, mastitis, loss of milk production and mortality, which rarely exceeds 3% but can be up to 40%(2). The morbidity in natural outbreaks is high, up to 100%(2). It ranks with foot and mouth disease as having the potential for rapid spread and severe economic losses(3).

Field observations and supporting evidence indicate that the disease is transmitted by biting flies, although transmission can occur in the absence of insect vectors as well(2). Viable virus has been reported to be excreted in semen for 22 days following the febrile response and 42 days following experimental infection(4,5). Viral DNA was detected in semen for much longer, up to day 159 in one instance(4). Although no data exists indicating transmission of the disease by infected semen, the movement of semen from countries where LSDV occurs represents a potential hazard(6,7). Characterisation of the effect on semen quality showed an initial drastic deterioration in semen quality followed by recovery before the end of the period of virus excretion(8). Virus is also excreted in semen in the absence of clinical signs of lumpy skin disease(5). This raises the possibility of the inadvertent use of infected semen for artificial insemination or *in vitro* embryo production. No studies have been done on the effect of use of such infected semen *in vivo* or *in vitro*.

In vitro embryo production is commonly used to produce bovine embryos. If semen infected with a pathogen is used for *in vitro* fertilisation (IVF), the embryo yield may be affected or the resulting embryos or culture media and rinsing fluids may be infected, posing a risk of spread of the disease(9). Embryos produced with semen contaminated with *Campylobacter fetus* subsp. *venerealis, Tritrichomonas foetus* and *Neospora caninum* develop normally and do not harbour infectious organisms after seven days of *in vitro* culture as long as the zona pellucida is intact(10,11,12). An extensive literature review concluded that embryos produced *in vitro* using semen infected with bovine virus diarrhea virus (BVDV), bovine herpesvirus-1 (BHV-1) and bluetongue virus are often contaminated, and that removal of viruses is less successful than with embryos produced *in vivo*(9). Despite this, transmission of these pathogens resulting in infection of recipients has never been demonstrated.

In addition to the potential for disease transmission, pathogens in IVF systems may affect the success of embryo production. The percentage of embryos undergoing fertilisation and developing to the blastocyst stage was lower when semen infected with BVDV or BHV-1 was used for fertilization than for a control group(13,14). Likewise, when semen contaminated with *Mycoplasma* spp. was used for fertilization, the cleavage, blastocyst and hatching rates were reduced and the organisms could be cultured from the resulting embryos, even after washing(15).

Semen is subjected to certain processing steps in preparation for use for IVF. The swim-up technique is a well-established step to obtain highly motile sperm samples, resulting in improved fertilisation rates. It involves taking a semen sample and overlaying it with a suitable medium, usually a tyrode-albumin-lactate-pyruvate (TALP) preparation, incubating it for a certain period, and then skimming off the upper layer of fluid. The swim-up procedure is capable of yielding a highly motile sperm sample from a relatively low yield of cells (16). Although swim-up processing is also utilised to remove or reduce pathogens(17), it failed to remove the infectivity of semen infected with BVDV(13) or *Mycoplasma* spp.(15).

There are no data on the use of bull semen infected with LSDV in IVP. The objectives of this work were to determine the virus status of naturally infected and spiked semen subjected to swim-up; to determine cleavage rate, blastocyst yield and hatching rate of embryos following use of infected semen for fertilisation of oocytes *in vitro*; and to determine the virus status of embryos produced after *in vitro* fertilisation using infected semen, and the virus status of culture fluids used in the process.

MATERIALS AND METHODS

The work was done in two experiments as follows:

Experiment 1: Semen processing

Frozen semen from two experimentally-infected bulls previously demonstrated by PCR to be excreting LSDV DNA in their semen at the time of freezing was used. Semen from another six uninfected bulls was used, with one straw being spiked with infectious LSDV and one straw acting as an uninfected control. All semen had been frozen in Triladyl cryoprotectant extender (Minitüb, Tiefenbach, Germany) in 0.25mL French straws.

Each straw was thawed in a water bath at 35 ºC. The contents of straws were spiked with an equal volume of tissue culture fluid containing 5 log $TCID_{50}$ of a virulent field isolate (V248/93) of LSDV. The sperm concentration and progressive motility of each semen sample was evaluated. Semen was then underlayed in 1mL of a FERT wash media consisting of 1mL of modified TALP medium including 100µL/mL gentamycin and 50µL/mL pyruvic acid (Sigma P3662, Sigma-Aldrich, Johannesburg, South Africa). Semen was incubated at 38.5 ºC for 1 hour after which the supernatant was removed and centrifuged for five minutes at 300 X g. The supernatant was discarded and the pellet was resuspended with 100µL TALP and evaluated for progressive motility and concentration. The semen was then submitted to PCR testing and virus isolation.

To rule out possible effects of cytotoxicity or inhibition of viral activity by components of the semen cryopreservation or swim-up media on the results, a series of ten fold dilutions of LSDV were made and each dilution was tested against the Triladyl cryoprotectant extender and modified TALP. One 96-well microtiter plate containing bovine dermis cell monolayers was used to test the modified TALP and another the Triladyl. Each micro titre plate was divided into 3 columns containing 8 rows each. The cytotoxicity of the media were tested in the first column using 800µL of media, 100µL of minimum essential medium (MEM) and 100µL water in each well. Inhibition of the media against the serial dilutions of LSDV in the second column using 800µL of media, 100µL virus and 100µL water. Cytopathic effect of the series of dilutions of the virus was controlled in the third column using 800µL of MEM containing 8% fetal calf serum, 100µL virus suspension and 100µL water.

To further investigate the relationship between sperm cells and virus particles formed during co-incubation and swim-up, transmission electron microscopy was done on the supernatant after swim-up. The sample was fixed in 2.5% glutaraldehyde in Millonig's buffer, followed by post-fixation in 1% osmium tetroxide in Millonig's buffer, washed in buffer and then dehydrated through a series of graded alcohols, infiltrated with a mixture of propylene oxide and an epoxy resin and finally embedded in absolute resin at 60 ºC. The cells were pelleted after each step by centrifuging at 3000rpm for 3 minutes. After curing overnight, ultra-thin sections were prepared and stained with lead citrate and uranyl acetate and examined in a Philips CM10 transmission electron microscope operated at 80kV. A count of virions in the section was done and virions were classified as being associated with a sperm cell, associated with other cellular material or proteinaceous debris, or free.

Experiment 2: Embryo production

A method known to be effective for large-scale production of bovine embryos was used for *in vitro* embryo production(18) with the exception of embryo culture where synthetic oviductal fluid (SOF) was substituted for TCM-bicarbonate and granulosa cell coculture(19). The method is described below.

Bovine ovaries were sourced from an abattoir during winter when the risk of exposure to virus is low. Ovaries were collected within 30 minutes of slaughter and stored and transported in a thermos flask containing PBS at 32 ºC. All visible follicles up to 8mm in diameter were aspirated using a 10mL syringe and an 18ga hypodermic needle. Follicular fluid was then collected into 15mL tissue culture tubes containing 2mL tissue culture medium containing TCM-HEPES (TCM-199 (Sigma M0650 Sigma-Aldrich, Johannesburg, South Africa) with 2.5mM HEPES (H0763, Sigma-Aldrich, Johannesburg, South Africa), 5mM bicarbonate, 0.2mM sodium pyruvate, 2.5mg/mL amphotericin B supplemented with 2% bovine serum (Danish Institute for Food and Veterinary Research, Denmark) and 25µg/mL gentamycin (Sigma G1264 Sigma-Aldrich, Johannesburg, South Africa) and 20µg/mL L-glutamine, pH 7.4, 280 mOsm). The tubes containing the aspiration media and follicular fluid were held in a water bath at 35 ºC.

The pooled aspiration fluid from a batch of approximately 100 ovaries were homogenised and an aliquot inoculated onto cell monolayers for virus isolation and subjected to DNA extraction and PCR to confirm the absence of LSDV. Oocytes arising from any batches found to be positive by either diagnostic method were removed from the trial.

Cumulus-oocyte complexes (COC's) were identified under a stereomicroscope and were selected on the basis of visual morphology. Only oocytes with at least one complete layer of cumulus cells present and homogenous appearing protoplasm were used for the trial. Oocytes suitable for *in vitro* maturation were alternately placed in test and control Petri dishes during searching out of aspirated fluid, resulting in a random contribution of each ovary to the oocyte group in each container and an equal contribution of each batch of ovaries to each group. The selected COC's were then washed for a second time in TCM-bicarbonate (TCM-199 medium containing 25mM sodium bicarbonate, 0.2mM sodium pyruvate, 0.4Mm L-glutamine, 50µg/mL gentamycin, adjusted to pH 7.4 and 280mOsm, supplemented with 15% bovine serum, 10 IU/mL eCG and 5 IU/mL hCG (P.G 600, Intervet SA, Isando South Africa) to remove any follicular debris.

Groups of 20 COC's were then transferred to small Petri dishes each containing five 60 µL drops of *in vitro* maturation fluid consisting of TCM-bicarbonate supplemented with 100µL/mL Suigonan (Intervet SA, Isando South Africa) overlayed with mineral oil. The COC's was then matured for 22 - 24 hours at 38.5 ^{o}C in 5% carbon dioxide with 100% humidity.

The semen used for IVF was that of the control bull used routinely in the IVF laboratory of the Section of Reproduction, Onderstepoort, Faculty of Veterinary Science, University of Pretoria. The semen met the minimum criteria set for frozen bovine semen and is known to result in adequate fertilisation and embryo development rates.

Six 0.25mL straws of semen were thawed in a water bath at 35-37 ºC for at least 20 seconds. The semen was pooled and then split into two groups and a swim-up performed as described above. After centrifugation and removal of the supernatant an equal volume of a suspension of tissue culture fluid containing 5 log $TCID_{50}$ of virulent virus grown on bovine dermis cell culture was added to the treated pellet and an equal volume of virus-free tissue culture fluid was added to the control pellet. These were then resuspended with a modified TALP medium supplemented with 30µg/mL heparin, 30µM penicillamine, 1µM hypotaurine, 1µM epinephrine. The semen was then incubated for 30 minutes to permit virus-semen interactions. Ten µL of the sperm-virus suspension contained approximately 10^5 spermatozoa and 8.3-9.9 x 10^2 $TCID_{50}$ of LSDV.

Of the 985 matured oocytes, 497 were designated as controls and 488 as the treated group. Treated and control matured oocytes were processed at the same time using identical media, equipment and operators. Strict segregation, removal of disposables and sterilisation of glassware were practiced between groups to prevent cross-contamination. The matured oocytes were the washed twice with modified TALP and then replaced into 60µL drops of modified TALP under mineral oil, each containing 20 oocytes. Each drop was inseminated with 10 µL of control or treated semen and incubated for 22-24 hours at 38.5 ^{o}C in 5% carbon dioxide with a 100% humidity.

Sperm and cumulus cells were stripped from the presumptive zygotes 22-24 hours after insemination of the drops by pipetting the oocytes several times. These stripped zygotes were then washed once through TCM-H and then through SOF modified for *in vitro* culture (SOF_{IVC} containing 107.7 mM NaCl, 7.16mM KCl, 1.19mM KH_2PO_4, 1.71mM $CaCl_2$, 0.49mM $MgCl_2$, 25.07Mm $NaHCO_3$, 3.3mM sodium lactate, 0.3Mm sodium pyruvate, 1mM glutamine, 1% BME essential

amino acids solutions, 1% MEM nonessential amino acids solution, adjusted to pH 7.4 and 280mOsm) supplemented with 5% steer serum. Embryos were placed in 60µL droplets SOF_{IVC} supplemented with 5% steer serum and 25µg/mL gentamycin under mineral oil, at 38 ^{o}C in 5% carbon dioxide, 5% oxygen and 74% nitrogen cultured in a bag with 100% humidity for culture.

All presumptive zygotes were cultured to hatching stage, with re-feeding of the culture drops consisting of replacement of the SOF media by means of adding and withdrawing 30µL of media three times. Re-feeding was done on day five, at which stage cleavage was evaluated to determine fertilization rate. Re-feeding and counting and grading of embryos were done on day seven. Embryos were graded again on day ten to record the hatching rate. Fisher's Exact test was used to test for differences in numbers undergoing cleavage, development to day seven, and hatching between control and treatment groups (GraphPad InStat, GraphPad Software Inc.).

Samples from both control and treatment groups from batches consisting of fluids from five droplets representing 100 oocytes/presumptive zygotes were submitted for PCR and virus isolation for LSDV at various stages. Fertilisation media after removal of the COC's, fluid from the second wash after fertilisation, day five culture media, day seven culture media, and day 10 culture media was submitted. At the end of the culture period pooled batches of 100 presumptive zygotes were tested for LSDV by PCR and virus isolation. PCR testing and virus isolation were done according to published methods(20,4).

An aliquot of pooled follicular fluids from each of the two batches of ovaries was also tested for BVDV using a RT-nPCR test for BVDV sRNA, as were aliquots of pooled day seven embryo culture media from each batch of the control and treated embryos. The primers for the RT-PCR are as follows: OPES 13A: 5'-GCT AGC CAT GCC CTT AGT AGG A-3', OPES 14A: 5' ATC AAC TCC ATG TGC CAT GTA CAG C-3'; and for the nested step: OPES 11: 5'-TGA GTA CAG GGT AGTY CGT CAG TGG TTC-3'; OPES 12A: 5'-GGC CTT TGC AGC ACC CTA TCA G-3'(21).

RESULTS

Experiment 1: Semen processing

The two samples from experimentally-infected bulls were both negative on PCR and virus isolation following swim-up, as were all six of the control samples. However, the six spiked samples all tested positive on PCR after swim-up, but no viable virus could be demonstrated in tissue cultures, even after a second blind passage.

Neither Triladyl nor modified TALP showed any evidence of cytotoxicity to the cell cultures nor was any viral inhibition noted.

Electron micrographs illustrate the association between sperm cells and viruses after co-incubation and swim-up processing. Nine percent of 239 virions were closely associated with the sperm plasma membrane either outside (Fig 1) or inside (Fig. 2) the sperm cell. The remainder were associated with other cellular material or proteinaceous debris (72%) or free (19%).

Figure 1

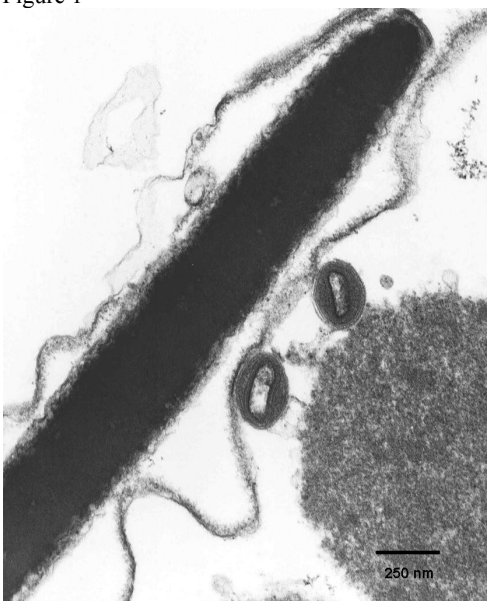

Figure 2

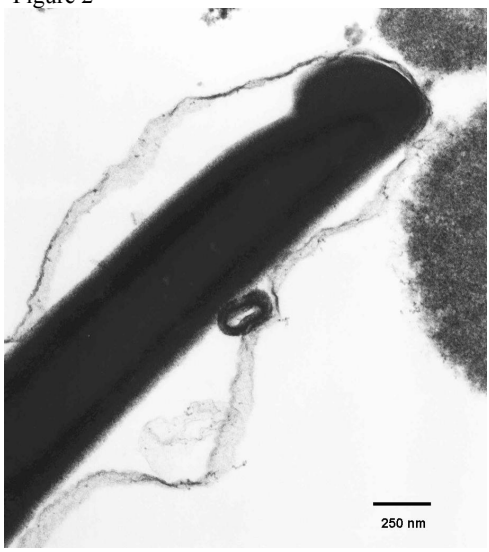

Figure 1. Virions in close association with the plasma membrane of a sperm cell
Figure 2. Virion between the plasma membrane and acrosome of a sperm cell

Experiment 2: Embryo production

Aspirated follicular fluid was found to be negative for LSDV by PCR. The samples were toxic to cell monolayers and virus isolation was therefore inconclusive.

Results of testing for lumpy skin disease virus is shown in Table 1. All batches of media from the treated group tested positive with the exception of one batch of IVF culture fluid and one of day seven refeeding fluid. Four out of five batches of treated embryos were positive on PCR.

Table 1. Results of PCR-testing for lumpy skin disease virus of media and embryos in batches of 100 cumulus-oocyte complexes fertilised with uninfected semen (control) and those fertilised using semen spiked with lumpy skin disease virus (treated)

	IVF culture fluid	2nd Wash fluid before IVC	Day 5 re-feed pool fluid	Day 7 re-feed pool fluid	Day 10 pooled embryos	Day 10 pooled IVC fluid
Control 1	-	-	-	-	-	-
Control 2	-	-	-	-	-	-
Control 3	-	± *	+	-	-	-
Control 4	-	±	+	-	-	-
Control 5	-	±	+	-	-	-
Control total	0	(3)	3	0	0	0
Treated 1	+ *	+	+	+	-	+
Treated 2	- *	+	+	+	+	+
Treated 3	+ *	+	+	+	+ *	+
Treated 4	+ *	+	+	+ *	+	+
Treated 5	+ *	+	+	-	+ *	+
Treated total	4 (5*)	5	5	4	4	5

*Samples also tested positive by virus isolation, all other samples were negative

Virus was isolated out of all five batches of IVF culture fluid from the treated group, one batch of day seven refeeding fluid and two batches of day ten embryos. Only one of these samples was negative on PCR testing.

Contamination of three batches of control presumptive zygotes with LSDV was detected at the start of culture at day two, when the PCR amplification product was barely discernible, and at day five when PCR was conclusively positive. This was confirmed by repeating the PCR and was deemed to be true contamination rather than a false positive result. These three batches were therefore included with the 'treated' group for the purposes of the following calculation of the day five cleavage rate. Culture of these three batches of presumptive zygotes was continued and the subsequent culture fluids and blastocysts were found to be negative for LSDV at days seven and ten, indicating that the virus had been washed off at the day five refeeding. These were therefore included in the 'control' group for the purposes of the following calculations. Virus was isolated from one of the control batches of control presumptive zygotes at day two.

Of the 187 control and 798 treated oocytes, 100 (53.5%) and 415 (52.0%) underwent cleavage ($p=0.75$). Of the 497 control and 488 treated presumptive zygotes, 146 (29.4%) and 173 (35.5%) developed to embryos by day 7 respectively ($p<0.05$). Of 146 and 173 embryos in the control and treated groups, 26 (17.8%) and 12 (6.9%) had hatched by day ten ($p<0.01$).

No BVDV was detected in follicular fluid or day seven embryo culture media by RT-nPCR.

DISCUSSION

Semen sanitising methods have been developed to enable the use of semen contaminated with pathogens. These methods, reviewed by Bielanski and Wrathall et al., include centrifugation through Percoll differential density gradients or Sephadex columns, and the use of trypsin washes and passage through density gradients incorporating trypsin(17,9). Swim-up and washing procedures have been relatively unsuccessful in removing viral pathogens from bovine semen, failing to remove BVDV, bovine leukaemia virus and bovine immunodeficiency virus(17). Complete removal of pathogens is unlikely for viruses such as bovine herpesvirus-1 which integrate into the sperm genome(17).

Swim-up and washing procedures may be more effective in removing viruses from 'spiked' samples than from samples with a true infection due to a closer association of the virus with the sperm cells in the case of the latter. We therefore attempted to obtain samples from bulls which were secreting virus in their semen following experimental infection in addition to spiked samples. Unfortunately the availability

of such samples is limited by the limited availability to suitable material and the fact that in many instances experimentally-infected bulls' semen quality had deteriorated by the time that virus appeared in semen, precluding successful cryopreservation of the semen. We were therefore only able to source samples from two out of six experimentally infected bulls which were frozen successfully at a time that the semen was positive for LSDV using a PCR test. Both of these samples had a satisfactory post-thaw sperm count and motility, demonstrating the potential for semen to be contaminated with LSDV while still meeting quality-control standards for commercial dissemination.

Both frozen-thawed semen samples from experimentally-infected bulls were negative for LSDV following swim-up despite the raw semen having been positive on PCR. This may be due to the dilution of semen during freezing and cannot be ascribed to the swim-up processing alone, although some reduction of virus load during swim-up cannot be discounted.

The discrepancy between PCR and virus isolation results after swim-up processing of spiked semen samples is noteworthy. Neither TALP nor Triladyl were found to inactivate LSDV, which, if it were the case, would explain the discrepancy. The discrepancy may be due to the differences in the detection limits of the two methods. Another possibility is that the virus is associated with the sperm cells in such a way that the virus is unable to infect tissue cultures although the DNA is detectable by PCR. The electron micrographs in this study reveal that many virions do adhere to and even enter the sperm cells, which explains why virus is detectable by PCR after swim-up. This association may however prevent them from causing cytopathic effects on cell cultures. However, many virions were not attached to sperm cells. These may be virions which were bound to sperm cells and released during centrifugation or other steps in the preparation of samples for electron microscopy, as occurs with bovine herpesvirus(22). Other viruses including herpesviruses, retroviruses and human hepatitis B virus are able to fuse with the sperm membrane and enter the nucleus(17). The adhesion of LSDV to spermatozoa increases the possibility of contamination of embryos resulting from IVF with infected semen. It cannot be assumed that viruses in naturally-infected semen will behave in the same way as spiked semen(9). Further investigation using semen from infected bulls is therefore required.

Lumpy skin virus in the semen used for IVF did not affect the cleavage and embryo development rates up to hatching. On the contrary, day seven embryo development rate was superior in the treated to that of the control group. In contrast, the hatching rate was lower in the treated than in the control group. Many

studies on pathogens in IVF systems conclude after seven or eight days of embryo culture and do not report hatching rates. Bielanski et al. found that *Tritrichomonas foetus* did not affect hatching rates at seven days(11). Mycoplasmas, on the other hand, were found to reduce cleavage, blastocyst and day seven hatching rates(15). The effect on hatching rates was particularly severe, which the authors speculated may affect post-transfer intrauterine development. A specific effect on hatching rates only as seen in this study is similar to findings with *Neospora caninum*, where collapse of the blastocysts and degeneration of trophoblastic cells were seen within three to five days of exposure of hatched blastocysts to tachyzoites(12). It also parallels the effect of bovine herpesvirus, which caused degeneration of hatched blastocysts(23).

The fact that LSDV was found in the later stages of embryo production suggests that either the virus adheres to the zona pellucida and is not removed during routine washing and refeeding or the initial titer of virus was high enough to still leave detectable amounts of virus all the way through despite the dilution of fluids at each step. The presence of virus permits normal embryo development while the zona pellucida is intact. The deleterious effect on embryos seen once the zona is opened may be due to cytopathic effects of the virus on the embryonic cells. The fact that virus could be isolated from two batches of embryos at day ten but from no batches of day ten culture fluid indicates that the virus is predominantly associated with the embryos, although not exclusively, as indicated by the detection of viral DNA in the culture fluids by PCR.

Based on the inability to isolate virus from most batches of embryos and all of embryo culture media we conclude that viable virus is present at low levels. This may imply a low risk of infectivity to recipients of embryos, but the risk of infection of susceptible recipients by introduction of LSDV into the uterus needs to be studied before firm conclusions can be drawn. The likelihood of embryos being infected is expected to be further reduced if infected semen is subjected to a swim-up before use, but this needs to be tested. The embryos produced from LSDV-infected semen in this study were not subjected to the IETS washing procedure. The ability of washing to cleanse LSDV from *in vitro*-produced embryos needs to be tested.

These results suggest a low risk attached to the use of LSDV-infected semen for *in vitro* embryo production, with no viable virus detected in semen following swim-up and no viable virus detected in most batches of embryos and embryo culture media and washing fluids after IVF with semen spiked with LSDV. The export of IVP embryos may therefore be a viable method of utilising the genetics of bulls

from LSD-endemic areas. Semen imported from LSD-endemic countries may also be safe for use in IVP systems providing that swim-up is incorporated into the semen preparation.

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Chapter 8

Absence of lumpy skin disease virus in semen of vaccinated bulls following vaccination and subsequent experimental infection.

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ABSTRACT

Twelve serologically negative bulls were used, six were vaccinated with a modified live LSD vaccine and six unvaccinated. All were then experimentally infected with a virulent field strain of LSDV. No clinical abnormality was detected following vaccination, and mild clinical signs were seen in four vaccinated bulls following challenge. Virus was not found in semen of vaccinated bulls. Two of the unvaccinated bulls developed severe LSD and four showed mild symptoms, all excreted the virus in the semen following challenge. This study confirmed the ability of LSD vaccination to prevent the excretion of LSDV in semen of vaccinated bulls.

Key words: Lumpy skin disease virus; Neethling strain vaccine; Semen

INTRODUCTION

The widespread use of artificial insemination in cattle production has facilitated the exchange of genetic material at the national and international level and assisted with the control of sexually transmitted diseases.

Lumpy skin disease (LSD) is caused by a virus in the genus *Capripoxvirus* of the family *Poxviridae*. It is an acute, subacute or inapparent disease in cattle and affects all ages and breeds [8,15]. The disease is characterized by pyrexia, generalized skin and internal pox lesions which can be seen as firm eruptions of circumscribed nodules in the skin, and generalised lymphadenopathy [8,15,19. The disease causes significant economic loss due to hide damage, loss of milk production, mastitis, infertility and death [26]. The morbidity rate in natural occurrence varies from 3-85%. About 40-50% of experimentally infected animals exhibit the clinical signs, and mortality is usually less that 3% [2].

Lumpy skin disease is primarily transmitted by biting insects, particularly blood feeding insects such as the mosquito [5]. Contact transmission between animals may occur at low rate but cannot be considered to play a significant role in transmission during epizootics [2,5]. Lumpy skin disease virus is excreted in the semen of susceptible bulls following experimental infection [14, 26]. This is a potential risk in the movement of semen from countries where LSD occurs to countries free of the disease, although there is no evidence to suggest transmission via infected semen.

The disease was considered a “List A” disease by the Office International des Epizooties (OIE) due to its potential for rapid spread and ability to cause severe economic losses. It is a constraint in international trade of live animals and their products.

The most widely used and viable means of controlling the disease in endemic countries is by vaccination [12]. The Neethling strain of LSDV, which is a live strain of *Capripoxvirus,* has been used successfully in a vaccine for the control of LSD in southern Africa [12, 26]. This vaccine is developed by attenuation of a field isolate in tissue culture and on the chorioallantoic membranes of embryonated hens’s egg [26]. According to the manufacturers, immunity to LSD starts developing 10 days after vaccination and reaches its peak after 21days. However, the vaccine does not necessarily confer absolute immunity to all animals vaccinated [12].

Although immunity to LSD is mainly cell-mediated, the production of antibodies is a useful indicator of the response to vaccination [12, 16, 26]. However, literature has shown the absence of detectable levels of antibodies after vaccination in some animals which were nevertheless immune to LSD when challenged [26].

The possibility of the vaccine virus being excreted in the semen following vaccination cannot be ruled out due to the fact that the vaccine virus replicates in the body of the animal. Shedding of vaccine virus in semen has been reported using a live attenuated PRRS -vaccine in boars [23].

The first objective of this study was to determine whether LSD vaccine virus is excreted in semen following vaccination with the modified live vaccine. The second objective was to determine the efficacy of vaccination in preventing LSDV excretion in semen of experimentally infected vaccinated bulls. Furthermore, the study aims at incorporating vaccination into biosecurity protocols for the export of bull semen from infected countries.

MATERIALS AND METHODS

Animals and housing
Twelve unvaccinated postpubertal Dexter bulls were acquired from herds where vaccination against lumpy skin disease is not practiced. All bulls were tested to be seronegative using the serum virus neutralizing test (SNT). They were also tested for their ability to produce semen before purchase, and again before the onset of the experiment. The bulls were housed in groups of three in isolated pens within an insect proof house with concrete floor covered with bedding. The bulls were provided with forage and water *ad-libitum*. All bulls were between 11 and 16 months of age. Ejaculation was induced in all bulls by electrical stimulation. The experimental trial was divided into 3 periods i.e. periods of acclimatization, vaccination and challenge.

Vaccine and vaccination procedure
Six bulls were vaccinated using the attenuated Neethling vaccine (Lumpy skin disease vaccine for Cattle, Onderstepoort Biological Products Ltd., Onderstepoort, Pretoria, Republic of South Africa) after a 2-3 week acclimatization period. The vaccine was reconstituted and a 5 ml dose was administered subcutaneously to each bull according to the manufacturer's instructions. Three weeks after the first vaccination, none of the bulls had detectable antibody titers, causing us to consider

the possibility of vaccine failure. The animals were therefore vaccinated again on day 21 with a different batch of vaccine to ensure effective vaccination.

Challenge-virus and procedure
Twenty seven days after the second vaccination, the six vaccinated and six unvaccinated bulls were experimentally infected. The experimental material used for inoculation was a virulent South African field isolate strain V248/93 of LSDV., The virus was prepared by 3 times passaged culture on bovine dermis cell monolayers, with an infective dose titre of 10^5 $TCID_{50}$ ml^{-1}. All bulls were inoculated intravenously with 2 ml of this virus suspension.

Clinical observation
The bulls were regularly examined throughout the trial. Clinical examination and rectal temperature was done daily during the period of acclimatization and vaccination and twice daily during the period of challenge. Clinical parameters observed and recorded were the general health of the bulls, superficial inguinal lymph nodes, left and right prescapular lymph nodes, rectal temperature, skin lesions and scrotal circumference. A febrile response was defined as a rise in rectal temperature above 39.5°C.

Blood sampling and semen collection
During the vaccination period, blood samples were collected twice a week and tested using the SNT. In the challenge period, blood samples were collected every other day and tested using virus isolation (VI) and SNT.

Semen samples were collected by electro-ejaculation twice a week during the vaccination period and every second day during the challenge period. Samples were tested for LSDV using PCR.

All ejaculates were collected into newly graduated sample tubes using collection funnels. Cross-contamination between animals was avoided by using different funnels for each animal for collection of all samples. Between collections, funnels were washed thoroughly with clean water, and rinsed with hot distilled water. Semen samples were frozen at –20 ^oC until PCRs were done.

Serum neutralization test
The SNT was done by using a 96-well, flat bottomed cell culture microtitre plates. The test sera were diluted to 1:5 in a minimum essential medium (MEM) containing 5% foetal calf serum and 0.05 ml gentamycin (stock 50 mg/ml) and inactivated at 56 ^oC for 30 minutes. A series of two-fold dilutions of the inactivated test serum was prepared and 100 µl of the serum was added in duplicate to each of

the wells. The titre of the LSDV used was determined and 100 µl of a 100 $TCID_{50}$ was then added to each of the wells.

As cell control, 200 µl of MEM was added to 12 wells. For virus control, three, ten-fold dilutions of antigen (100 $TCID_{50}$) were made and 100 µl of each dilution was added to 100 µl of MEM in each well. The microtitre plate was incubated at 37 ^{o}C for 1 hour. Following incubation, 80 µl of bovine dermis cells at a concentration of 480 000 cells/ml were added to all the wells.

The microtitre plates were further incubated at 37 ^{o}C in an incubator containing 5% CO_2. Using an inverted microscope, the monolayers were examined daily for seven days for evidence of cytopathic effect (CPE). The cell control indicated how long the cells remained viable and for how long it was possible to read the test before cell degeneration. The absence of CPE was an indication of neutralization and therefore a positive antibody reaction.

Virus isolation
Bovine dermis cells at ± 50% confluence grown in 25 cm^2 culture flasks were infected with 0.5 ml heparinized blood. After 24 hours the medium was removed and the cells were washed twice with buffered phosphate saline containing Mg^{2+} and Ca^{2+} (PBS+) containing 0.05 ml gentamycin (stock 50 mg/ml). The medium was replaced with MEM containing 5% foetal calf serum and gentamycin (stock 50 mg/ml). The cell cultures were observed daily for CPE. After 14 days, negative cultures were frozen briefly at –70 ^{o}C and thawed. The flasks were shaken gently to break up the cell material and to release the cell-bound virus. A second passage was done and observed for 14 days. Positive cell cultures were stored at -70 ^{o}C.

PCR
The extraction method used was a modification from the method described by Gubbels *et al.,* (1999) and Schwartz *et al.,* (1997). The semen samples were tested by the PCR using primers developed from the gene for viral attachment protein (Ireland and Binepal, 1998), and had a forward primer of 5'-d TTTCCTGATTTTTCTTACTAT 3' and reverse primer: 5'-d AAATTATATACGTAAATAAC 3'.The size of the amplicon was 192bp.

A positive control of bovine semen spiked with LSDV was used; negative semen controls consisting of bovine semen as well as a water control were included in the PCR. Amplified products were analyzed on 1.5% agarose gels using a 100 bp DNA ladder (Whitehead Scientific Ltd.) as a molecular marker. Amplicons were visualized using an UV transilluminator at a wavelength of 300 nm and positive

amplicons were indicated by the presence of bands corresponding to those of the positive control sample.

RESULTS

Clinical signs

Unvaccinated bulls
Two of the unvaccinated bulls developed severe LSD and four showed mild to inapparent infections. In the severely affected group fever started on day 6 and 7 post infection (p.i). and lasted for 12 and 16 days. Multiple skin lesions developed 5 days later and corneal opacity was observed 2 to 3 weeks after the appearance of skin lesions. These bulls were febrile for 9 days and became depressed and inappetant. Symptoms in the other bulls were limited to transient febrile reactions and few skin lesions which did not persist.

Vaccinated bulls
No clinical abnormalities were detected following vaccination, and clinical signs were limited to mild lymph node enlargement in four bulls following challenge of the vaccinated bulls.

Serum neutralization test

Vaccinated bulls
Three of the vaccinated bulls were serologically positive by day 48 post vaccination (p.v) and four bulls by the end of the trial. One animal was found to be serologically negative at the end of the trial having been positive at day 48 p.v. Antibody titers were low following both vaccination and challenge, with maximum titers of 1:3 and 1:6 respectively.

Viraemia

Unvaccinated bulls
Virus was isolated from heparinized blood samples from both of the severely affected bulls on multiple occasions, in one seven times during days 9- 21 p.i. and in the other eight times on days 9- 23 p.i., these being two and one days after the onset of the fever reaction respectively and a day after the appearance of skin lesions. In the mildly affected group, virus was not isolated from one bull but was

isolated from the three other bulls on days 15, 17 and 19 p.i., days 11, 15 and 25 and from one other bull on day 7 only (Table 1).

Table 1. Viraemia following experimental infection of unvaccinated bulls, as determined by virus isolation.

Clinical Signs		Days Post Infection											
	Bulls	7	9	11	13	15	17	19	21	23	25	27	29
Severe	D	-	+	+	+	+	+	+	+	-	-	-	-
	E	-	+	+	+	+	+	+	+	+	-	-	-
Mild	A	-	-	-	-	+	+	+	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-
	C	+	-	-	-	-	-	-	-	-	-	-	-
	F	-	-	+	-	+	-	-	-	-	+	-	-

(-) = negative for virus isolation, (+) = positive for virus isolation.

Vaccinated bulls
None of the vaccinated bulls were found to be viraemic after vaccination or experimental inoculation.

Virus in semen

Unvaccinated bulls
The semen of all bulls except one with an inapparent infection tested positive on one or more occasions using the PCR. The presence of virus in semen is depicted in Table 2. Viral nucleic acid was detected in the severely affected bulls from day 10 p.i. until the end of the trial on day 28 p.i.. The onset of the shedding of virus in semen corresponded with the day when the peak of the febrile response.

One semen sample from one of the mildly affected animals tested positive on day 27 p.i. and this bull was not found to be viraemic at any stage. The other mildly affected bull tested positive on four occasions between days 12 and 27 p.i. while two other bulls tested positive once each, on days 16 and 18 p.i. (Table 2).

Table 2. Presence of LSDV in semen following experimental infection, as determined by the PCR.

Clinical Signs		Days Post Infection									
	Bulls	10	12	14	16	18	22	24	26	27	28
Severe	D	+	+	+	+	+	+	+	+	+	+
	E	+	+	+	+	+	+	+	+	+	+
Mild	A	-	-	-	-	+	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	+	-
	C	-	+	+	-	-	-	+	-	+	-
	F	-	-	-	+	-	-	-	-	-	-

(-) = negative for PCR, (+) = positive for PCR.

Vaccinated bulls
Viral nucleic acid was not detected in the semen samples of the vaccinated bulls on any day following vaccination or experimental infection.

DISCUSSION

The duration of viraemia among the unvaccinated bulls varied considerably and was similar to a recent study [25] where virus was isolated within a day of the onset of fever. No vaccinated bulls were found to be viraemic after experimental infection with the same strain of LSDV used in the unvaccinated bulls. It is therefore evident that vaccination did prevent viraemia.

In this study, three of the six vaccinated bulls seroconverted by the end of the vaccination period and four bulls by the end of the trial, with a rise in antibody titre in four bulls. The very low level of antibody formation found in this study is characteristic of the response of LSDV [3, 16]. The data suggest that high antibody titres were not necessary to prevent or reduce the manifestation or severity of the clinical signs of the disease in the bulls when experimentally infected. This is thought to be due to the fact that immunity to LSD infection is predominantly cell mediated [3, 17]. Tuppurainen et al. [25] also demonstrated that the level of antibody titre is proportional with the severity of clinical disease observed in experimentally infected bulls.

In this study, the failure of any of the six bulls to seroconvert by day 21 p.v. was unexpected as the manufacturer's report indicated that immunity starts developing about ten days after vaccination and should be fully developed after 3 weeks [12]. This prompted us to consider the possibility of vaccine failure in this instance, a phenomenon which is well documented [12,20]. For this reason we decided to repeat the vaccination using a different batch of vaccine in an attempt to ensure effective protection, a deviation from our original study design. Whether different results would have been obtained had the vaccination not been repeated cannot be determined.

Previous work by this group indicated that testing for the presence of LSDV in bull semen by PCR is much more sensitive than virus isolation, due largely to the toxicity of bovine semen to tissue cultures[14, 25]. While centrifugation and serial dilution of samples reduce the toxicity and do allow for observation of cytopathic effects [1], the sensitivity of the test is reduced by these methods. Due to the improbability of identifying additional infected samples using virus isolation and the labour and cost implications of doing virus isolation on large numbers of samples it was decided to use PCR in this study. While it is not possible to infer the presence of infective LSDV in semen from these results, no superior diagnostic method is currently available to achieve this goal. The bioassay used by Givens et al to demonstrate the presence of infective bovine virus diarrhea virus in semen utilising live susceptible calves [10] was not feasible for this study due to the difficulty in sourcing known susceptible animals and the cost implications of sourcing and housing calves.

In this study, all six experimentally unvaccinated bulls excreted the virus in their semen at one point during the course of the trial. This is in agreement with the observation of Irons *et al.* [14]. No viral nucleic acid was detected by PCR in the vaccinated group at any point till the trial was terminated. Since the pathogenesis of seminal shedding of LSDV is not known, it is not known how vaccination prevents shedding. The skin nodules which are characteristic of LSD contain a very high concentration of the virus [6]. As nodules often develop within the genital tract in severely affected animals it is possible that LSDV is released from nodules within the genital tract into the semen during passage through the testes or epididymal ducts. However, the fact that there was transient shedding even in bulls that did not develop any clinical signs following challenge suggests that there are other mechanisms of voiding of virus in semen.

Shedding of vaccine virus in semen has been shown to occur in boars vaccinated against PRRS [23] but not in boars vaccinated against Pseudorabies [4].

Vaccination was found to reduce seminal shedding of PRRS virus in boars [23] and EAV in stallions after challenge [24].

A possible explanation for the presence or absence of virus in the semen samples could arise from semen being contaminated with preputial secretions at the time of collection, or cross-contamination between samples from different bulls in subsequent collections. The semen collection technique in the present study was aimed at minimizing contamination of semen samples by trimming the preputial hairs and cleaning with clean water prior to collection. Also, different funnels were used for each animal, and between collections, these funnels were washed thoroughly with clean water and later rinsed with hot distilled water and kept in a rack to dry. Contamination of semen samples with blood during sampling has been implicated in the presence of BLV in semen [7]. No semen samples in the present study apart from samples from two unvaccinated bulls on days 13, 17 and 19 p.i were visibly contaminated with blood, but the presence of small amounts of blood in other samples cannot be ruled out.

Comparison with results obtained from the experimentally infected vaccinated bulls and those from the non-vaccinated bulls showed that vaccination prevented the excretion of LSDV in the semen of experimentally infected vaccinated bulls. Transient shedding of virus in semen of the vaccinated bulls from days out of the peak periods chosen cannot be excluded but is deemed highly unlikely. Should vaccinated animals experience severe clinical signs following infection as may likely occur [12], the possibility of seminal shedding cannot be ruled out.

The results of this study provide preliminary data on the abilities of the Neethling strain vaccine to prevent seminal shedding of LSDV when challenged. This study supports the inclusion of LSD vaccination with this vaccine as a biosecurity measure when semen is moved from LSD infected areas. It also illustrates further, based on these findings that double vaccination of clinically normal bulls against LSD would prevent the possible presence of LSDV in semen collected for export to LSD-free countries during the period immediately following the vaccination. Bulls should nevertheless be monitored for clinical signs of LSD while semen is being collected for export as an additional safeguard.

This study provides the first evidence of the absence of LSDV in bull semen following vaccination. It also illustrates the ability of LSD vaccination to prevent the excretion of LSD viral particles in semen when vaccinated bulls are experimentally infected.

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Summarising discussion

Breeding soundness evaluations of bulls are conducted to enable owners and bull buyers to make appropriate bull selection and management decisions. A systematic evaluation of the bull and a representative semen sample can identify potentially subfertile or infertile animals and thus reduce the risk of unsatisfactory breeding results. Such a breeding soundness evaluation (BSE) should be simple, repeatable and unambiguous. Identification of carriers of infectious agents is part of the evaluation procedure. The approach to bull breeding soundness evaluations described in Chapter 2 was adopted as the industry standard in southern Africa and has proven to be a robust means of screening bulls. The standard set here is higher than either the Australian or the Society for Theriogenology (SFT) models, which were used as points of comparison.

The classification of bulls according to the proposed use of the bull is a unique aspect of this standard. This is in line with the reasoning that bulls to be used as semen donors and those high-value animals which are insured should be subjected to more stringent requirements. This aspect is touched on in the Australian standard, where a top rating in both categories of semen quality tested imply that the bull's semen has a high probability of freezing successfully. As the use of breeding soundness certificates to add value to an animal at the point of sale is considered a positive selling point, the philosophy of the certificate is one of quality assurance. For this reason it was decided that only animals which satisfy the full set of criteria should be certified, which is a departure from the Society for Theriogenology and Australian forms, where the client receives a breeding soundness evaluation report indicating parameters tested and outcomes, and, in the case of the SFT form, the final classification of the bull in the bottom corner.

The criteria for semen quality are more stringent than those of either of the other two standards. The SFT criteria for motility are that an individual motility of 30% is acceptable. On the other hand, the Australian model makes the election of which tests to apply the prerogative of the client, and indicates that a large proportion of users of the report do not elect to have sperm morphology evaluated(1). This is regarded as a serious deficiency, as poor sperm morphology is a common cause of failure of a breeding soundness examination(2) and morphology is consistently related to fertility(3). Our model goes beyond either of these by requiring a full semen evaluation in all cases, setting the criteria for all parameters at realistic levels based on available research, and including a requirement for a semen smear to be examined for the presence of foreign cells, which neither of the others require.

Scrotal circumference is regarded as an important characteristic in bull breeding soundness due to its' relationship with sperm production and quality and with the age of onset of puberty (4). The Australian system of evaluation does not specify criteria for this parameter, stating that those of the relevant breed society should be utilised. In the southern African context this would not by inadequate as not all breed societies have standards and the standards are regarded as too low in some cases.

Further research into reliable methods of predicting bull fertility is required. The procedure described in this work is exacting and time-consuming and not comprehensive enough to detect some causes of reduced fertility. While no single test is ever likely to be adequate given the complexity of the processes involved in sperm delivery and fertilisation, ancillary tests which increase the predictive value of a breeding soundness examination are required.

The prevention of introduction of infectious diseases into a herd by means of bull introductions is an important aspect of the breeding soundness evaluation. The question of which diseases to include in such a screening process is often debated, given the range of diseases which a bull may carry. While testing for bovine leukaemia virus (BLV) was previously included, the lack of evidence of transmission through semen, the high incidence of the disease in dairy herds and the lack of associated disease in most herds led to requests to remove it. On the other hand, the increasing awareness of the role of the persistently-infected carrier of bovine virus diarrhoea virus led to the inclusion of testing for bovine virus diarrhoea virus (BVDV) antigen on either blood or skin samples from bulls tested for breeding soundness. Bovine brucellosis and tuberculosis continue to be important controlled diseases and are therefore included by default. The venereal diseases trichomonosis and bovine genital campylobacteriosis continue to be important causes of infertility in southern Africa. As such, the confirmation of the negative status of bulls is an integral part of ensuring the reproductive success of breeding herds in which the bulls are used. Unfortunately, diagnostics for these diseases is problematic, with test sensitivity of as low as 70% in many instances. Sample contamination is a common problem, leading to bacterial or fungal overgrowth of cultures. The organisms are also fastidious and require rapid transport to the laboratory or the use of enriched transport media. The research described in this thesis is an attempt to provide solutions to the problems of the low sensitivity and specificity of culture-based diagnostics. Sample collection methods and transport conditions for preputial samples for testing by both traditional culture methods and a modern molecular assay were evaluated in an attempt to improve the reliability of laboratory diagnostics.

In Chapter 3 the diagnostic sensitivity of culture-based diagnosis for Trichomonosis was shown to be very high (97%) when bulls were sampled under ideal conditions but somewhat lower (83%) when bulls were sampled in the field. This is consistent with published findings in general, and illustrates the practical difficulties in obtaining good quality samples under field conditions and getting them to the laboratory in good time. It was not surprising to find that the diagnostic accuracy of the two methods for collection of preputial samples did not differ, as this is consistent with the findings of other groups. However, the practical advantages of preputial scraping over washing makes this the method of choice, and since this research and the series of training courses presented over recent years this method has gained in popularity amongst southern African practitioners, some of whom favour a custom-made collection apparatus while others use the dry pipette method as described in this work. The use of disposable sample-collection apparatus is regarded as being particularly advantageous in the light of the increased use of highly sensitive molecular assays where sample contamination is a real problem.

What was surprising was the degree to which the operator affected the outcome. Despite the fact that the trial was not designed to test differences between operators and the numbers are low, a significant effect was noted. As sheath scraping causes discomfort to the animal, operators who do not apply sufficient restraint will be likely to spend less time obtaining samples, resulting in a less representative sample. Sheath-washing has been the method of choice for many years in southern Africa and training of veterinarians in this method has been of a consistently high standard. This points out the necessity of thorough training of operators to ensure a sample of consistently high quality. This action has been embarked upon by the author and a colleague and a number of training courses have been presented for practitioners to date.

The results of the investigation into sample-taking and –handling for PCR testing for trichomonosis reported in Chapter 4 were somewhat surprising, given that the conventional wisdom is that PCR testing has a high sensitivity and detects infections for longer than culture when testing is delayed as it does not require live organisms. The work reported compared detection of trichomonosis on preputial samples by PCR to that of culture for the infectious agent, and found the sensitivity of the two test methods to be the same with prompt testing. It was hoped that PCR testing would have a sufficiently high sensitivity to enable classification of an animal on a single test, which was not the case. The sensitivity of the PCR assay was inferior to that reported elsewhere using other PCR primers or sample extraction methods (5,6). The loss of nucleic acid during the extraction process

employed in this work was a likely explanation for this relatively low sensitivity. The reason for the additional loss of sensitivity with time despite the addition of the guanidinium thiocyanate, which inactivates nucleases, binds DNA and preserves its helical structure, is not known. Possibilities are continued degradation of DNA, accumulation of inhibitory compounds or contamination of the samples. A PCR-assay using preputial fluid without a DNA-extraction step and with samples held in normal saline did not suffer from a similar decline in sensitivity with time(7). This indicates that the loss of assay sensitivity in our work may be related to the loss of nucleic acid during DNA extraction.

The high specificity of the PCR test in diagnosing *T. foetus* is encouraging, given the potential for mis-identification of other organisms found in preputial samples. Nothing in this work suggests that such an error occurred in either samples from the test bulls or field samples. The test can therefore be advocated as a confirmatory follow-up test to culture-based diagnostics as employed by other researchers(8,5).

The value of having results within 24 hours of receiving samples, as is possible with the PCR test, should not be underestimated. With the culture-based test, the first test of the required three must be done three weeks before the certification is required in order to allow for the required week between tests and the week required to complete the last test. With PCR, this can be reduced by 6 days. There are many other situations where the rapid completion of the test is a significant advantage. Based on this work the following procedure is recommended for certification of bulls as free of trichomonosis: Three tests should be done at weekly intervals by the preputial scraping method. Samples should be submitted in buffered saline to reach the laboratory within 24 hours. If positive by culture, confirmation using PCR should be done to rule out false positives.

Further research is required in the field of trichomonosis diagnostics. A singly highly reliable test still needs to be developed, which is robust enough to maintain reliability despite variations in sample quality. PCR-based tests show the most promise at this stage.

Lumpy skin disease virus is endemic to Africa. It occurs wherever cattle are kept and animals generally have a degree of immunity. It is therefore not a disease which may be meaningfully included in breeding soundness standards with a view to preventing spread of the disease. Bulls showing clinical signs of the disease would not be considered to be breeding sound. Those who have had a severe bout of clinical disease in the preceding months would have poor semen quality and

therefore not be considered sound. The potential for disease transmission is therefore limited to those cases where semen is to be exported to countries where the disease does not occur and where semen is frozen at a time that the bull has an inapparent infection and virus is present in the semen.

The data presented in Chapter 5 and 6 representing experimental challenge of 12 susceptible bulls with virulent virus demonstrates a number of instances in which bulls produce semen of good quality which is infected with lumpy skin disease virus. This occurs in bulls with mild or inapparent infection, bulls with severe lumpy skin disease before the drop in semen quality, and bulls with clinical lumpy skin disease after the recovery of semen quality. In the latter two instances one would expect that the animal's condition would be apparent to the collector and therefore semen would not be collected and frozen, but in the first instance this is not the case, resulting in the potential for virus to be found in semen. The semen from bulls which have recovered from severe dysspermatogenesis as a result of clinical lumpy skin disease may also not freeze successfully (R de la Rey, personal communication).

The majority of the testing of semen for the presence of lumpy skin disease virus was done using a PCR test, which we showed in Chapter 5 to be of superior sensitivity to virus isolation. This has prompted the question whether viable virus was present in these samples. This was discussed in Chapter 5, but the rationale will be briefly repeated here. The sensitivity of virus isolation from semen samples is low, even when highly sensitive methods are used(9). Using a biological test consisting of inoculation of susceptible calves with raw semen these authors demonstrated that semen samples were infected with viable bovine viral diarrhoea virus for prolonged periods although they were unable to isolate the virus. This occurred during the period when the semen was positive on PCR, suggesting that the PCR test was detecting the presence of viable virus. Likewise, Kupferschmeid *et al*. reported using the same 'modified Cornell semen test' to show that imported semen was infected with bovine herpesvirus-1 despite the inability to isolate the virus in cell culture(10). A bioassay consisting of the inoculation of piglets with semen and testing for seroconversion is also used to test for the presence of viruses in boar semen(11,12). We did not have the facilities to perform bioassays to detect the presence of virus in semen and for that reason used PCR as the most sensitive test at our disposal, in the knowledge that this does not provide proof of the presence of viable virus.

In our work, where semen was tested for the presence of lumpy skin disease virus using tissue cultures semen was found to be toxic to the bovine dermis cells, which

made the detection of virus impossible. The semen therefore had to be diluted to prevent this, which unavoidably reduced the sensitivity of the virus isolation. Other possible methods to reduce toxicity to tissue cultures include centrifugation, filtration and the addition of neutralising agents such as kaolin. A trial was done to investigate these options, and dilution was found to result in the optimal balance between reduction in toxicity and maintenance of cytopathic effects(13). This work was reported elsewhere using the samples generated for Chapters 6 and 8 and is not included in this work.

Viraemia was not an accurate predictor for the development of persistent shedding as one bull with inapparent infection and transient shedding of virus in semen had a viraemia of similar duration to that of the animals which developed persistent shedding. Antibody responses were similar in time of onset and duration for bulls which shed the virus during the acute phase of the disease only and those which became persistent shedders, although the magnitude of antibody responses was greater for bulls which became persistent shedders(14).

Age of the animals in the experimental infection studies did not influence the development of persistent seminal shedding of virus, nor did sexual maturity as judged by the scrotal circumference.

The site of persistence of lumpy skin disease virus was sought in an attempt to explain the phenomenon of persistent shedding in semen. Only severely-affected bulls shed virus in their semen for a prolonged period in the study reported in Chapter 6. This matches what was seen in the previous study reported in Chapter 5. In addition, only severely-affected bulls developed lesions in their testes and epididymides, as confirmed by regular ultrasonographic examination. Virus was only found in the epididymides and testicular tissues using PCR testing, and virus was localised to the necrotic areas in the testes and in lesions in the epididymides using immunohistochemical evaluation. This circumstantial evidence supports the hypothesis that persistent shedding is due to release of virus from necrotic lesions in the testes into the seminiferous tubules, or from necrogranulomatous lesions into the epididymal ducts, or from both. This is clearly distinct from the mechanism of persistent shedding in some other viral infections. In the testicular carrier state for bovine virus diarrhoea virus, virus is found within the seminiferous tubules adjacent to the basement membrane (9). Bovine herpesvirus-1 is not found in the testes but is released into the semen from the lower tract during ejaculation(15,16). A practical implication of this mechanism is that prevention of the formation of lesions would be expected to prevent shedding of virus. Viral shedding would also

be expected to cease once full resolution of necrotic areas had taken place, although, as was shown in Chapter 5, this could be longer than five months.

The mechanism of viral shedding in the acute phase of the disease as seen in all twelve of the bulls experimentally infected for Chapters 5 and 6 and the mechanism which leads to persistent shedding as seen in the four severely-affected bulls appears to differ. Bulls which shed semen in the acute phase of the disease do not necessarily have clinical signs or develop lesions in the reproductive organs. Of the four such bulls for which data is available, in only one was virus found in the cell-rich fraction of the ejaculate. In bulls which become persistent shedders, virus is most consistently found in the cell-rich fraction. It would therefore appear that the association with the sperm cells is less consistent and that the semen may become infected with virus from the accessory glands during ejaculation rather than in the testes or epididymides, as appears to be the case in bulls which develop persistent shedding. The abovementioned disparity in the mechanisms of viral shedding during the acute and persistent phases is similar to that seen in BVDV(17). For that virus, viral excretion during the acute phase is ascribed to infection of the secondary sex glands whereas prolonged excretion is due to infection of cells within the seminiferous tubules.

The consequences of the use of semen infected with lumpy skin disease virus for *in vitro* embryo production were explored in the study reported in Chapter 7. The ability of swim-up processing of semen to eliminate viable virus was tested in the first part of the trial. Swim-up processing is standard practice in many IVF laboratories with the purpose of filtering out many immotile sperm cells and contaminating micro-organisms. Our finding of the processed semen being PCR-positive but virus isolation-negative is difficult to explain. We considered the possibility that elements of the cryoprotectant or swim-up media inactivated the virus or were toxic to the cell culture system, but neither affected viral replication or tissue cultures in any way. An alternative explanation is that the virus is adsorbed onto the sperm or even taken up into the sperm cell in such a way that the virus is inactivated but the DNA is still detectable by PCR. This explanation is consistent with the observations of the association between virus particles and sperm cells seen with electron microscopy on spiked semen samples after swim-up. Whether this is the case for infected semen from naturally-infected bulls requires further investigation as it has important implications.

The adhesion of virus particles to sperm cells increases the likelihood of direct contact between the virus and the female gametes during fertilisation *in vitro* as

well as *in vivo*. It may also complicate attempts to remove the virus from infected semen.

In the *in vitro* embryo production part of the trial, abattoir ovaries were used in accordance with common practice for the commercial production of IVF embryos. The high risk of microbial contamination of the IVF system from such ovaries necessitated the immediate PCR test of the follicular fluid to detect lumpy skin disease virus and the testing for the presence of BVDV and BHV-1 by RT-nPCR and tissue culture respectively.

This is the first ever report on the effect of lumpy skin disease virus on embryo production. The presence of viable lumpy skin disease virus in semen used for IVF does result in the production of hatched blastocysts with detectable amounts of LSDV in the absence of any specific washing process(18). The efficacy of such washing regimes in removal of lumpy skin disease virus from *in-vitro* produced embryos should be tested. While virus was isolated from the day ten embryos, it could not be isolated from wash fluids after IVF or from day five refeeding fluid, and was only isolated from one of five batches of day 7 refeeding fluid. This indicates that the virus is closely associated with the presumptive zygotes. The fact that the virus could only be isolated from the minority of batches of embryos on tissue cultures would indicate that the risk of infection of susceptible recipients is probably low. However, this assumption must be tested experimentally and by monitoring of recipients after transfer of potentially infected IVF embryos.

In Chapter 8, the question of how to prevent viral shedding in semen of challenged bulls is addressed. In this trial, vaccination was shown to be a promising method of preventing the shedding of lumpy skin disease virus in bull semen. This work was carried out in a small number of experimental animals, and results therefore need to be confirmed in larger numbers of animals under a variety of conditions. However, this work confirms the efficacy of the Neethling strain modified-live virus vaccine in preventing clinical lumpy skin disease, as reported elsewhere(19,20). Whereas the vaccine was reported to confer lifelong immunity and recommended for use once in an animal's life, subsequent experience showed that this regime was not always adequate and annual revaccination is now recommended (21,22). Vaccination of animals with a modified-live virus vaccine was shown to result in seminal shedding of virus and prolonged testicular infection with BVDV irrespective of whether the bulls were pre- or postpubertal(17). This is obviously clearly different from the situation in our study, although we did not examine the reproductive tissues at the end of the trial for the presence of virus. However, given the absence of virus in semen, it is very unlikely that virus would have been

detected in testicular or accessory sex gland tissues. There was also no effect on semen quality following vaccination of these bulls. There is therefore no contra-indication to use of the modified live Neethling strain vaccine in breeding bulls and bulls destined to be semen donors.

Another note of caution regarding the applicability of the results of the vaccination and experimental challenge work reported in Chapter 8 are that we vaccinated these bulls twice prior to challenge, as apposed to the single dose recommended by the manufacturers. This was due to the absence of detectable antibodies in any of the vaccinated animals three weeks after vaccination as determined by the serum neutralisation test. Available information stated that immunity starts to develop ten days after vaccination, antibodies reach a high titre by three weeks, and that only a small percentage of animals fail to develop an antibody response following vaccination(20,22). Based on this, we considered it likely that there had been a vaccine failure and decided to repeat the vaccination with a different batch of vaccine for the purposes of this trial. Following the second vaccination, three bulls seroconverted, confirming efficacy of the vaccine. As immunity to this disease is primarily cell-mediated, the absence of a detectable serological response does not equate to susceptibility to disease. However, antibodies are regarded as a reliable measure of response to vaccination(23). The fact that not all animals seroconverted following the second vaccination and yet none shed lumpy skin disease virus in their semen confirms that seroconversion is not necessary for the prevention of shedding of virus in semen. The fact that vaccine virus was not found in semen was also encouraging. Live virus vaccines may result in the shedding of viable virus in semen. The vaccine strain of BVDV was detected in testicular tissue of vaccinated bulls at 134 days after immunisation at which stage the bulls were castrated(17). Vaccine virus was detected in semen samples from boars vaccinated with a live attenuated porcine reproductive and respiratory syndrome virus vaccine(11). The absence of lumpy skin disease vaccine virus in semen is further circumstantial evidence for the relationship between lesions in the testes or accessory sex glands and excretion of virus in semen discussed in Chapter 6.

The formulation of a protocol to prevent the export of genetic material containing lumpy skin disease virus from endemic areas is not recommended based on the limited scope of the information contained in this work. However, from this preliminary data the following are suggested: A thorough clinical examination should be done and any bulls with lymphadenopathy or skin lesions consistent with lumpy skin disease should be excluded from semen donation for six months or each batch of semen should be tested using the PCR test used in this work. Bulls should also be vaccinated twice with a three week interval prior to semen

collection. A single vaccination with subsequent seroconversion would also be an indication of adequate protection. Embryos produced by IVF from bulls in infected countries may be safe for transfer to susceptible recipients.

Major topics for future research on the risk posed by lumpy skin disease virus in semen are to confirm the duration for which viable virus can be shed in semen. This will most likely have to be done using a bioassay. The consequences of inseminating susceptible females with viable virus must also be studied as an important element of determining the likelihood of transmission in infected semen. Whether the virus can be washed off *in-vitro*-produced embryos and the consequences of transferring such embryos into susceptible females also need to be ascertained.

This work has provided significant new insights into the diagnosis of Trichomonosis, the behaviour of lumpy skin disease virus in bull semen, and the testing of bulls for breeding soundness.

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Samenvating

Het onderzoek beschreven in dit proefschrift betreft de rol die de stier vervult bij de overdracht van geslachtsziekten. Sommige van deze aandoeningen kunnen tevens onvruchtbaarheid tot gevolg hebben.

Het eerste hoofdstuk is een inleiding op dit onderwerp, geeft een overzicht van de literatuur en licht de doelstellingen toe van het onderzoek beschreven in de daarop volgende hoofdstukken. Om de vruchtbaarheid van een stier die voor de fokkerij bestemd is van te voren in te kunnen schatten worden een gestandaardiseerd klinisch onderzoek evenals een aantal aanvullende laboratoriumtesten uitgevoerd met als doel de meest voorkomende oorzaken van onvruchtbaarheid te kunnen uitsluiten bij het gebruik van de stier voor de fokkerij. Dergelijke gestandaardiseerd onderzoek wordt in vele landen routinematig uitgevoerd. De waarde van de uitkomsten is mede afhankelijk van de uitgebreidheid en de diepgang van het klinisch en laboratoriumonderzoek, de wijze waarop stieren vervolgens geclassificeerd worden en de wijze van rapportage. Infectieziekten zijn een belangrijk aandachtspunt bij het vruchtbaarheidsonderzoek.

De stier kan een grote rol spelen bij het overdragen van infectieuze aandoeningen binnen een kudde maar ook door gebruik van geïnfecteerd sperma voor kunstmatige inseminatie of voor *in vitro* fertilisatie en daaropvolgende embryotransplantatie. Twee aandoeningen zijn daarbij in het bijzonder in zuidelijk Afrika specifiek van belang. Daar is de betekenis van trichomoniasis toegenomen bij de fokkerij van vleesrunderen en algemeen wordt aangenomen dat deze aandoening de meest belangrijke infectieuze oorzaak is van productieverlies met betrekking tot het aantal kalveren geboren. Voor de internationale handel is het virus dat lumpy skin disease veroorzaakt van betekenis en bemoeilijkt de export van sperma vanuit Zuid-Afrika. Het onderzoek vermeld in dit proefschrift is gericht op het vruchtbaarheidsonderzoek van de stier met name op de situatie in zuidelijk Afrika, op de diagnose van trichomoniasis en op een aantal aspecten van de aanwezigheid van lumpy skin disease virus in sperma van de stier.

In hoofdstuk twee is de doelstelling toegelicht van en wordt de procesgang beschreven die geleid heeft tot het certificaat voor de vruchtbaarheidsevaluatie van een stier van de Zuid-Afrikaanse Maatschappij voor Diergeneeskunde. In het eerste deel van het certificaat verklaart de dierenarts dat de stier voldoet aan de minimumeisen die gesteld worden met betrekking tot het doel waarvoor de stier

gebruikt zal worden, namelijk voor natuurlijke dekking, als spermadonor voor latere inseminaties of dat het onderzoek betrekking heeft op een verzekeringsovereenkomst. Deze vorm van klassenindeling is uniek voor een dergelijk certificaat. Vervolgens worden gegevens vermeld met betrekking tot de stier, de eigenaar en lijst van aanbevolen onderzoek en testen die in verband met het toekomstige gebruiksdoel zijn uitgevoerd. Blanco certificaten zijn beschikbaar in boekvorm waarbij aan het certificaat een verklarende bijlage en tevens minimum standaardeisen zijn toegevoegd. Na invulling blijft een doorslag ter archivering in het boek achter. De helderheid en volledigheid van het document zijn waardevol. Alleen dieren die geheel aan de eisen voldoen krijgen een certificaat. De gestelde eisen zijn hoger dan die van zowel de Amerikaanse (Society for Theriogenology) als Australische standaard. Informatie wordt vermeld betreffende betrokken partijen zodat in geval nadere informatie wordt gewenst contact opgenomen kan worden ter voorkoming van meningsverschillen.

Het derde hoofdstuk beschrijft twee experimenten uitgevoerd om de gevoeligheid en de praktische uitvoerbaarheid van een nieuwe testmethode voor de diagnostiek van trichomoniasis te onderzoeken. Daarbij werd materiaal van de voorhuid verzameld van met *Tritrichomonas foetus* geïnfecteerde stieren door dit af te schrapen. In het eerste experiment, werden bij drie geïnfecteerde stieren en bij een controlestier monsters tegelijkertijd verzameld door zowel wassen als schrapen van de voorhuid, en wel tien maal in een periode van vijf weken. In een tweede experiment werden zes maal monsters verzameld op beide manieren bij vijf geïnfecteerde stieren en acht controledieren welke laatste drie maal werden bemonsterd. De monsters werden gekweekt in een medium specifiek geschikt voor Trichomonas. Bij het eerste onderzoek gaven 29 van de 30 monsters van besmette stieren een positieve uitslag. In het tweede onderzoek was 83% van de monsters bij beide methoden positief. Geen van de monsters van de controledieren was positief. De afschraapmethode bleek snel en veilig en had als voordeel dat, in tegenstelling tot de voorhuid-spoelmethode, een verontreiniging van het monster met urine voorkomen werd. Het monster had een kleiner volume, was daarom meer geconcentreerd en ook was er minder besmetting vanuit de omgeving hoewel er ook een duidelijk effect waarneembaar was op de uitslag van degene die de bemonstering uitvoerde. Er kan geconcludeerd worden dat het afschrapen van de voorhuid als methode tot een even goed diagnostisch resultaat leidt als de voorhuidspoeling en daarom een bruikbaar alternatief is bij het directe onderzoek op en kweek van *Tritrichomonas foetus*.

Het vierde hoofdstuk is getiteld: ‘Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: Effects of sample collection method,

storage and transport medium on the test.' Er werd onderzocht wat de ondergrens is bij de PCR-detectie methode waarbij het infectieuze agens, *Tritrichomonas foetus*, nog kan worden aangetoond. Hiervoor werden verdunningsreeksen gemaakt en het bleek dat bij aanwezigheid van 100 organismen het agens nog kon worden gedetecteerd. Ook werd de invloed van de methode van bemonstering en van bewaren van het monster op de testuitslag bestudeerd bij vijf besmette en acht controle dieren waarvan 29, respectievelijk 28 monsters werden genomen. Monsters werden opgesplitst, daarna gekweekt of geëxtraheerd voor DNA in aan- of afwezigheid van GuSen dat een preserveringsmiddel is. Extractie van monsters vond plaats na bewaren gedurende maximaal zes uur, dertig uur of vijf dagen bij een temperatuur van vier graden Celsius. PCR-bepaling en agarose gelelectrophorese werden vervolgens uitgevoerd. Een two-tailed Chi kwadraat test werd uitgevoerd om statische verschillen tussen behandelwijzen aan te tonen. De PCR bepaling had een specificiteit van 98%. De gevoeligheid nam af in de loop van het bewaren van 90% op zes uur tot 31% na vijf dagen. De methode van bemonstering noch aan- of afwezigheid van GuSCN had effect op de gevoeligheid van de bepaling. Vergelijkbare resultaten werden behaald met 193 monsters die vanuit de perifere praktijk naar het laboratorium waren toegezonden.

Het vijfde hoofdstuk beschrijft de frequentie en duur van de uitscheiding van lumpy skin disease virus (LSDV) in het ejaculaat van experimenteel geïnfecteerde dieren, welke gevoelig zijn voor deze aandoening. Zes stieren, tussen 11 en 20 maanden oud, die geen specifieke afweerstoffen in het bloed hadden tegen LSDV werden geïnfecteerd met een virulent veldisolaat van LSDV. De dieren werden vervolgens onderzocht op het voorkomen van klinische symptomen, er werd bloed afgenomen voor serologisch onderzoek tot 90 dagen na infectie en sperma gewonnen: om de dag tot dag 18 en vervolgens twee maal per week tot dag 63 en dan twee maal per maand tot dat twee opeenvolgende monsters negatief getest waren met behulp van de PCR-test op LSDV. Bovendien werd een deel van elk positief getest monster geënt op een celcultuur om het virus op deze manier te kunnen isoleren.

Twee stieren ontwikkelden een ernstige mate van Lumpy skin disease, twee stieren vertoonde milde klinische symptomen terwijl bij twee dieren slechts een kortdurende temperatuurverhoging waarneembaar was. Meerdere monsters gaven met de PCR methode een positieve uitslag bij de beide stieren met ernstige symptomen en bij een van de stieren met milde symptomen: respectievelijk tussen dag 10 en 159, tussen dag 8 en 132 en tussen dag 10 en 21. Slechts een maal werd bij elk van de andere stieren een monster positief getest met PCR. Virusisolatie was alleen succesvol bij dieren met ernstige symptomen en wel 1 respectievelijk 5

maal. Wij hebben dus aangetoond dat uitscheiding van LSD virus in rundersperma gedurende langere tijd na infectie plaats kan vinden, zelfs als duidelijk klinische symptomen niet meer waarneembaar zijn.

Het onderzoek beschreven in hoofdstuk 6 had tot doel vast te stellen waar het virus vandaan kwam als dit gedurende langere tijd in het sperma werd uitgescheiden, in welke fractie van het ejaculaat het virus aanwezig was en tevens de laesies die zich in het geslachtapparaat ontwikkelden te onderzoeken. Zes volwassen dieren welke geen antistoffen tegen LSDV in het bloed hadden werden geïnfecteerd met een virulent veldisolaat van LSDV. De PCR werd uitgevoerd op voorhuidspoelsels, vloeistof uit de zaadblaasjes, en op het supernatant en de celrijke fractie van een ejaculaat gewonnen op dag 10 en dag 26 na infectie (p.i.). Stieren waarvan het ejaculaat monster positief was op dag 26 p.i. werden geslacht en weefselmonsters van het geslachtsapparaat verzameld voor histologisch onderzoek, virusisolatie en PCR.

Twee stieren kregen na infectie daadwerkelijk LSD en bleken virus uit te scheiden in het sperma op dag 28 p.i..Viraal DNA kon worden aangetoond in alle ejaculaatfracties van alle stieren, maar vooral in de celrijke fractie van de meest zieke dieren. De PCR uitslag was positief op testis en bijbal weefsel van de deze twee dieren. Ook bleek er histologisch necrose in deze organen te zijn. Deze bevindingen onderbouwen de hypothese dat via dergelijke necrotische laesies virusafscheiding plaats kan vinden. Een praktische consequentie hiervan zou zijn dat bij het tegengaan van het ontstaan van zulke laesies virusuitscheiding voorkomen kan worden. Virusuitscheiding zou stoppen wanneer necrotische plekken zijn genezen maar dit zou, zoals in hoofdstuk 5 is aangetoond, langer kunnen duren dan vijf maanden.

De risico's betrokken bij het gebruik van met LSDV besmet sperma voor *in vitro* fertilisatie zijn onderzocht in hoofdstuk 7. Aan zes doseringen van diepgevroren/ontdooid sperma van controledieren werd virus toegevoegd, tevens werden twee doseringen gebruikt afkomstig van experimenteel geïnfecteerde dieren welke virus-uitscheider waren. Monsters werden behandeld volgens de swim-up procedure en daarna getest op aanwezigheid van virus met de PCR- en kweekmethode. Monsters afkomstig van uitscheiders gave een negatieve uitslag maar die waaraan virus was toegevoegd bleken positief met PCR maar niet via kweek. Viruspartikels werden in de monsters aangetoond met behulp van electronenmicroscopie. Van deze partikels bleek negen procent te zijn gehecht aan zaadcellen.

Spermamonsters werden door toevoeging van virulent LSDV besmet alvorens te worden gebruikt om eicellen *in vitro* te bevruchten. Deze eicellen waren afkomstig van op het slachthuis verzamelde eierstokken. De controle en behandelgroep bestonden respectievelijk uit 497 and 488 eicellen. Aanwezigheid van lumpy skin disease virus werd door middel van PCR onderzocht in de opeenvolgende fases van embryogenese bij de behandelde groep en daarnaast in drie controle groepen van elk 100 presumptieve zygoten op dag twee en dag vijf na bevruchting. Virus werd aangetoond in twee batches van dag 10 embryo's. Er was geen verschil tussen controle en behandeling in klievingspercentage van de zygoten geëvalueerd op dag vijf maar het percentage embryo's op dag zeven was hoger en het percentage embryo's dat uit de zona pellucida was gekomen was lager bij de controlegroep dan bij de behandelde groep.

Wanneer sperma, gebruikt voor IVF, is besmet dan blijft virus aanwezig in de kweek en is dit ook aantoonbaar bij de embryo's. De besmettingsgraad is echter laag en dit vormt dan ook maar een gering risico voor de ontvangster. De embryo-ontwikkeling wordt negatief beïnvloed door het virus als het embryo eenmaal buiten de zona pellucida is.

In hoofdstuk 8 wordt aangetoond dat na vaccinatie en een daaropvolgende experimentele besmetting LSDV niet in sperma aantoonbaar is. Hiervoor werden 12 stieren gebruikt die geen specifieke antilichamen tegen LSDV in hun bloed hadden. Zes dieren werden gevaccineerd en geïnfecteerd, bij zes werd deze vaccinatie niet uitgevoerd. De vaccinatie leidde niet tot klinische symptomen en na infectie waren bij vier dieren milde klinische verschijnselen waarneembaar. Bij deze dieren was LSDV niet aantoonbaar in het ejaculaat. Bij twee niet gevaccineerde stieren ontwikkelden zich na infectie ernstige klinische symptomen van LSD, bij de overige vier dieren waren de verschijnselen iets minder ernstig. Al deze dieren scheidden LSDV in het sperma uit. Dit onderzoek toonde dus aan dat vaccinatie tegen LSD een eventuele latere uitscheiding van LSDV kan voorkomen.

Tenslotte zijn de resultaten van het onderzoek als geheel samengevat en bediscussieerd in hoofdstuk 9 en wordt aangegeven waar nader onderzoek wordt gewenst. Vooral is zulk onderzoek nodig naar betrouwbare voorspellende methoden betreffende de vruchtbaarheid van stieren. De huidige methoden zijn vaak tijdrovend en niet voldoende nauwkeurig om sommige oorzaken van sub-fertiliteit te identificeren.

De volgende procedure wordt aanbevolen om te kunnen verklaren dat stieren niet besmet zijn met *Tritrichomonas foetus*: drie maal bemonsteren door middel van het

schrapen van de voorhuid met een wekelijks interval. Monsters, bewaard in een gebufferde oplossing, moeten binnen 24 uur op het laboratorium worden verwerkt. In geval van een positieve kweekuitslag dient een PCR test te worden uitgevoerd om vals-positieve resultaten uit te kunnen sluiten. Nader onderzoek is gewenst om de diagnostiek betreffende dit agens te optimaliseren. Er is behoefte aan een test waarbij eenmalige bemonstering voldoende is en waarbij enige variatie in monsterkwaliteit geen rol speelt.

Het opstellen van een protocol om te voorkomen dat LSDV via internationaal handelsverkeer wordt verspreid is niet mogelijk op grond van de huidige, nog beperkte onderzoeksresultaten. Toch kunnen op grond van de onderzoeksresultaten de volgende aanbevelingen worden gegeven: na zorgvuldig klinisch onderzoek moeten stieren, bij wie specifieke afwijkingen aan de lymfeknopen evenals huidlaesies worden gediagnosticeerd, niet worden gebruikt voor spermawinning gedurende een periode van zes maanden of elk ejaculaat dient gecontroleerd te worden op afwezigheid van LSDV met de door ons gebruikte PCR test. Stieren zouden twee maal gevaccineerd moeten worden met een tussentijd van drie weken voordat men het sperma gaat winnen. Een eenmalige vaccinatie met een goede seroconversie zou ook voldoende zijn. De kans op verspreiding van LSDV via export van embryo's uit landen waar LSD endemisch is wordt niet groot geacht

Nader onderzoek is nodig om vast te kunnen stellen hoe lang na infectie virusuitscheiding in het ejaculaat plaats vindt. Dit zal waarschijnlijk met behulp van een bio-assay moeten worden onderzocht. Ook is nog niet bekend hoe groot het risico op ziekte-overdracht is na inseminatie van met LSDV besmet sperma. Evenmin is nog bekend wat de risico's zijn van het transplanteren van met LSDV besmette maar vervolgens gewassen embryo's, naar niet besmette recipiënten

Samengevat heeft het onderzoek beschreven in dit proefschrift belangrijke nieuwe inzichten verschaft betreffende de onderzoeksprocedure naar de vruchtbaarheid van de stier en vooral voor de diagnostiek van *Tritrichomonas foetus* en het voorkomen van lumpy skin disease virus in het sperma.

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Curriculum Vitae

Peter Charles Irons was born in Maseru, Lesotho in 1963. He matriculated from Springs Boys' High in 1981 and obtained his Bachelor of Veterinary Science from the University of Pretoria in 1987. He completed an internship in Preventive Medicine at the University of Florida's School of Veterinary Medicine before completing his national service at the University of Bophuthatswana in 1990. He then spent three years in mixed and large animal practice in England, during which he obtained the Royal College of Veterinary Surgeons' Certificate in Cattle Health and Production. He joined the Faculty of Veterinary Science, University of Pretoria in 1994, where he still serves as an Associate Professor in Reproduction. He became a Diplomate of the American College of Theriogenology in 1998 and obtained the MMedVet (Gyn) degree with distinction in 2002. He is an active member of the production animal veterinary profession in southern Africa, providing specialist clinical expertise and initiating an e-mail discussion group for rural practitioners which has grown to over 200 members. He founded Veterinary Fertility Services, a private specialist reproductive veterinary practice, in 2005. He is married with three daughters, is an avid birdwatcher and chairman of the Seringveld Conservancy.