# Semen quality and the excretion of lumpy skin disease virus in semen following vaccination and experimental challenge of vaccinated bulls

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# SEMEN QUALITY AND THE EXCRETION OF LUMPY SKIN DISEASE VIRUS IN SEMEN FOLLOWING VACCINATION AND EXPERIMENTAL CHALLENGE OF VACCINATED BULLS

**BY**

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**Submitted in partial fulfilment of the requirements for the degree of Master of Science (Veterinary Science) in the Department of Production Animal Studies**
**Faculty of Veterinary Science**
**University of Pretoria**

**Date submitted: March 2006**

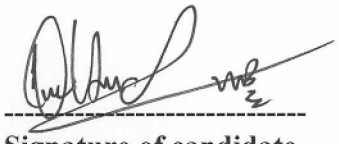
To my unborn child

## DECLARATION

I, Uchebuchi I. Osuagwuh, do hereby declare that the research presented in this dissertation, apart from the assistance received as reported in the acknowledgement represent the original work of the author.

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

This dissertation is presented in partial fulfillment for the requirement for the MSc in Production Animal Studies.



**Signature of candidate** **Date** 16 / 3 / 2006

## ACKNOWLEDGEMENTS

First of all, my profound gratitude goes to **The Sugmad**, **The Eck** and **The Mahanta** for whom life and total guidance is being given unto me.

I would like to thank my supervisor Dr. P.C. Irons, Section of Reproduction, Department of Production Animal Studies for giving me the opportunity to be part of his team. This is to say that your criticisms, suggestions, assistance and intellectual guidance were very useful. Although I made mistakes but learnt from every one of them, and when it's all said and done, I bet I'm a better one. Sir, I say thank you for all you did.

I am also grateful to my Co-Supervisor Prof. E.H. Venter, Department of Veterinary Tropical Diseases, for her support, assistance and patience, and also putting in bits of her knowledge in making this project a success. I say a big thank you.

I wish to thank Dr. C.H. Annandale, Joost, Michiel for helping in sample collection during my most difficult times and Dr. V. Bagla for the sample processing.

I am also grateful to all the staff and colleagues of the Section of Reproduction, University of Pretoria, for making my stay in the Department a memorable one.

I am indebted to my parents who provided moral support especially my late Dad, Professor A.I.A. Osuagwuh who introduced me to this 'game'. It's been one year since you left and couldn't wait to see the end of what we started. Sometimes it's hard to wake up in the morning, my mind full of memories of you, I know you still watching and smiling as you always did. I want you to know that I will continue to grow as long as I live and follow no one else's shadow. I hope I will be able to make you proud, being the man I have become under your guidance.

To my siblings, Uchebundu, Uchebuike, Ucheneake, thank you all for being there for me, we are a team and always will be till time fades. One day we shall all laugh back at the memories we once had. You all are very much appreciated and I love you all.

I am also grateful to all my friends, because true friends make one feel real good. Though I haven't flashed a smile in a long while, my only friend was my misery, my attitude got me

walking solo, right all alone, watching the whole world move slow motion. Besides observing, the only thing I did well was observing. To others, well, know that nowadays things have changed, and everyone is ashamed than they used to because the truth looks strange. Wipe your eyes and see clearly, there is no need for you to discriminate others, only if you take your time to hear them, maybe you can learn to cheer them, and is not about being black or white because we are all human.

To my one and only best friend, Jo Thomas, you are the very best, thank you for your love, support, care and encouragement throughout this period. I love you and appreciate your being there always.

Finally, I want to thank the Faculty of Veterinary Science and Department of Production Animal Studies, University of Pretoria, for their financial assistance towards my research.

This material is based upon work supported by the National Research Foundation under Grant number 2054210.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AGIP | Agar-gel immunodiffussion test |
| AKV | Adult vervet monkey kidney cell |
| BD | Bovine dermis |
| BHK-21 | Baby hamster kidney cell |
| BTV | Bluetongue virus |
| BVDV | Bovine viral diarrhea virus |
| CPE | Cytopathic effect |
| dATP | Deoxy adenine triphosphate |
| dCTP | Deoxy cytocine triphosphate |
| dGTP | Deoxy guanidine triphosphate |
| DNA | Deoxiribonucleic acid |
| dUTP | Deoxy uracil triphosphate |
| DVTD | Department of Veterinary Tropical Diseases |
| EDTA | Ethylendiamine tetra-acetic acid |
| FAO | Food and Agricultural Organization |
| FCS | Foetal calf serum |
| FMDV | Foot and mouth disease virus |
| HIV | Human immunodeficiency virus |
| hrs | Hours |
| IBRV | Infectious bovine rhinotraechietis virus |
| KCl | Potassium chloride |
| LSDV | Lumpy skin disease virus |
| MEM | Minimum essential medium |
| $MgCl_2$ | Magnesium chloride |
| mins | Minutes |
| mM | Milli mole |
| NaCl | Sodium chloride |
| ng | Nano gram |
| OIE | Office International des Epizooties |
| p.i. | Post inoculation |
| p.v. | Post vaccination |
| PCR | Polymerase chain reaction |
| PCV | Porcine circovirus |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| RNA | Ribonucleic acid |
| SGP | Sheep and goat pox |
| SNT | Serum neutralization test |
| SOP | Standard operative procedure |
| $TCID_{50}$ | Tissue culture infective dose 50 |
| Tris-HCl | Tris hydrochloride |
| UDG | Uracil DNA glycosylase |
| UPBRC | University of Pretoria Biomedical Centre |
| USA | United States of America |
| VI | Virus isolation test |
| µl | Micro littre |
| µM | Micro molar |

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# SUMMARY

## SEMEN QUALITY AND THE EXCRETION OF LUMPY SKIN DISEASE VIRUS IN SEMEN FOLLOWING VACCINATION AND EXPERIMENTAL CHALLENGE OF VACCINATED BULLS

BY

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The aim of this study was to determine the efficacy of vaccination in preventing LSDV excretion in semen and negative effects on semen quality.

Lumpy skin disease (LSD) is caused by a virus in the genus *Capripoxviru*s of the family *Poxviridae.* The virus has been reported to be excreted in the semen of experimental infected nonvaccinated bulls. Nevertheless, vaccination has been the most widely used method to reduce and prevent the spread of the disease. This work was done to determine the efficacy of lumpy skin disease vaccination in preventing the excretion of lumpy skin disease virus (LSDV) in semen of experimentally infected vaccinated bulls. It also determined further the effect of vaccination and experimental infection on semen quality.

Six serologically negative bulls 11-16 months of age were vaccinated with an attenuated Neethling strain of LSD vaccine, and a repeated dose of vaccine was given twenty one days later. These bulls were then experimentally infected by intravenous injection with a virulent field strain of LSDV (V248/93). Six unvaccinated bulls were similarly infected to act as controls. All animals were observed for clinical signs, blood and semen was collected and evaluated twice a week until day 40 post-

vaccination and every two days until day 28 post-infection when the trial was terminated. Serology was done using the serum neutralization test and viraemia was determined by virus isolation. Semen was examined by polymerase chain reaction (PCR) for the presence of virus. Semen evaluation was done visually and microscopically.

Two of the unvaccinated controls developed severe LSD, two showed mild symptoms and two were asymptomatic. 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. There was a significant difference ($P<0.05$) in semen quality after experimental infection of the unvaccinated bulls. In the vaccinated bulls, semen quality showed no significant difference ($P>0.05$) following vaccination and challenge. Three of the vaccinated bulls were serologically positive at the time of experimental infection and four at the end of the trial. Five unvaccinated bulls were found to be viraemic during the course of the trial. No vaccinated bulls were found to be viraemic at any stage. Four unvaccinated bulls excreted the virus in their semen during the course of the trial. Viral nucleic acid was not detected in any semen samples following vaccination or challenge in vaccinated bulls.

This study provides evidence that vaccination against LSD prevented the excretion of viral particles in semen. It also illustrated that LSD vaccination prevented any effect on semen quality after experimental infection with virulent virus.

# CHAPTER 1

# INTRODUCTION

## 1. Overview

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 (Kitching and Taylor, 1985; Davies, 1991). The virus causing LSD is classified with goat and sheep pox in the genus *Capripoxvirus* (Fenner *et al.*, 1987). 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 which usually undergo necrosis and generalised lymphadenopathy (Prozesky and Barnard, 1982; Kitching and Taylor, 1985; Davies, 1991).

The disease causes significant economic loss due to hide damage, loss of milk production, mastitis, infertility and death (Weiss, 1968). Clinical signs include necrotic plaques in the mucous membranes of the mouth, respiratory tract, vulva and prepuce, and swelling of peripheral lymph nodes. The severity of the disease depends on the isolate of the virus (Woods, 1988).

Lumpy skin disease is considered a “List A” disease by the Office International des Epizooties (OIE) due to its rapid spread and economic implications. It is a constraint in international trade of live animals and their products. This disease is endemic in most parts of sub-Saharan Africa. It was first reported outside Africa in 1989 with an outbreak in Israel which was controlled and eradicated by quarantine and slaughter policies. Arnaud *et al.*, (1992) also reported a case of the disease in a 2 year old female Arabian Oryx in Saudi Arabia in 1989.

The disease was included in the Council Directive 82/894/EEC on the notification of animal diseases by the European Economic Cooperation (EEC) / European Union, thus making it compulsory to report any primary outbreak of the disease to the commission and all other member states within 24 hours.

Transmission of the disease is by biting flies, although this has yet to be proven conclusively (Haig, 1957; Weiss, 1968; Davies, 1991; Carn and Kitching, 1995a). Other possible means of transmission include contact with infected animals or contaminated objects or materials by clinically sick animals (Henning, 1956; Haig, 1957; Weiss, 1968). Unpublished observations indicated that the virus was excreted in the semen for 22 days after the fever reaction following experimental infection (Weiss, 1968).

Two live attenuated strains of capripoxvirus are being used for vaccination and these are the South African Neethling and Kenya sheep and goat poxvirus strains. The South African vaccine was developed by attenuation of a field isolate of the Neethling strain which is now used as a major control measure for further outbreaks (Weiss, 1968).

This study addresses the risks associated with exporting and importing bovine semen from vaccinated bulls between countries by investigating the role of vaccination against lumpy skin disease virus (LSDV). Specific research questions were:

- The possibility of excretion of the vaccine virus in semen of vaccinated bulls,
- The effect of vaccination against LSD on semen quality,
- The ability of vaccination to prevent shedding of virulent LSDV when challenged,
- The possible protective effect against virulent LSDV on semen quality of vaccinated bulls.

# LITERATURE REVIEW

## 1.1. History

Weiss, (1968), reviewed the early history of the disease. In 1929, a new disease of cattle was reported in Zambia which manifested the appearance of skin nodules. At that time the infectious nature of the disease was not recognized. It was then believed that the lesions resulted from bites of insects, hence the condition was called "pseudo-urticaria" (Weiss, 1968).

The disease spread gradually within Zambia for 13 years and during that time it was called "lumpy skin disease" which was thought to be caused by plant poisoning by (Weiss, 1968). An outbreak of the disease was seen in cattle in 'Ngamiland' (Botswana) and was called 'Ngamiland Cattle Disease' (Von Backstrom, 1945). The disease continued to spread and resulted to a panzootic that lasted for a number of years, affecting cattle in most southern African countries. By 1944, the disease had spread to South Africa (Thomas and Mare, 1945).

The first case was cited in the Marico District of Western Transvaal (now North West Province) where it was called 'knopvelsiekte' (Afrikaans for lumpy skin disease) (Thomas and Maré, 1945). This was a panzootic in South Africa, which lasted until 1949, affecting over eight million cattle and consequently causing enormous economic losses (Diesel, 1949).

Lumpy skin disease was first recognized in Kenya in 1957 (MacOwen, 1959) when it appeared as an epizootic, Sudan in 1971 (Ali and Obeid, 1977), Chad and Niger in 1973 (Ali and Obeid, 1977), Nigeria in 1974 (Nawathe *et al.*, 1978) but the clinical diagnosis of lumpy skin disease in Nigeria was first reported by Woods, (1988).The disease was also reported in Somalia in 1983 (Ali *et al.*, 1990).

From 1929 to 1986 the disease became endemic within the sub-Saharan African countries and Madagascar (Davies, 1991). The disease was reported in Ismailia (Egypt) where there was an outbreak in 1988. Since then it has become endemic despite all measures to eradicate and control the disease (Ali *et al.*, 1990).

An outbreak of LSD was reported for the first time outside the shores of Africa in Israel in 1989. It was believed that the disease spread from the Egyptian outbreak through insect vectors carried by wind or by vehicles of cattle traders (Yeruham *et al.*, 1995). The disease was later completely eradicated in Israel by quarantine measures, ring vaccination programme in a radius of 50 km of the outbreak and rapid slaughtering of all infected and in contact cattle, sheep and goats. Ever since no further clinical cases of the disease has been reported (Yeruham *et al.*, 1995).

The epidemiology of LSD is characterized by the apparent disappearance of the disease from the region for months or even years and then a sudden reappearance with widespread outbreaks. Control of LSD is primarily by vaccination, although it tends to cause local reaction at the site of inoculation in some cattle, particularly imported breeds (Weiss, 1968). This frequently causes farmers to stop vaccination when the disease is absent, resulting over time in large susceptible population of animals. Therefore, the reappearance of LSD after long intervals of quiescence could be ascribed to the presence of unvaccinated animals.

### 1.2. Aetiology

Lumpy skin disease virus belongs to the family *Poxviridae*, genus *Capripoxvirus*. It is closely related antigenically to sheep and goat poxvirus. The LSDV primarily affects cattle but can affect sheep and goats, experimentally. The prototype strain of LSDV is the Neethling virus (Alexander *et al.*, 1957).

There is only one serotype of LSDV and it is serologically closely related to the virus of sheep and goat pox (SGP) and can be distinguished by routine virus neutralization or other serological tests (Burdin, 1959). Lumpy skin disease virus will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis cells (Alexander *et al.*, 1957; Prydie and Coackley, 1959).

Restriction endonuclease studies of capripoxviruses indicate that LSDV isolates are essentially identical with each other and with a Kenyan strain (0 240/KSGP) of sheep and goat pox virus (SGPV) (Kitching *et al.*, 1989). Other strains of SGPV from

Kenya were different from the 0 240/KSGP strain but similar to each other and resembled strains of SGPV from the Arabian Peninsula. The Kenyan group of SGPV strains showed differences when compared with those from India, Iraq and Nigeria (Kitching *et al.,* 1989).

Lumpy skin disease virus is very resistant to physical and chemical agents and is stable between pH 6.6 and 8.6. The virus persists in necrotic skin for at least 33 days and remains viable in lesions in air-dried hides for at least 18 days at ambient temperature (Weiss, 1968). The virus also can remain viable in the lesions and superficial epidermal scrapings from such lesions of air-dried portions of hides kept at room temperature (Weiss, 1968). The virus can remain viable for at least 10 years in tissue culture fluid kept at -80 $^{o}$C (Weiss, 1968).

## 1.3. Laboratory identification

### *1.3.1. Virus isolation*

LSD virus can be isolated using a wide variety of tissue cultures and these include lamb and calf testis, lamb and calf kidney, calf fetus muscle cells, sheep kidney cells, lamb and calf adrenal and thyroid cultures, sheep embryonic kidney and lung, adult vervet monkey kidney cell line (AKV 58) and baby hamster kidney cells (BHK/21) (Alexander *et al.,* 1957; Prydie and Coackley, 1959). The development of cytopathic effects (CPE) is characterized by rounding, shrinking and detachment of cells to give a moth-eaten appearance to the monolayer.

Capripoxvirus causes a characteristic cytopathic effect (CPE) and intracytoplasmic inclusion bodies (Alexander *et al.,* 1957; Prydie and Coackley, 1959; Plowright and Witcomb, 1959; Munz and Owen, 1966) and it is distinct from the virus of pseudo-LSD (Allerton-herpes mammillitis) which is a herpesvirus producing syncytia and intranuclear inclusion bodies. Lumpy skin disease virus causes the development of macroscopic lesions (pocks) in the chorioallantic membranes of embryonated chicken eggs (Alexander *et al.,* 1957; Van Rooyen *et al.,* 1969), and production of generalised skin lesions in rabbits (Alexander *et al.,* 1957).

In South Africa, LSDV has never been isolated in the field from goats or sheep, although under laboratory conditions, the virus is able to grow to high titres in lamb cells (Alexander *et al.*, 1957; Prydie and Coackley, 1959; Weiss, 1968). Virus isolation is essential in the confirmation of clinical disease and determining the isolate. The newly isolated virus can be used in vaccine production if an established vaccine has failed to control the spread of the disease.

### *1.3.2. Other diagnostic methods*

Electron microscopy, being technically simple, provides a rapid method for detecting LSD virus in tissue samples (Kitching and Smale 1986) but in large parts of endemic areas in Africa this facility is not available. The presence of immune complexes has no effect on the detection of viral DNA or RNA. A PCR - based test for the detection of capripoxvirus in biopsy samples described by Ireland and Binepal, (1998) has better analytical and diagnostic sensitivity than the antigen trapping ELISA described by Carn, (1995). DNA isolated from the virus can be used to identify any changes in the nucleotide sequence (Fenner *et al.*, 1987). Nucleic acid hybridization techniques and the polymerase chain reaction (PCR) are now widely used for detection and characterization of many viruses (Forsyth and Barrett, 1995; Browning and Begg, 1996; Uwatoko *et al.*, 1996).

The PCR has been demonstrated to be more sensitive in detecting virus in semen than virus isolation in semen samples of bulls experimentally infected with LSDV (Irons *et al.*, 2005). In that study, it was also reported that PCR was more sensitive in detecting lower numbers of LSD viral nucleic acid in the semen of infected bulls than virus isolation. The sensitivity of PCR in detecting herpes viruses in semen and other specimens over virus isolation has also been reported by various authors (Sharma *et al.*, 1992; Lawrence *et al.*, 1994; Xia *et al.*, 1995; Rocha *et al.*, 1998; Wald *et al.*, 1999).

### 1.4. Transmission

The virus has been isolated from nasal, ocular, and pharyngeal secretions, semen, milk and blood (Thomas and Maré, 1945; Weiss, 1968; Irons *et al.*, 2005). Lumpy skin disease is not particularly contagious, and direct transmission by contact between animals is inefficient (Barnard *et al.*, 1994). Infection by contact can occur, though it is said to be at a low rate and not considered a major role in transmission during epizootics. Biting flies have been incriminated in most epidemics, which have been well defined and have occurred at regular intervals (Barnard *et al.*, 1994). A report by Davis and Otema, (1981) alluded to the possibility of the involvement of arthropod vectors but also suggested that husbandry methods where cattle are crowded together would predispose them to aerosol transmission.

Experimental and field evidence indicates that LSD is transmitted by an insect vector (Weiss, 1968; Kitching and Mellor, 1986; Carn and Kitching, 1995a). Epidemics of the disease are associated with rainy seasons, and pools of water are very conducive to insect multiplication (Davies, 1991; Haig, 1957; Mac Owen, 1959; Weiss, 1968). The virus has also been recovered from *Biomyia fasciata* and *Stomoxys calcitrans* caught while feeding on infected cattle, therefore, confirming that insect vectors appear to be the main mode of transmission (Hunter *et al.*, 2001). A more recent experimental report implicates blood feeding insects such as the mosquito (Chihota *et al.*, 2003). These authors demonstrated that LSDV is transmitted to susceptible animals if the mosquito was interrupted during the course of feeding on an infected animal, and then completed its feeding on another animal. Poxviruses are highly resistant and may remain viable in infected tissue for at least four months and probably longer.

### 1.5. Host range

The host range of lumpy skin disease virus includes sheep, goats and cattle breeds of all ages and sexes, while some wildlife have also been implicated. Experimental infection of sheep and goats with lumpy skin disease virus has been reported (Weiss, 1968). The disease was reported to be present in the Asian water buffalo (*Bubalus*

*bubalis*) in Egypt, in which five buffaloes from the three hundred buffaloes investigated, showed clinical signs of LSD, and multiple dermal nodules appearing within the first week of pyrexia following an outbreak of LSD (Ali *et al*., 1990). The authors further indicated that domesticated buffaloes (*Bubalus* species) appeared to be more susceptible to LSD than wild buffaloes (*Syncerus* species), and this report agrees with the findings of Ali and Obeid, (1977) reporting that wild buffaloes in Sudan showed no clinical signs or lesions when placed together with affected cattle.

Arnaud *et al*., (1992) reported a case of the disease in a 2 year female Arabian Oryx (*Oryx leucoryx*) in Saudi Arabia which occurred in 1989, and showed that the clinical signs observed were similar to those seen in cattle affected with LSD and had an antibody titre of 1:64 when tested for antibodies against the disease. Furthermore, the authors concluded that, the disease could have resulted from the importation of live sheep into Saudi Arabia from countries endemic with the disease such as Sudan and Somalia. A study carried out by Young *et al*., (1970) on wildlife, indicated that impala and giraffe may be susceptible to LSD. In this study, the authors showed that the impala and giraffe (*Giraffa camelopardis*) manifested typical signs and lesions of LSD, while the two buffaloes and adult black wildebeest showed no clinical reaction when all the animals were experimentally infected with the Neethling strain of LSDV.

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

### 1.6. Clinical signs

Lumpy skin disease virus causes inapparent to severe diseases in cattle of all ages (Weiss, 1968; Ayre-Smith, 1960; Davies, 1991; Carn and Kitching, 1995b). Reports on the clinical signs of both natural and experimentally infected animals have been reported (Thomas and Maré 1945; Haig, 1957; Alexander *et al.*, 1957; Capstick *et al.*, 1959; Weiss, 1968; Prozesky and Barnard, 1982; Davies, 1991; Barnard *et al.*, 1994;

Carn and Kitching, 1995b; Irons *et al.*, 2005). Weiss reported that only 40 to 50 % of experimentally infected animals develop generalized skin lesions.

The incubation period of LSD under field condition is two to four weeks (Haig, 1957), and experimental infection is between 4 - 14 days (Prozesky and Barnard, 1982; Carn and Kitching, 1995b). A biphasic fever of 40 - 41.5 °C can occur in animals that have developed clinical disease. Animals remain febrile for a total period of 4 -14 days during which inappetence, salivation, lachrymation and mucoid or mucopurulent nasal discharges may develop (Prozesky and Barnard, 1982; Carn and Kitching, 1995b).

Lachrymation may be followed by conjunctivitis, resulting in subsequent corneal opacity and blindness (Thomas and Maré, 1945; Haig, 1957; Alexander *et al.*, 1957; Capstick *et al.*, 1959). Skin nodules are characteristic of the disease; appearing before or during a second rise in body temperature 4 -10 days after the initial fever response (Weiss, 1968; Prozesky and Barnard, 1982; Davies, 1991; Carn and Kitching, 1995b). The skin nodules are 10-50 mm in diameter, well circumscribed, firm, and raised, and are particularly conspicuous in short haired cattle unlike long haired animals in which it is only recognized when skin is palpated or moistened (Henning, 1956).

Ulcerative lesions may appear on the conjunctiva, muzzle, nostrils, mucous membrane of the mouth, larynx, trachea (Prozesky and Barnard, 1982; Davies, 1991; Carn and Kitching, 1995b; Tuppurainen *et al.*, 2005). In some cases nodules and ulcers are seen in the mucous membranes of the reproductive and respiratory tract (Weiss, 1968; Davies, 1991). Subcutaneous swelling of the legs may occur (Thomas and Maré, 1945; Barnard *et al.*, 1994). Nodules may be present in the skin of the udder and teats, which become swollen and tender, and this may lead to mastitis. Pregnant cows may abort or have prolonged anoestrus (Weiss, 1968). Davies, (1991) reported intrauterine infection of late-term fetuses of which calves were born with LSD lesions.

Temporary sterility in bulls may result from the fever or lesions in the testes (Irons and Gerber, 2004). Secondarily infected necrotic tissue in the upper respiratory tract may be inhaled resulting in pneumonia and stenosis of the trachea following healing of lesions (De Boom, 1948). Secondary bacterial infections may occur. Large

nodules may become fibrotic and persist for several months; this is referred to as “sit fast” (Thomas and Maré, 1945; Haig, 1957; Alexander *et al.*, 1957; Capstick *et al.*, 1959; Weiss, 1968; Davies, 1991; Carn and Kitching, 1995b). The scars remain indefinitly thereby destroying hides and skin (Green, 1959).

### 1.7. Pathology

The gross lesions of LSD are well described (Thomas and Maré, 1945; De Boom, 1948; Haig, 1957; Burdin, 1959; Weiss, 1968; Prozesky and Barnard, 1982). Skin nodules are congested, hemorrhagic, edematous and necrotic. All layers of the epidermis, dermis and subcutaneous tissue are involved, and in some, musculature is affected. Skin nodules appear whitish-grey on cut section. Soft yellowish-grey nodules may appear on the muzzle, nostril, and mucous membrane of the mouth, digestive and respiratory tract, vulva, prepuce and scrotum (Thomas and Mare, 1945; Prozesky and Barnard, 1982; Barnard *et al.*, 1994; Tuppurainen *et al.*, 2005).

Histologically, skin lesions in the acute stage are characterized by vasculitis, perivasculitis, lymphangitis, thrombosis, oedema, necrosis and infarction (Thomas and Maré, 1945; Prozesky and Barnard, 1982). Lymphocytes macrophages, plasma cells and fibroblast proliferation appear in the later stages, and if secondary infection occurs, necrosis, polymorphonuclear leukocytes and red cells are seen (Thomas and Maré, 1945; Burdin, 1959). There is cuffing of blood vessels by leukocytes, and eosinophilic, intracytoplasmic pox inclusion bodies may be seen in the epitheliod cells, and cells of hair follicles, smooth muscle and skin gland (Thomas and Maré, 1945; Burdin, 1959; Prozesky and Barnard, 1982).

### 1.8. Economic importance

Lumpy skin disease is an economically important disease of cattle in Africa because of the effects it has on cattle herds. In severely affected animals, damage to hide greatly reduce the value and in turn affects the leather industries (Green, 1959; Weiss, 1968). Milk production may be reduced in milk herds as well as becoming debilitated; beef herds become emaciated and useless as beef producers; and bulls as

well as cows may become infertile after contracting LSD (Green, 1959; Weiss, 1968; Irons *et al.*, 2005).

### 1.9. Immunity

Animals which have recovered from the disease develop neutralizing antibodies which persist for at least 5 years (Wood, 1988). The immunity to reinfection is predominantly cell mediated (Kitching and Hammond, 1992). A proportion of cattle vaccinated with attenuated capripox strains develop neutralizing antibody 10 days after vaccination. In some animals, the antibody levels are too low to demonstrate, but are still resistant to challenge (Weiss, 1968). Neutralizing antibodies to LSDV persist for at least 2-3 years after vaccination, and calves born to immunized cows will have passive immunity for up to six months (Weiss, 1968).

The SNT is the most specific serological test, but because immunity to LSD infection is predominantly cell mediated, some animals that have had contact with LSD virus only developed low levels of neutralizing antibody (Manual of Diagnostic Test and Vaccines for Terrestrial Animals, 2004).

The agar gel immunodiffusion test and indirect fluorescent antibody test are less specific due to cross-reaction with other poxviruses. Western blotting is both sensitive and specific, but too expensive and difficult to perform (Kitching and Smale, 1986). For this reason, a history of no clinical disease in the herd of origin and or zone is needed for export as recommended by the OIE (Office International des Epizooties, 1996).

### 1.10. Prevention and Control

The most likely mode of entry of LSD into a new area is by the introduction of infected animals and contaminated materials (Weiss, 1968; Haig, 1957; Carn and Kitching, 1995a). Since it is considered that LSD will probably continue to be endemic after an outbreak, certain measures have been used with limited success, and these include proper hygiene, quarantine methods, slaughter policies and vaccination.

These methods/measures should be judiciously applied in order to minimize and control the spread of the disease.

Vaccination has been used successfully for prophylaxis against LSD. Four live strains of *Capripoxvirus* have been used as vaccines for the control of LSD (Capstick and Coakley, 1961; Carn, 1993); these are the (strain) Kenya sheep and goat poxvirus and a Neethling strain from South Africa. The sheep and goat pox vaccine is a freeze-dried live vaccine based on a local strain of sheep and goat poxvirus produced at the Veterinary Research Laboratory, Kabete, Kenya. It has been used in the field since 1959 also to immunize cattle against LSD (Capstick and Coackley, 1961). The authors reported that the clinical effects produced after vaccination was a transient local reaction, and very occasionally, systemic reactions but there were no generalized lesions and shedding of live virus in any form.

The first work on a homologous vaccine against LSD was carried out in South Africa, following severe epizootics of the disease in the 1940s and 1950s (Capstick and Coackley, 1961). The Neethling strain of LSDV has been used to produce an attenuated vaccine. The strain proved to be innocuous and immunogenic for cattle, although local reactions do occur in a high proportion of animals at the vaccination site (Weiss, 1968). The vaccine is a freeze-dried product produced by the Onderstepoort Biological Products, Onderstepoort, South Africa, since 1960. Vaccination causes local reaction at the site of injection in 50 % of cattle, as well as an occasional transient drop in milk yield in dairy cows (Weiss, 1968). The use of this vaccine has been the most successful means of control or reduction in the spread of LSD (Capstick and Coakley, 1961; Weiss, 1968; Carn, 1993).

Two other strains of heterologous virus have been used in cattle in the control of the outbreaks in Egypt and Israel; the Egyptians used the Romanian strain of sheep pox, and the Israelis used the RM65 strain (Davies, 1991). There were no recorded complication with the use of these vaccines and they appeared to be immunogenic in the field (Davies, 1991).

### 1.11. Effect of vaccines on semen quality

Various vaccine may affect semen qaulity in various ways. Young *et al.,* (1955) (cited by Radhakrishnan *et al.,* 1975) reported that vaccination generally may affect prenatal development. Das, (1967) (cited by Radhakrishnan *et al.,* 1975) reported that vaccination may cause a reduction in milk yield in the female. Similarly, in the male, vaccination can increase the incidence of abnormal spermatozoa and decrease spermatozoa resistance to cold shock and their metabolic activity (Radhakrishnan *et al.,* 1975). Similar studies on vaccination have been carried out in different species and breeds of animals, showing that some vaccination affect semen quality.

Reddy *et al.,* (1991), showed that vaccination of Ongole and Jersey bulls against FMD using an inactivated vaccine had an adverse effect on semen quality. There was an increase in sperm abnormalities but a marked reduction in the initial sperm motility, mean sperm concentration, live sperm count and percentage of cold shock resistant sperm. This reduction gradually became normal 80 days after vaccination. Similar studies on FMD in buffalo bulls (Singh *et al.,* 2003), Holstein Friesian and Jersey breeds of bulls (Manqurkar *et al.,* 2000) and Surti breeds (Kammar and Gangadhar, 1998) also showed that semen quality after vaccination was adversely affected but the duration of its effect differed from breeds of bulls. Vaccines used in these studies contained inactivated viruses.

Murugavel and Veerapandian, (1998), reported the effects of vaccination against blackquarter and FMD on buffalo bulls. The bulls were vaccinated simultaneously with blackquarter vaccine (formalinised and alum precipitated) and FMD vaccine (tissue culture inactivated) at different sites. The vaccination had no significant effect on sperm volume and sperm concentration but caused a reduction in the percentage of live sperm and initial motility for up to 3 weeks post vaccination while the sperm abnormalities increased for up to 7 weeks post vaccination The frequency of abnormalities returned to normal, 8 weeks post vaccination.

Semen quality has also been found to be affected by vaccination against rinderpest using live vaccines in certain breeding bulls. There was a gradual recovery to normal, 40 days post vaccination. These cross-bred bulls were maintained under semi

intensive management and vaccinated at the recommended dose with rinderpest goat tissue vaccine. The secretory activity of the accessory reproductive glands remained unaffected (Radharkrishnan *et al.,* 1975).

Dewey (2002), citing unpublished data showed that there was a decrease in the semen quality of boars after vaccination against Porcine Reproductive and Respiratory Syndrome (PRRS) using a modified live virus vaccine. Semen quality normalized 7 weeks later but there was no shedding of the vaccine virus in the semen when tested using the PCR. However, shedding of vaccine virus in the semen has been indicated using the modified live PRRS vaccine in boars (Dewey, 2002).

In a similar study, Swenson *et al.,* (1995) showed that in addition to the adverse effects of an inactivated PRRS virus vaccine on semen quality, there was also shedding of the vaccine virus in the semen and the duration of shedding varied from boar to boar. In another study, Thomas *et al.,* (1997) showed that despite the shedding of PRRSV in semen of unvaccinated boars, the level and duration of shedding was reduced in vaccinated boars after challenged when compared to the unvaccinated boars. The statement made by Thomas *et al.,* (1997) agrees with findings of Fukunaga *et al.,* (1991) and Timoney, (1992) on EAV in stallions and Swenson *et al.,* (1995) on PRRSV in boars, suggesting that vaccination may reduce or eliminate seminal shedding of virus when challenged.

Castro *et al.,* (1992) reported that there was no clear difference observed in the quality of semen collected from a boar up to 49 days post vaccination using live or attenuated vaccines against Pseudorabies (Aujeszky's Disease) in the Belgian large white boars. In that study, the authors reported that vaccine virus was not excreted in semen of boars vaccinated against Pseudorabies disease. The diagnostic test used was the virus isolation technique. The authors further elaborated on the inconsistent results reported from previous studies. One possible explanation for the inconsistency is that the presence or absence of virus may reflect strain differences in pathogenicity or that semen samples may have been contaminated at the time of collection from preputial secretions.

Reports have shown a local reaction on site of inoculation following vaccination

against LSD using the attenuated Neethling strain vaccine (Weiss, 1968), and severe clinical signs with a temperature of 40 to 41 $^{o}$C, when using a live attenuated Kenya sheep and goat pox strain 0240 vaccine (Yeruham *et al.*, 1995) in cattle. On the other hand, the same vaccine strain (Kenya sheep and goat pox strain 0240) has been used effectively without any severe clinical signs in sheep and goats (Kitching, 1986). Impairment of spermatogenesis by elevated temperature has been expressed as increased sperm abnormalities and reduced sperm output and viability (Cassady *et al.*, 1953; Johnston *et al.*, 1963; Skinner *et al.*, 1966; Ross and Entwistle, 1979; Meyerhoeffer *et al.*, 1985; Vogler *et al.*, 1993).

There has however, been no report indicating the effect of LSD vaccination on semen quality, its duration and the shedding of the vaccine virus in semen, as well as the efficacy of vaccination in preventing any adverse effects on semen quality in challenged bulls.

## 1.12. Excretion and transmission of viruses in semen

### *1.12.1. Other viruses*

The two main concerns associated with the widespread use of semen in artificial insemination are: the dissemination of undesirable genetic traits and transmission of diseases. The latter will be addressed in our present study.

Reviewed literature has shown that some viruses are excreted in bovine semen of infected bulls, and examples are FMD virus, bluetongue virus (BTV), bovine leucosis virus (BLV), bovine herpesvirus-1 (BHV-1), LSDV, human immunodeficiency virus (HIV), and bovine viral diarrhoea virus (BVDV) (Kahrs *et al.*, 1980).

Some published data has shown that some viruses may be transmitted via semen (Bowen and Howard, 1984; Meyling and Jensen, 1988). Gajendragad *et al.*, (2000) reported a case of an outbreak of FMD in regularly vaccinated cattle, and showed that semen samples collected from randomly selected animals were found to be positive for FMDV. The authors concluded that despite the presence of virus in the semen, its

role in the transmission of the disease is not known. In another study where three gilts were inseminated with semen infected with FMD virus and swine vesicular disease virus (SVD), no signs of infection occurred (McVicar and Eisner, 1977). Sellers *et al.*, (1968) also showed that FMDV is excreted in the semen of infected bulls prior to the onset of clinical signs, while Giefloff and Jacobsen, (1961) demonstrated that the virus could survive for up to one month in frozen semen.

Bovine leukosis virus was reported to be present in the semen of an 8-year bull (Lucas *et al.*, 1980), suggesting that BLV could be transmitted through the semen. In contrast to the previously reported excretion of BLV, a more recent study showed the absence of BLV in the semen of 79 seropositive bulls using PCR and Agar Gel Immunodiffusion (AGID) throughout the course of the disease (Choi *et al.*, 2002). The authors suggested that the previous positive result of BLV may have resulted from blood contamination due to traumatic collection technique resulting in tissue trauma and infiltration of leukocytes associated with inflammation.

Bovine Herpesvirus 1 was detected in the semen of two experimentally inoculated bulls before onset of clinical signs using PCR with Southern blot hybridization, virus isolation and dot-blot hybridization (Xia *et al.*, 1995). The authors concluded that PCR with Southern blot hybridization was the most sensitive of the three methods used and that PCR detected virus for the longest period. Van Engelenburg *et al.*, (1995) also demonstrated that the PCR detected BHV-1 much longer than virus isolation in the semen of eight intrapreputially infected bulls.

Bluetongue viruses have been isolated from bovine semen from a non-clinical persistent infected bull for up to 300 days after exposure (Bouters and Vanderplassche, 1964, Branny and Zembala, 1971, Breckon *et al.*, 1980, Bowen and Howard, 1984). Parsonson *et al.*, (1994), reported a study carried out using BTV serotype 11 in eight cows. Four cows were mated naturally to a bull that was reported to be shedding BTV in semen and another four cows were inoculated by intradermal subcutaneous route with the same virus strain isolated from the same bull used for mating. The authors showed that the mated cows did not show any infection to BTV but cows inoculated with the virus strain isolated from the same bull showed infection. It was concluded that there was no evidence of transmission via semen from

the bull with BTV-11. Bowen and Howard, (1984) showed when nine heifers were inseminated with 0.5 ml of processed, frozen-thawed semen which contained BTV, that six became pregnant and three became viraemic and developed antibodies. They also noted that there was no evidence of foetal infection in one heifer that became infected and pregnant, and the five heifers that became pregnant showed no signs of infection. This demonstrated BTV may be transmitted via semen.

Bovine viral diarrhoea virus has been demonstrated to be transmitted to susceptible cattle via semen (Meyling and Jensen, 1988; Niskanen *et al.,* 2002). In this study, Meyling and Jensen, (1988), reported that all twelve heifers that never had any antibodies to BVDV seroconverted within 2 weeks after insemination with semen infected with the virus, but showed no clinical signs of the disease. It was concluded that BVDV is transmitted via semen.

In addition to FMDV and SVDV already mentioned, some viruses in pigs have also been reported to be transmitted via semen. Classical Swine Fever virus (CSFV), PRRS virus and African Swine Fever virus (ASFV) are good examples (Guerin and Pozzi, 2005).

### *1.12.2. Excretion of lumpy skin disease virus in semen*

The excretion of LSDV in semen has been reported (Weiss, 1968; Irons *et al.,* 2005). Weiss (1968) demonstrated that the virus was excreted in semen for 22 days after the fever reaction following experimental infection.

Recently, Irons *et al.,* (2005) reported the presence of LSDV in semen using PCR and virus isolation and showed that the virus was detected 159 days post infection, and for a much longer period with PCR than virus isolation. The authors also showed that the semen quality had recovered in some bulls by the end of the experiment. However, no data has been published demonstrating the transmission of LSDV via semen.

Lumpy skin disease virus which is excreted in semen could lead to a source of transmission. These factors are a potential hazard in international trade of bovine semen used for artificial insemination.

Irons and Gerber, (2004) showed that LSD had a dramatic effect on semen quality, which may lead to temporary sterility following experimental infection of bulls with the virus. These authors suggested LSDV could affect semen quality by disturbance of thermoregulation in the testes due to scrotal skin thickening or by direct effects on testicular tissue and more work on the exact mechanism is required. However, there has not been any report evaluating the effect of LSD vaccination on semen quality.

## AIMS AND OBJECTIVES

The aim of the study was to investigate the use of vaccination with the Neethling strain in the prevention of dissemination of LSDV via semen.

The objectives of the study were

- Ø To determine the possibility of excretion of vaccine virus in semen and its duration of shedding.
- Ø To identify the possible effects of vaccination against LSD on semen quality.
- Ø To assess the ability of vaccination to prevent the excretion of the virus in experimentally infected bulls.
- Ø To assess the ability of vaccination to prevent the effects of a virulent strain of LSD virus on semen quality in experimentally infected bulls.

# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Overview

Twelve bulls were used in this study and purchased from herds where vaccination against LSD is not practiced. Prior to purchase, the bulls were tested for their ability to produce semen and to ensure that they were seronegative using the serum neutralization test (SNT). The animals were placed in an insect proof house where all procedures used in this study were carried out. The experiment consisted of three periods namely: (A) Period of acclimatization in which the animals were allowed to acclimatize. (B) Period of vaccination; here, six bulls were vaccinated, and (C) the period of challenge in which the twelve bulls were experimentally inoculated.

During the course of the various periods, clinical examination and rectal temperature were carried out daily. Blood samples were collected three times a week during the first two periods and every other day in the last period. Antibody detection was done using the SNT, and virus isolation (VI) was done during the period of challenge for the presence of virus. Semen samples were also collected with the aid of an electro-ejaculator three times a week during the first two periods and every other day during the last period. A PCR was done on semen samples during the periods of vaccination and challenge for the presence of either vaccine nucleic acid or virulent viral nucleic acid. Semen evaluation was done three times a week within the period of acclimatization and vaccination and once weekly during the period of challenge.

### 2.2. Experimental animals and housing

Twelve post-pubertal Dexter bulls between 11 and 16 months of age were used in this experiment. Before purchase and again before the onset of the experiment all bulls were confirmed to be seronegative using the SNT. Their ability to produce semen was determined.

The bulls were housed in groups of three in isolated pens within an insect proof house with concrete floor covered with bedding at the University of Pretoria Biomedical Research Center (UPBRC), Faculty of Veterinary Science, University of Pretoria. The bulls were provided with forage and water ad-libitum.

### 2.3. Experimental Material and Procedures

The animals were allowed to acclimatize for 2-3 weeks in the new environment before the onset of the experiment. During the acclimatization period, general handling and procedures needed in the trial were carried out to accustom the animals to the procedures and to establish baseline parameters for semen quality.

The experimental bulls were treated with antihelmintics as per the manufacturer's instruction (1% Ivermectin, Virbamec®) and an ectoparasiticide (Flumethrin). The weights of individual bulls were taken and identification was done using ear tags. Semen and blood were collected during this time at the intervals illustrated in Figure1. All procedures took place between 8 am and 1 pm.

### 2.4. Clinical examination

The bulls were regularly examined during the three periods. Clinical examination and rectal temperature was done daily during the period of acclimatization and vaccination and every other day during the period of challenge. All data were recorded in a data capture sheet as shown in Appendix A. Clinical parameters observed and recorded were the general health of the bulls, left and right sub scapular lymph nodes, rectal temperature, skin lesions and scrotal circumference.

### 2.5. Period of acclimatization

In this period, the twelve bulls were allowed to get used to their new environment as well as handling and all procedures adopted in this study. Clinical examination and rectal temperature were carried out daily; blood and semen samples were collected three times a week. Semen samples were collected on days -10, -8, -6, -3, as well as day 0, denoted as the day of vaccination, while blood samples were collected on days -10, -8, -3 and 0. To determine antibody titres, collected blood was subjected to the SNT while semen evaluation was done on the semen samples. Days at which individual samples were evaluated are as shown in Figure 1.

### 2.6. Period of vaccination

On the first day of this period, denoted as day 0, six bulls were vaccinated using the attenuated Neethling vaccine called lumpy skin disease vaccine for cattle (Onderstepoort Biological Products Ltd., Onderstepoort, Pretoria, Republic of South Africa). The material was reconstituted according to the manufacturer's instructions. A 5 ml dose was administered subcutaneously (SC) to each of the six bulls. Thereafter, collections of blood and semen samples were carried out.

During the course of this period, clinical examination and rectal temperature was done daily and blood and semen samples collected twice a week. Semen samples collected during this period were tested using the PCR on days 7, 11, 14, 18 and 21 while semen evaluation was done every 3-4 days from day 4 to day 42 post vaccination (p.v.) Blood samples collected were tested using the SNT on days 4, 7, 11, 14, 18, 21, 25, 28 and 48. Three weeks after the first vaccination, none of the bulls had detectable titers on SNT. Since effective protection of bulls was a requirement; the six animals were vaccinated again on day 21. A different batch of vaccine was used in order to reduce the likelihood of vaccine failure. Blood and semen samples were then collected and evaluated as previously described. Days on which all these procedures were done in this period are as shown in Figure 1.

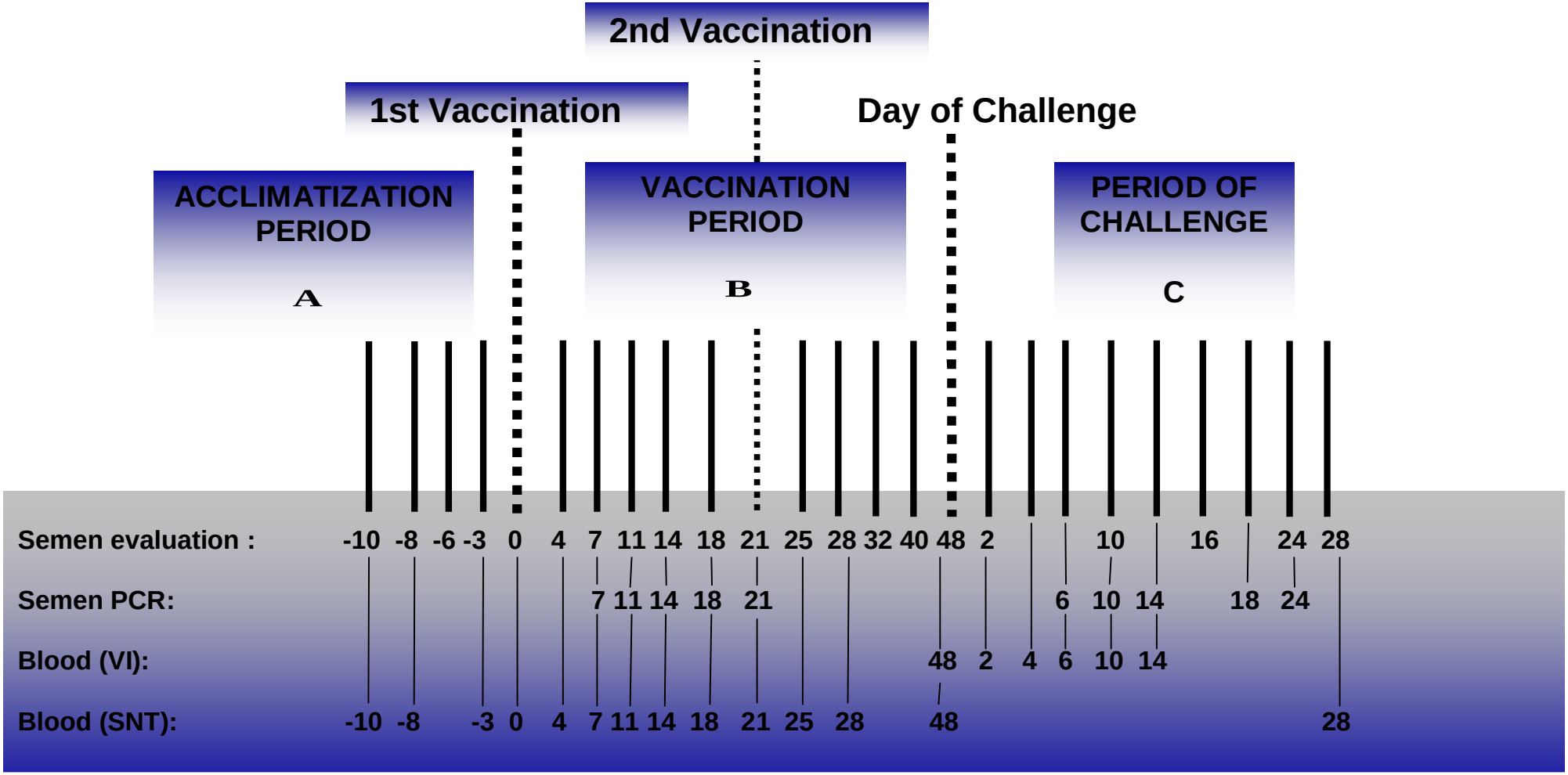


Figure 1. Summary of experimental procedures for the vaccinated bulls.

## 2.7. Period of challenge

### *2.7.1. Experimental infection*

Both groups of bulls i.e. six vaccinated and six unvaccinated bulls were experimentally infected with a virulent field strain of LSD virus on the first day of this period, this being day 48 after vaccination (Figure 1). Therefore, day 48 p.v. corresponds to day 0 post inoculations (p.i.) which denotes day of experimental inoculation (Figure 1). The experimental material used for inoculation was a virulent, South African, 3 times passaged, field isolate strain V248/93 of LSD virus. This was obtained from the Department of Veterinary Tropical Disease (DVTD). It was harvested on bovine dermis cell monolayers, at a titre of $10^5$ $TCID_{50}$ /ml. Both the six vaccinated and six unvaccinated bulls were inoculated intravenously (IV) with 2 ml of this virus suspension.

Clinical examination as well as rectal temperature was done every other day. Heparinised blood samples were collected for VI and serum collected for SNT. The blood samples were tested using VI on days 4, 6, 10 and 14 p.i. and SNT for antibody detection on day 28 p.i. denoting end of the trial. Semen collected was tested using PCR for the presence of viral nucleic acid on days 6, 10, 14, 18 and 24 p.i. Semen evaluation was done once weekly on days 10, 16, 24 and 28 p.i. Days at which procedures were done on various samples are as shown in Figure 1.

## 2.8. Semen and blood collection

Semen samples were collected with the aid of an electro-ejaculator (EL Torro, Electronic Research Group, Midrand, Republic of South Africa). This was achieved by first restraining the bull in a crush with minimal movement, using cross bars placed cranially and caudally. The preputial hairs were cut short and cleaned with water to prevent contamination of the collected semen. Feaces were removed during rectal palpation to allow good contact of the rectal probe of the electro-ejaculator on the rectal mucosa. The accessory glands were palpated and massaged to pre-stimulate the animal before electrical stimulation began.

The rectal probe of the electro-ejaculator was lubricated and inserted into the rectum with the electrode placed ventrally to touch the rectal mucosa over the accessory glands. The electrical stimulator was switched on and regulated from low to high until ejaculation occurred. In order to keep the semen sample viable for evaluation, the ejaculate was collected in a graduated plastic tube attached to a rubber cone placed in a warm water jacket. The initial ejaculate, i.e. pre-sperm fraction was discarded until the sperm-rich fraction was obtained. The collected semen samples were evaluated immediately. Two thirds of each semen sample collected was submitted immediately to the laboratory where it was frozen at –20 $^{o}$C for virus detection using PCR.

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 later rinsed with hot distilled water and kept in a rack to dry.

Blood samples were collected via the tail vein, using a 4.5 ml plain and heparinsed vaccutainers inserted at an angle of 90 degrees. This was achieved by raising the tail to an angle of 90 degrees and inserting the vaccutainer in a vertical manner. Heparinised blood samples collected were delivered to the laboratory for VI and plain (clotted) samples for SNT.

## 2.9. Diagnostic methods

### *2.9.1. Serum virus neutralization test*

The SNT was done by the Virology laboratory of the Department of Veterinary Tropical Disease (DVTD). This was done by using a 96-well, flat bottomed cell culture microtitre plate. The test sera were diluted to 1:5 in MEM containing 5% foetal calf serum and 0.05 ml gentamycin (stock 50 mg/ml) and inactivated at 56 $^{o}$C for 30 min. A series of two-fold dilutions of the inactivated test serum was prepared and 100 µl of sera were added to the wells. The titre of the LSDV used was determined and 100 µl of a 100 $TCID_{50}$ were added to each well.

As cell control, 200 µl of MEM was added to 12 wells. For antigen 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 then incubated at 37 $^{o}$C in an incubator containing 5% $CO_2$. Using an inverted microscope, the monolayers were examined daily 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 results were recorded accordingly.

### *2.9.2. Virus isolation*

Virus isolation was done according to the standard operating procedures of the Virology laboratory, DVTD. Bovine dermis cells at ± 50% confluence 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+) and 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. Isolates were stored at -70 $^{o}$C.

### *2.9.3. Polymerase chain reaction (PCR)*

The PCR was done by the Biotechnology laboratory of the DVTD. The extraction method used was a modification from the method described by Gubbels *et al.,* (1999) and Schwarts *et al.,* (1997). A volume of 200 µl semen sample frozen at –70 $^{o}$C was thawed and suspended in a 100 µl lysis buffer containing 0.378g KCl, 1ml Tris HCL (1.0 M, pH 8), 0.5 ml Tween 20 and 60% guanidine thiocynate. Protein bands were digested by adding 1 µl of Protienase K (20 mg/ml Invitrogen) sample. The samples

were then incubated at 56 $^{o}$C overnight and were heated for 10 min at 100 $^{o}$C to denature the enzyme.

A solution of Phenol:Choroform:Isomylalcohol (25:24:1, v/v, Invitrogen) was added to each sample at a 1:1 volume and mixed while incubating at room temperature for 10 min. After centrifugation (13 000 rpm for 15 min) the upper, aqueous phase was collected and two volumes of ice cold, 100% ethanol was added. The sample was then place at –20 $^{o}$C for 1 hour, centrifuged at 13000 rpm for 15 min and the pellets washed with 70-75% etanol. After centrifuging at 13000 rpm for 1 min, the supernatants were discarded and the pellets were dried thoroughly. The pellets were suspended in 30 µl of distilled water.

The semen samples were tested by the PCR using primers developed from the gene for the viral attachment protein (Ireland and Binepal, 1998), and had the following sequences:

Forward primer: 5'-d TTTCCTGATTTTTCTTACTAT 3'
Reverse primer: 5'-d AAATTATATACGTAAATAAC 3'
The size of the amplicon was 192bp.

A Platinium® Quantitative PCR SuperMix-UDG (2X) reaction mixture was used for amplification of nucleic acid templates. The mixture contained deoxiribonucleotide triphosphate with dUTP, Platinium® Tag DNA polymerase for DNA amplification and urasil DNA glycosylase (UDG) for preventing reamplification of PCR carryover products. DNA amplification was carried out in final volume of 25 µl containing 12.5 µl Platinium® Quantitative PCR SuperMix-UDG, 1 µl of 0.20 mM of each primer, 9.5 µl distilled water and 1 µl DNA sample.

The reaction was carried out in a Perkin-Elmer Gen Amp 9600 PCR system. The PCR started with one cycle of 42 $^{o}$C for 2 min and 94 $^{o}$C for 10 min. This step allows the UDG component of Platinium® Quantitative PCR SuperMix-UDG to remove any urasil residues from the PCR mixture. The initial cycle was 94 $^{o}$C for 1 min, 50 $^{o}$C for 30 seconds and 72 $^{o}$C for 1 min, and a final elongation step of 72 $^{o}$C for 1 min to complete the extension of the primers.

A positive control of bovine semen spiked with LSDV was used, a negative semen control consisting of bovine semen as well as a water control were included in the PCR. Amplified products were analyzed using a 100 bp DNA ladder (Whitehead Scientific Ltd.) 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.

All laboratory procedures were done according to the standard operating procedures of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort.

**2.10. Semen Evaluation**

Semen collected was evaluated both macroscopically and microscopically. During macroscopic evaluation, the volume, odour, colour, consistency, and the pH of semen samples collected were recorded. This examination was done visually; the pH was recorded using a pH paper. Materials used were first pre-warmed on a warm stage at a temperature of 37 $^{o}$C before coming in contact with the collected semen.

Mass motility was determined by placing a drop of semen on a cover slip and inverted on warm slides. Examination of droplets for mass motility were carried out under phase contrast microscopy at low power, and scores ranging from 0 – 5 were given. Analysis was done and recorded in a data capture sheet (Appendix B). The evaluation of mass motility which ranges from 0-5 is classified on a six point scale according to the standard operating procedure of the Section of Reproduction, Faculty of Veterinary Science, Onderstepoort. University of Pretoria, South Africa;

0 - No movement other than Brownian movement.

1 - Slight movement of individual sperm.

2 - Distinct local movement of individual sperm but no waves.

3 - Slow round wave mostly seen in the middle of the droplet.

4 - Strong waves reaching the end of the droplet.

5 - Strong waves reaching the edge of the droplet, forming whiplash effects at the edge.

Individual sperm motility was done by diluting semen samples with a semen extender which constitute Moleculed water (Bodene (PTY) Ltd. Port Elizabeth, South Africa), Equex STM (Nova Chemical Sales, Scituate, MA, USA), egg yolk and Tryladyl (Taurus Co-operative, Pretoria) and reconstituted in the Section of Reproduction, Faculty of Veterinary Science, Onderstepoort. The dilution of the semen extender with the semen sample is at the ratio of 1:4 depending on the thickness of the semen sample. A semen drop was placed on a cover slip inverted on a glass slide, and 10 microscopic fields were examined. Phase contrast microscopy (Olympus Optical Co. Ltd.) using 200x magnification was used for assessment. Observations were scored as average percentages of progressively motile sperm (relatively straight, linear motility), abnormally or aberrant motile sperm (oscillatory, tight, circular and reverse) and non-motile sperm. All analysis was done and recorded in a data capture sheet as shown in Appendix B.

Thin smears of each collected semen sample were made for morphological study on slides with frosted ends on which the bull's details and date were inscribed in pencil. Smears were done by diluting a drop of the individual semen samples with 2-3 drops of warm Eosin/Nigrosin (pH 8.4) and drying the slide with a hair drier. All smears were left on a warm stage to dry out completely. Slides thereafter were viewed under a light microscope using a x200 magnification to find a suitable area of good quality on the smear to evaluate. This area was now evaluated under immersion oil using x1000 magnification and phase contrast. One hundred sperm cells were counted with a sheep-counter and evaluated as they came into view. Data was recorded in a data capture sheet as shown in Appendix C. Specific cell abnormalities were identified using classification of Barth and Oko, (1984) as adopted by the standard operating procedures of the Section of Reproduction, Faculty of Veterinary Science, Onderstepoort , University of Pretoria .

Semen concentration was done using a Neubauer haemocytometer (Merck NT Laboratory Supplies (PTY) Ltd.). The sperm count was determined using the method described by the European Society of Human Reproduction and Embryology Monographs (2002).

### 2.11. Data capture and analysis

The results of clinical examination were recorded in the data capture sheet adopted for this study (appendix A), and analyses were done descriptively. Data on semen quality recorded on each day of semen collection from the six vaccinated bulls were as follows: seminal volume, pH, mass motility, linear motility (%), sperm concentration, total sperm count, percentage normal sperm and percentage live sperm. Data obtained from linear motility and morphology were analyzed using repeated measures analysis of variance (ANOVA) to determine whether days of collection had any effect on the parameters of individual bulls. Once established that there was no significant difference ($P>0.05$) within the variables and that days had no effect on the variables, the mean percentage of the six bulls on each day was established and analysed for any significant difference ($P>0.05$). Thereafter, all mean percentages in the various periods were pooled together and a paired comparison between periods were done using 'T' test for the establishment of significance ($P<0.05$). This was to establish any significant difference in regards to vaccination and experimental inoculation on parameters in question. All statistical analyses were conducted using the Statistical Analysis System software package (SAS®, Cary, NC, 2004).

# CHAPTER 3

## RESULTS

### 3.1 Clinical signs

***Unvaccinated bulls* (controls)**

Following experimental infection, the six bulls fell into three groups according to the severity of clinical signs. Bulls D and E (group 1) showed severe generalized lesions, bulls B and C in group 2 showed mild clinical disease and in group 3 (bulls A and F) showed inapparent infection.

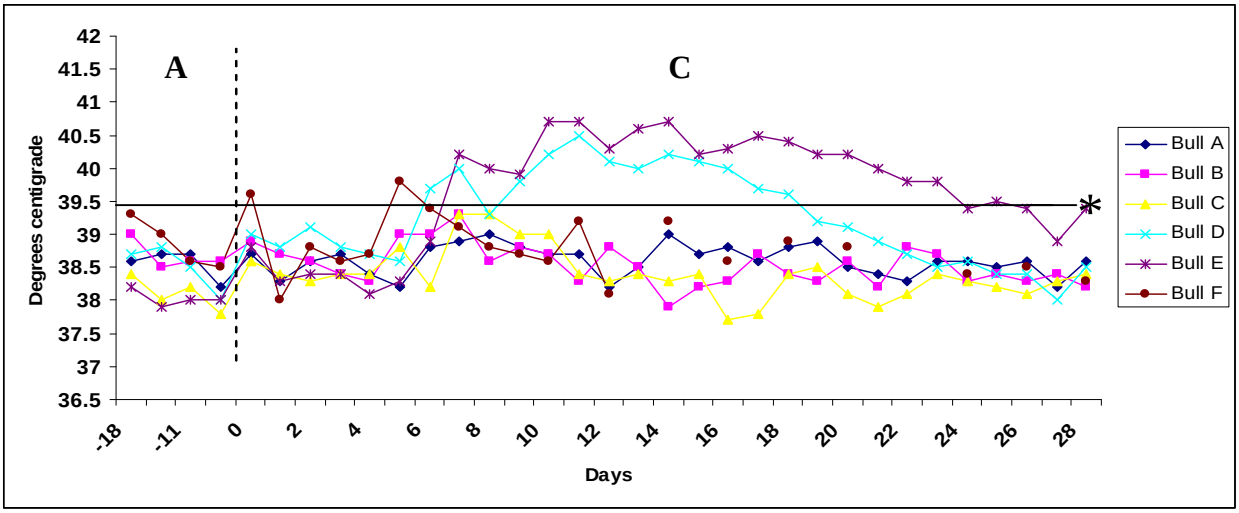


**Figure 2. Rectal temperature of individual unvaccinated bulls experimentally infected with LSD virus.**

*A = Acclimatization period; C =Period of challenge*

* *= level of 39.5 °C or more is indicative of a fever reaction.*

In group 1: There was an increase in rectal temperature following experimental infection. In bull D, the fever reaction started on day 7 p.i. and persisted for up to day 18 p.i. after which there was a steady decline, and in bull E for up to day 24 p.i. after which the trial was terminated (Figure 2). Temperatures as high as 40.3 °C was recorded on day 12 p.i. for bull D and 40.7 °C for bull E on day 11 p.i. In addition, bull D developed circumscribed nodules on the flank, paralumbar fossa, ventrum,

penis and scrotum on day 8 p.i., and became generalized five days later with corneal opacity developing on day 23 p.i. In bull E, skin nodules were first seen on both flanks on day 8 p.i. As infection progressed, the skin nodules became suppurative with ulceration around the muzzle and bucal mucosa. Corneal ulceration was also observed on day 29 p.i. The superficial lymph nodes were also enlarged in both bulls.

In group 2: Bulls A, B, F and C showed a mild form of clinical disease. The highest rectal temperature recorded in bull F was 39.9°C on day 5 p.i. Bull B was 39.3 °C on day 8 p.i. while in bull C the highest rectal temperature recorded was 39.3 °C on day 8 p.i. (Figure 2). In bull B, ulcers were observed on the muzzle and a few around the mouth on day 16 p.i, while in bull C, a few scattered nodules were observed along the flanks. Localized skin nodules were seen around the tail in bull A on day 8 p.i. only and no skin lesions was seen in bull F.

***Vaccinated bulls***

No clinical abnormalities were detected following vaccination, and clinical signs were limited to mild lymph node enlargement in the four bulls following challenge of the vaccinated bulls. Throughout the course of the trial, there was no significant rise in temperature above 39.5 °C, which is regarded as fever (Figure 3).

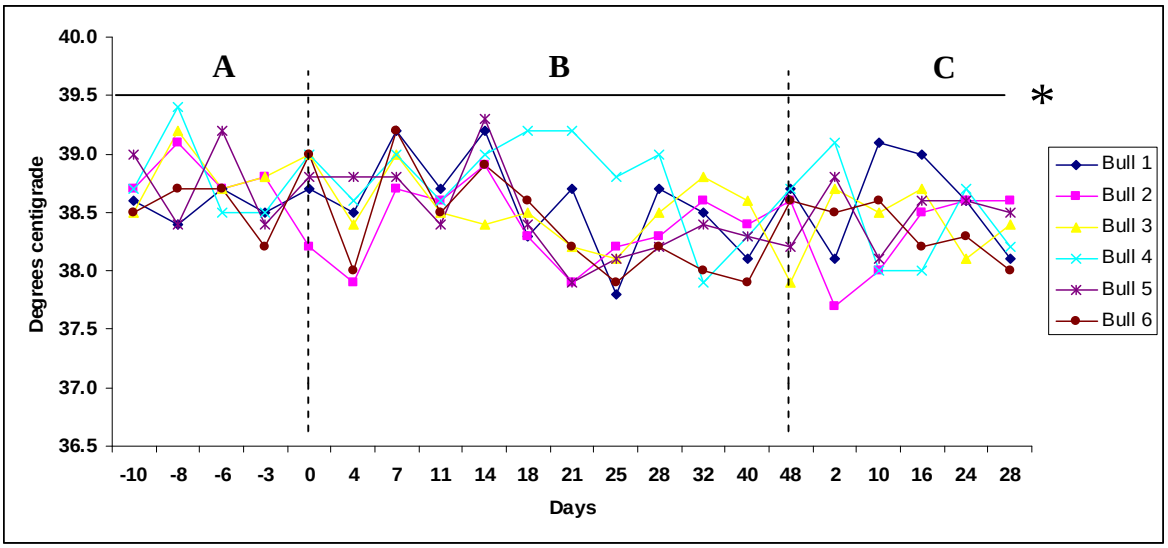


**Figure 3: Rectal temperature of individual bulls vaccinated and experimentally infected.**

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

### 3.2. Virus isolation

#### Heparinized blood samples

***Unvaccinated bulls***

In group 1, virus was isolated from blood samples from bull D on days 9, 11, 13, 15, 17, 19 and 21 p.i. In bull E, virus was also isolated from blood on days 9, 11, 13, 15, 17, 19, 21 and day 23 p.i. (Table 1).

In group 2, virus was isolated from blood samples from bull A on days 15, 17 and 19 p.i. while virus was not isolated from blood samples from bull B (Table 1). In bull C, virus was only isolated from blood samples on day 7 p.i. while in bull F, virus was isolated on days 11, 15 and 25 p.i. (Table 1).

**Table 1. Virus isolated from heparinized blood samples following experimental infection of unvaccinated bulls, as determined by virus isolation on cell cultures.**

**Days**

| Bulls | 7 | 9 | 11 | 13 | 15 | 17 | 19 | 21 | 23 | 25 | 27 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | - | - | - | - | + | + | + | - | - | - | - | - |
| **B** | - | - | - | - | - | - | - | - | - | - | - | - |
| **C** | + | - | - | - | - | - | - | - | - | - | - | - |
| **D** | - | + | + | + | + | + | + | + | - | - | - | - |
| **E** | - | + | + | + | + | + | + | + | + | - | - | - |
| **F** | - | - | + | - | + | - | - | - | - | + | - | - |

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

***Vaccinated bulls***

None of the vaccinated bulls were found to be viraemic after experimental infection on days 0, 2, 4, 6, 10 and 14 as determined by virus isolation on cell cultures.

### 3.3. Serum virus neutralization test

***Vaccinated bulls***

Despite the low antibody titre, three of the vaccinated bulls were serologically positive by day 40 p.v. which is at the time of experimental infection and four bulls by the end of the trial. Bull 3 was found to be negative at the end of the trial having had a titre of 1.0 at day 40 p.v. This is shown in Figure 4.

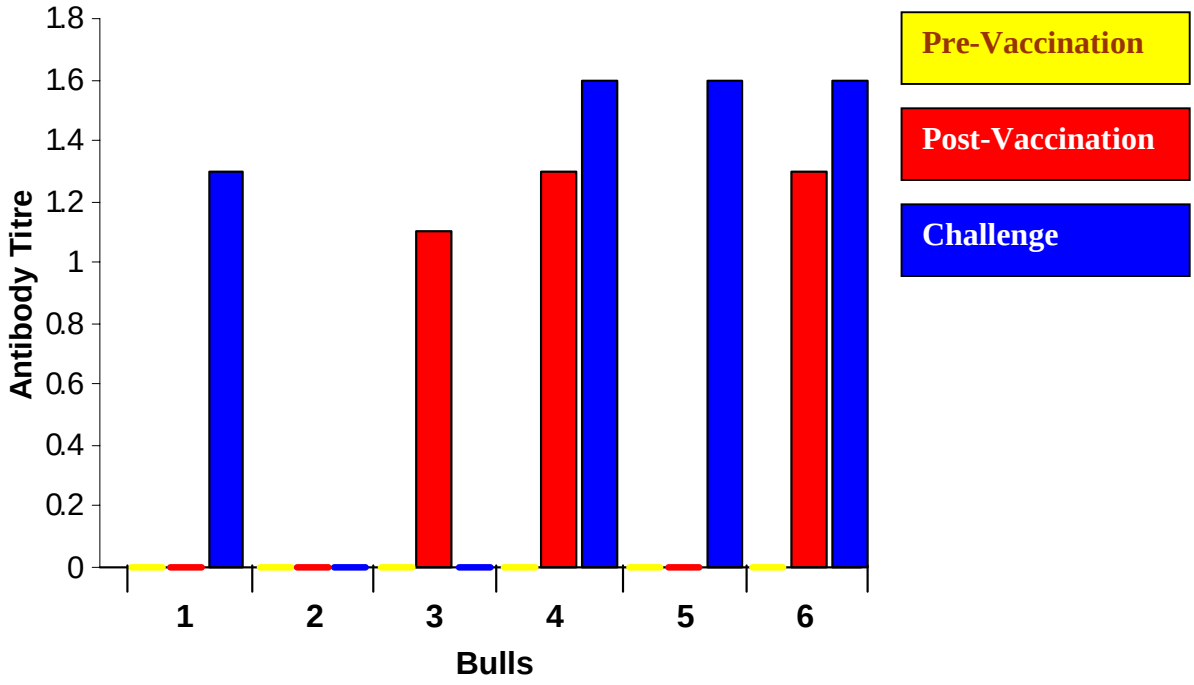


**Figure 4. Serum neutralization titres of the six vaccinated bulls.**

### 3.4. Polymerase chain reaction

#### Semen samples

***Unvaccinated bulls***

The semen of all bulls tested positive on one or more occasions using the PCR. In group 1, viral nucleic acid was detected in bulls D and E from day 10 p.i. until day 28 p.i. which was the termination day for the trial (Table 2). The viral nucleic acid was first detected on day 10 p.i., which corresponds to the day when the rectal temperature was the highest (Figure 2).

In group 2, bull A tested positive only on day 18 p.i. and bull B tested positive only on day 27 p.i., all other samples were negative (Table 2), and in this bull, virus was not isolated on culture from blood samples (Table 1). Bull C tested positive on days 12, 14, 24 and 27 p.i., and became negative until the termination date while viral nucleic acid was detected in bull F only on day 16 (Table 2).

**Table 2. Presence of viral nucleic acid in semen following experimental infection, as determined by the PCR in unvaccinated bulls.**

| | | Days post infection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Bulls** | **10** | **12** | **14** | **16** | **18** | **22** | **24** | **26** | **27** | **28** |
| **A** | - | - | - | - | + | - | - | - | - | - |
| **B** | - | - | - | - | - | - | - | - | + | - |
| **C** | - | + | + | - | - | - | + | - | + | - |
| **D** | + | + | + | + | + | + | + | + | + | + |
| **E** | + | + | + | + | + | + | + | + | + | + |
| **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.

### 3.5. Semen quality

***Unvaccinated bulls***

Although the semen volume varied widely throughout the course of the trial, each bull tended to maintain a similar volume from one ejaculate to the next. There was no significant difference before and after experimental infection amongst the bull when individual values were analysed i.e. bulls A (*P = 0.233), B (P = 0.6547), C (P = 0.7656), D (P = 0.3711), E (P = 0.3075) and F (P = 0. 5778).*

***Vaccinated bulls***

The semen volume varied widely throughout the course of the trial, although each bull tended to maintain a similar volume from one ejaculate to the next. There was no significant difference in the average semen volume from the six bulls during pre-vaccination *(P = 0.0849*), vaccination (*P = 0.3666)* and challenged period *(P = 0.2327)*. With regard to the values obtained from the total sperm count, there was a dramatic trend of variation observed in all animals (Figure 5).

The sperm count was not determined on days -13, -10 and -8 in the acclimatization period and days 21, 25 and 28 in the vaccination period. Although values obtained from each bull fluctuated within the different periods and throughout the trial, there was no statistical significant difference (*P = 0.2028, P = 0.4364, P = 0.5298)* observed from the six bulls per each day of collection in the various periods.

However, since days did not have any effect on individual values obtained per bull per collection in the various periods, a mean value was established within the six bulls. Analysis of the mean values showed no significant difference; *A vs B (P = 0.0647); B vs C (P = 0.0952)* seen across periods (Figure 6).

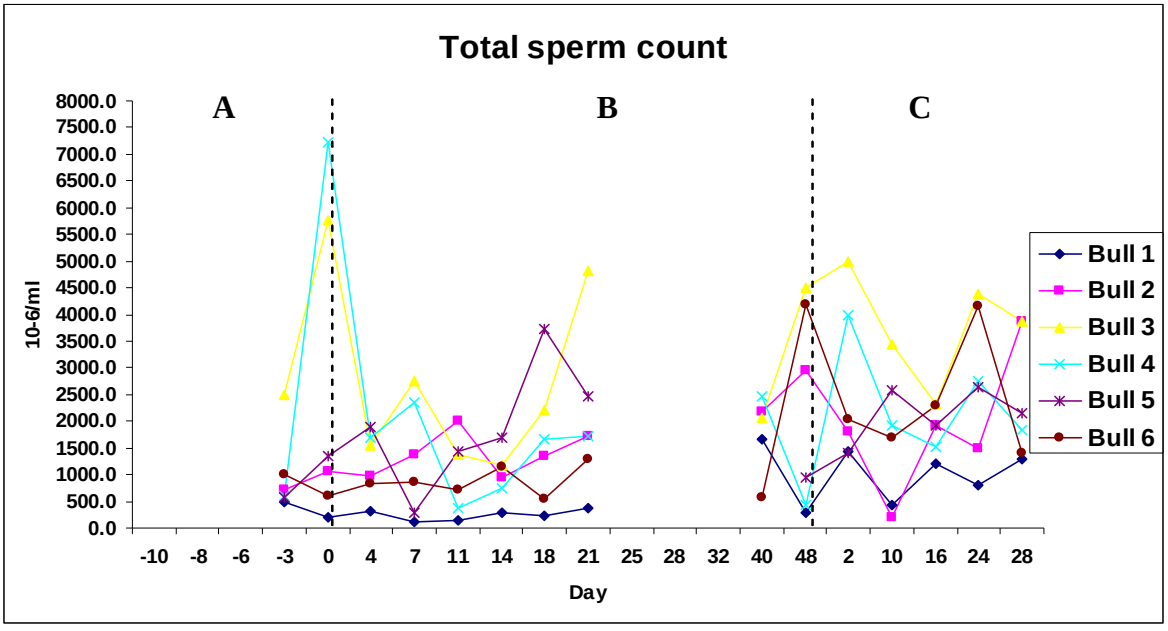


**Figure 5. Effect of vaccination and experimental infection on the total sperm count of bull's semen (*n=6*).**

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

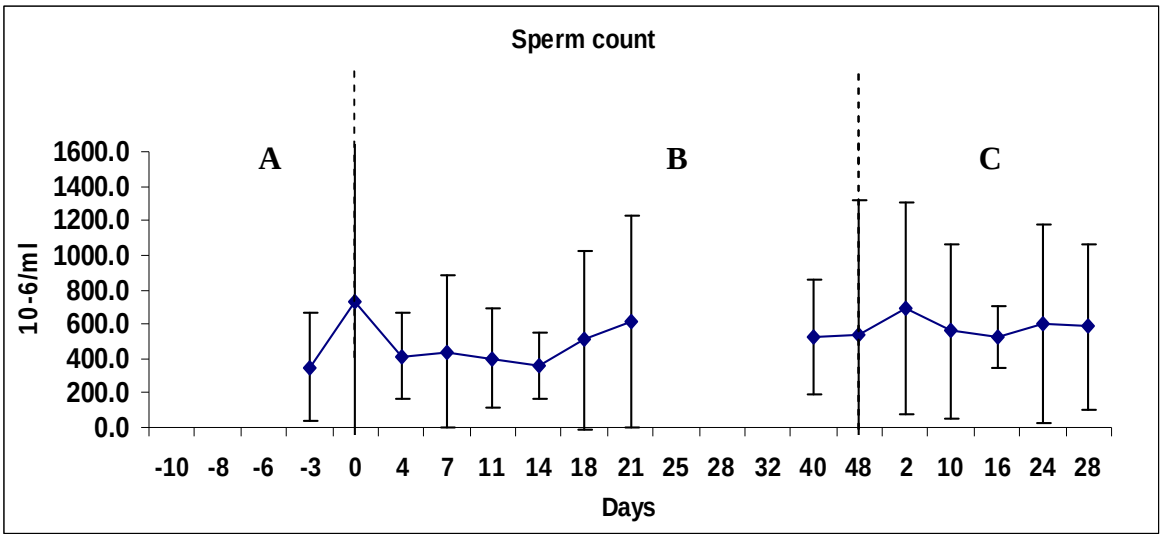


**Figure 6. Mean ± SEM sperm count of bull's semen (*n=6*).**

*P > 0.05 (A vs B; B vs C).*

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

***Unvaccinated bulls***

The values of the progressive linear motility (%) of six individual bulls after experimental infection are presented in Figure 7. While the progressive linear motility was relatively stable before experimental infection in some bulls (A, C, D, E and F), it varied dramatically from one sample to the next in bull B.

Analysis of values obtained before and after experimental infection in four bulls revealed no significant difference i.e. bulls A (*P = 0.3258), B (P = 0.6133), C (P = 0.3146) and F (P = 0. 8393)*. There was a significant difference in values obtained from two bulls after experimental infection i.e. bulls D (*P = 0.000018*) and E (*P = 0.00155*).

A mean percentage value was established from the six bulls for each day of collection (Figure 8). There was a statistically significant difference; *A vs B = (P = 0.00012)* between the mean percentage values obtained before and after experimental infection (Figure 8) for this parameter.

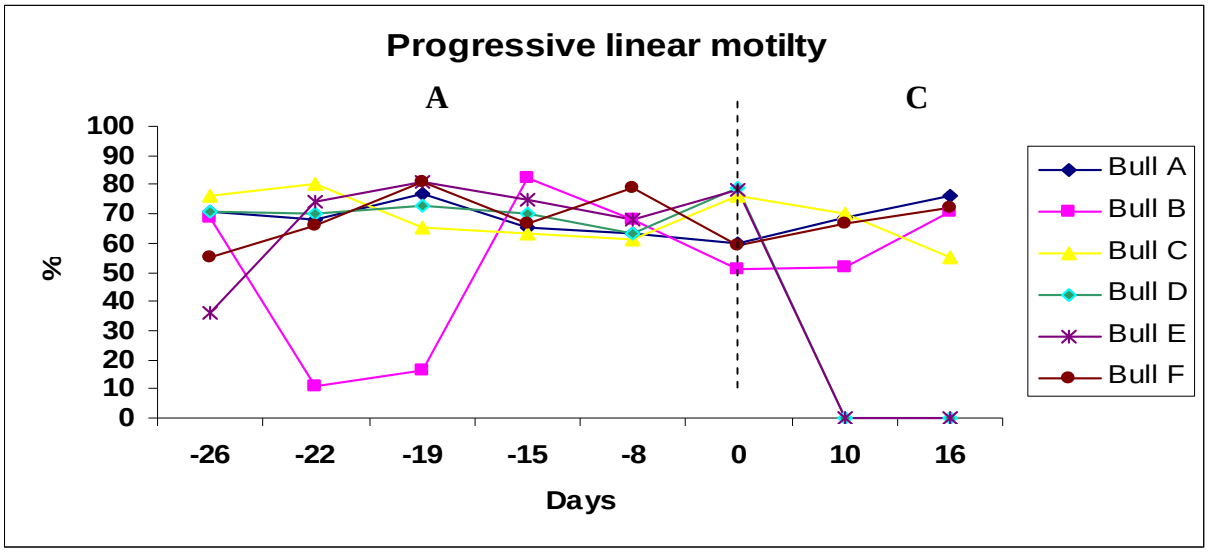


**Figure 7. Effect of experimental inoculation on progressive linear motility of unvaccinated bull's semen (*n=6*).**

*A = Acclimatization period*; *C =Period of challenge*

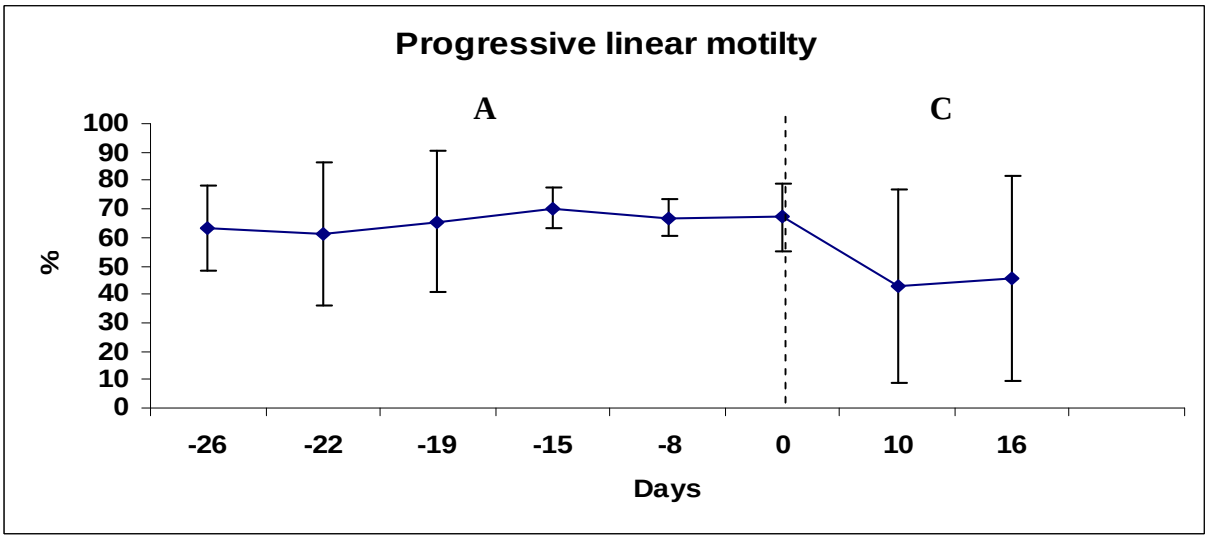


**Figure 8. Mean ± SEM Linear motility of unvaccinated bull's semen (*n=6*).**

*P < 0.05 (A vs B)*

*A = Acclimatization period*; *C =Period of challenge*

***Vaccinated bulls***

The values of the progressive linear motility (%) of six individual bulls following vaccination and experimental infection are presented in Figure 9. The linear motility of each bull varied widely throughout the study (Figure 9).

While the progressive linear motility was relatively stable in some bulls (3 and 4), it varied dramatically from one sample to the next in others (1, 5 and 6). Variations appear to be random, and not related in time to either vaccination or experimental infection. Analysis of values obtained from the six bulls revealed no significant difference in period A = (*P = 0.4829), B = (P = 0.7597 and C = (P = 0. 915).*

A mean percentage value was established for each day from the six bulls (Figure 8). There was also no statistically significant difference; *A vs B = (P = 0.089); B vs C = (P = 0.113)* between the mean percentage values obtained after vaccination and experimental infection (Figure 10) for this parameter.

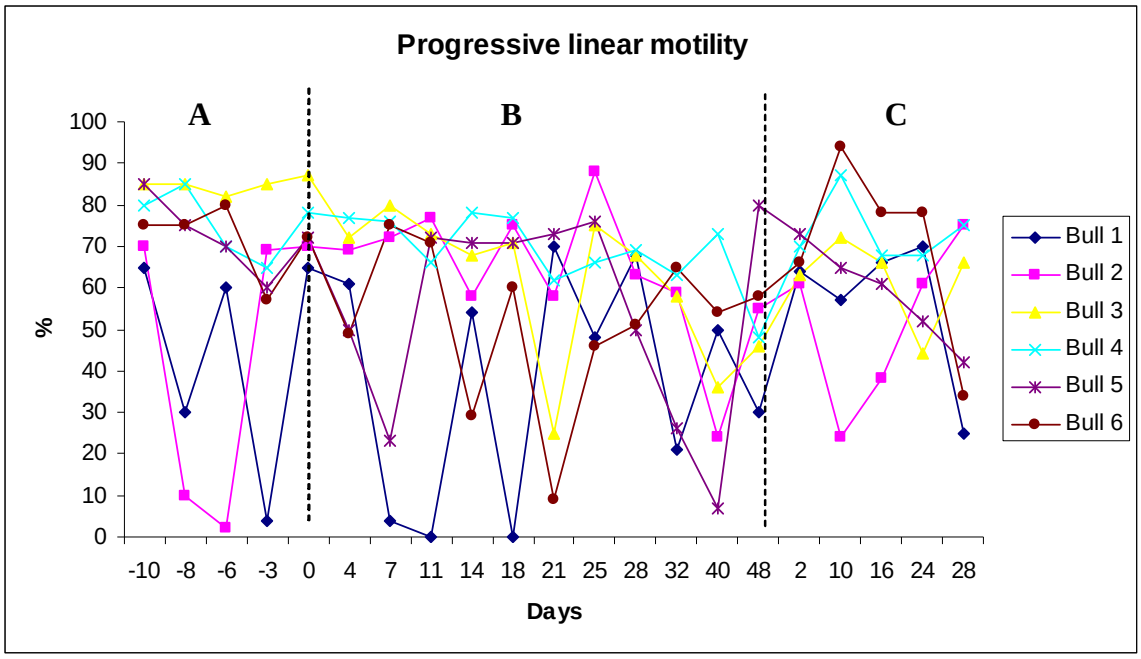


**Figure 9. Effect of vaccination and experimental inoculation on progressive linear motility of bull's semen (*n=6*).**

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

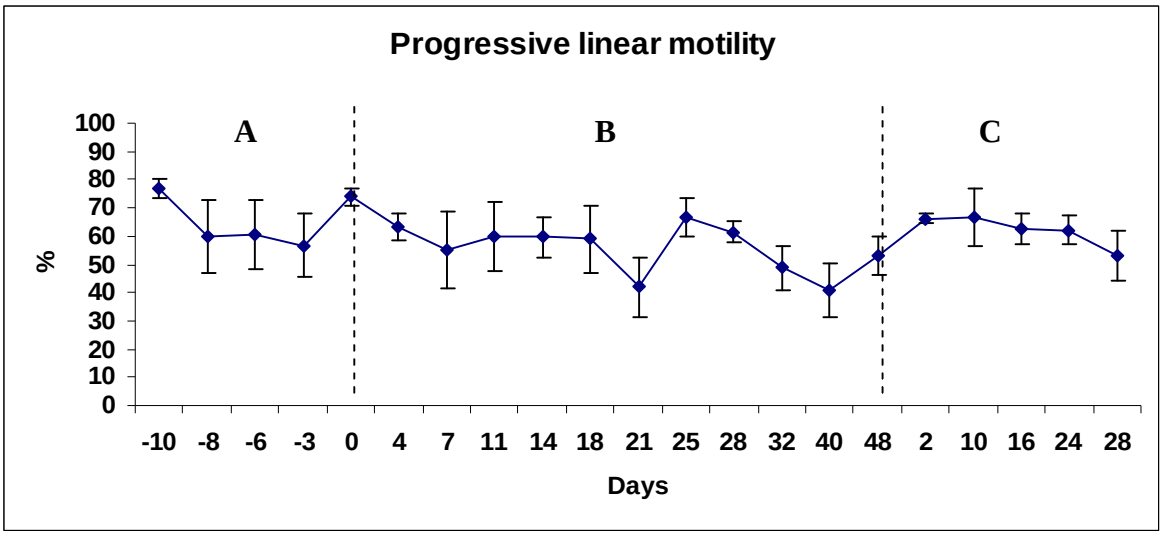


**Figure 10. Mean ± SEM Linear motility of bull's semen (*n*=*6*).**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

The mean percentages values were pooled together into the various periods and paired comparisons were done using the 'T'- test to observe whether vaccination or experimental infection had any effect on the progressive linear motility (Figure 11). Again results showed no significant difference between periods *A vs B = (P = 0.0509); B vs C = (P = 0.1092).*

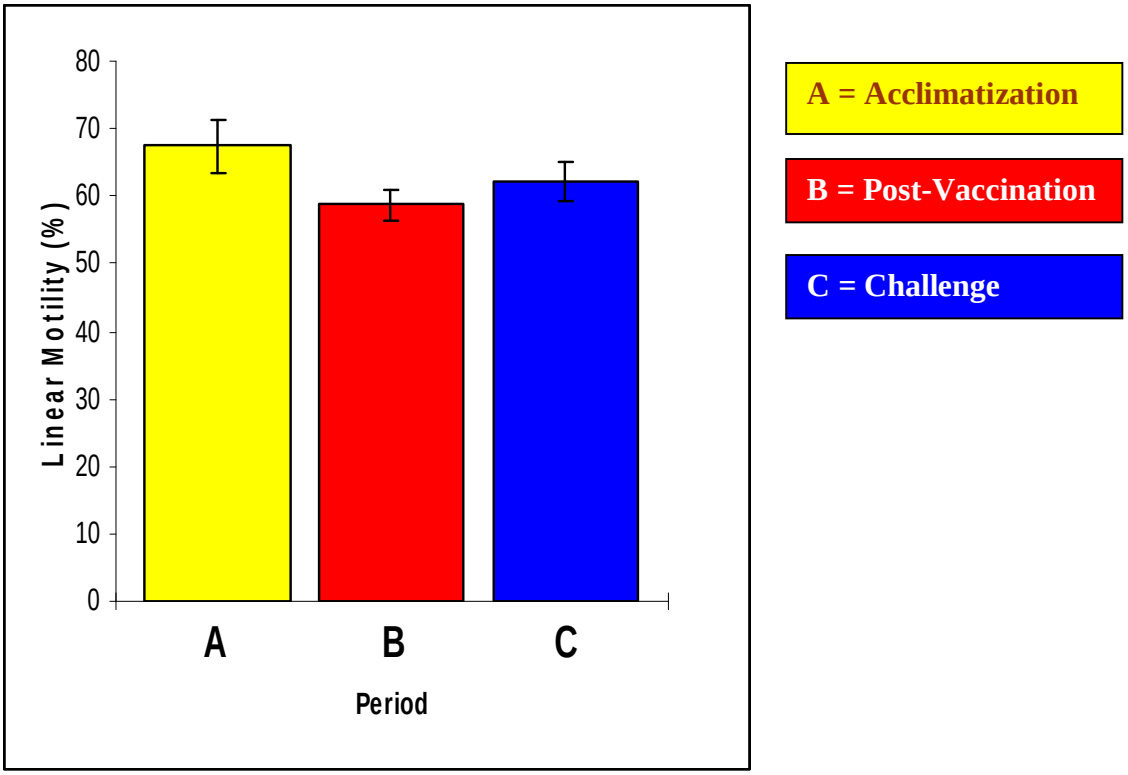


**Figure 11: Mean ± SEM Linear motility of bull's semen (*n=6*).**

*P > 0.05 (A vs B; B vs C)*

***Unvaccinated bulls***

The values of the morphologically normal spermatozoa (%) of six individual bulls after experimental infection are presented in Figure 12. Values of individual bulls were relatively stable before experimental infection in all six bulls but varied considerably from one sample to the next after experimental infection (Figure 12). There was no significant difference seen in three bulls after experimental infection i.e. bulls A (*P = 0.8825), C (P = 0.0821) and F (P = 0. 0607)* when values for each day of collection were analysed. There was a significant difference seen in the other three bulls i.e. bulls B (*P = 0.0080), D (P = 0.0216), and E (P = 0. 0183).* A mean percentage for each day of collection for the six bulls was therefore established and analysed (Figure 13). However, there was a statistically significant difference after experimental infection *A vs B = (P = 0.0214)* for the mean percentage value of the morphologically normal spermatozoa.

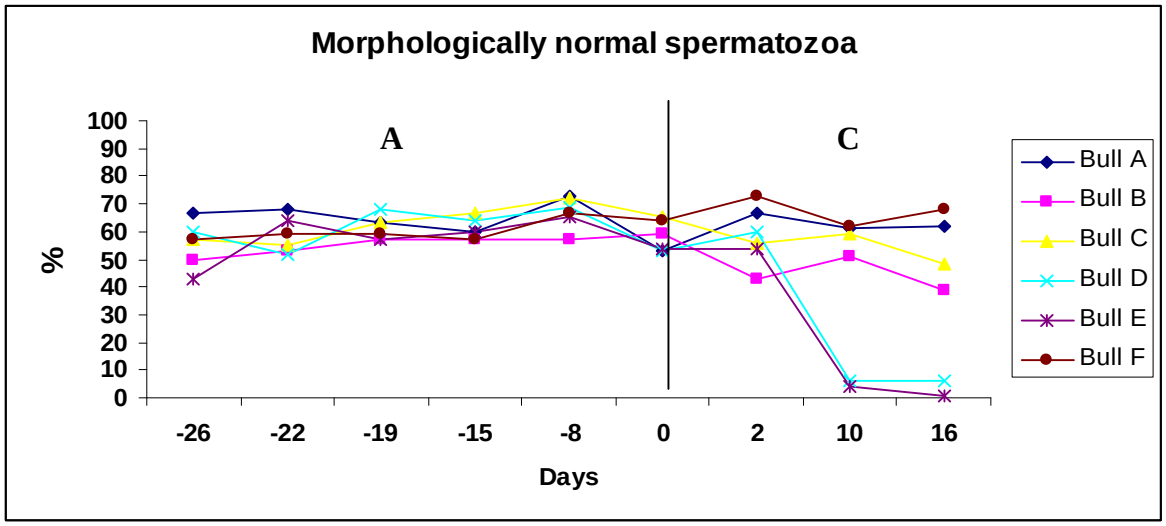


**Figure 12. Effect of experimental inoculation on progressive linear motility of unvaccinated bull's semen (*n=6*).**

*A = Acclimatization period*; *C =Period of challenge*

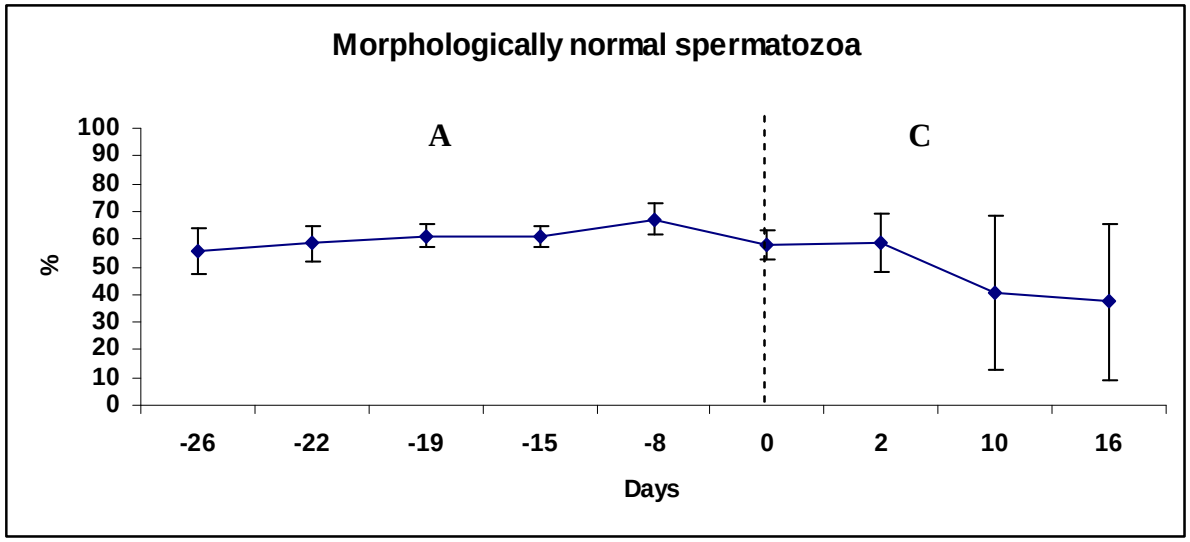


**Figure 13. Mean ± SEM Linear motility of unvaccinated bull's semen (*n=6*).**

*P < 0.05 (A vs B)*

*A = Acclimatization period*; *C =Period of challenge*

***Vaccinated bulls***

The values of the morphologically normal spermatozoa (%) of six individual bulls following vaccination and experimental infection are presented in Figure 14. Values of individual bulls varied widely at the acclimatization period but became steady

throughout the remainder of the trial (Figure 14). There was no significant difference seen in the various periods i.e. period A = (*P = 0.2062), period B = (P = 0.0652) and period C = (P= 0.5081)* when values for each day of collection for the six bulls were analysed.

A mean percentage for each day of collection for the six bulls was therefore established and analysed (Figure 15). However, there was no statistically significant difference *A vs B = (P = 0.9098); B vs C = (P = 0.9548)* observed for the mean percentage value of the morphologically normal spermatozoa.

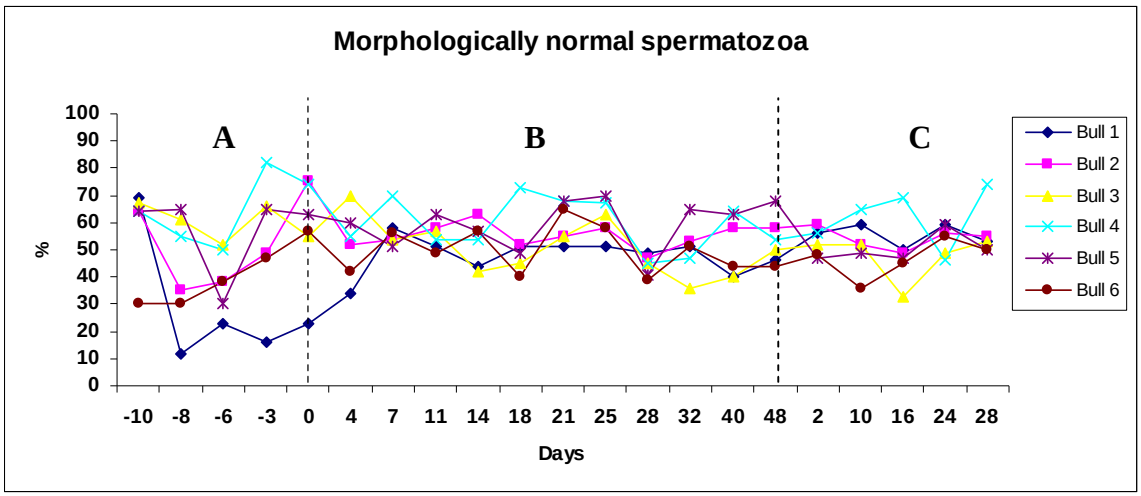


**Figure 14. Effect of vaccination and experimental infection on sperm morphology of six bulls.**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

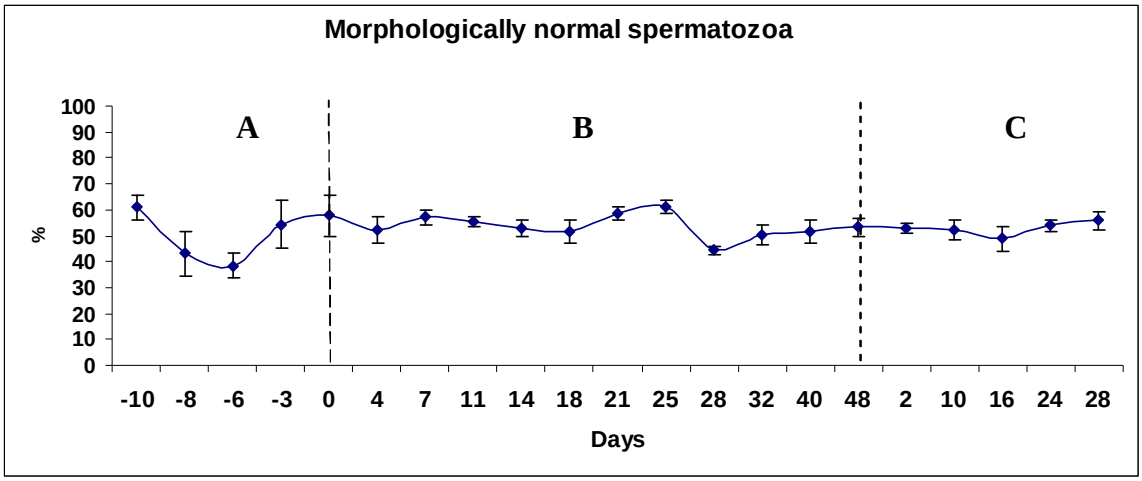


**Figure 15. Mean ± SEM Morphologically normal spermatozoa of vaccinated bulls (*n*=6).**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

The mean percentages values were pooled together into the various periods and paired comparisons were done using the 'T'- test to observe whether vaccination or experimental infection had any effect on the morphological normal spermatozoa (Figure 16). Results showed no significant difference between periods i.e. *A vs B = (P = 0.0918); B vs C = (P = 0.5422)*

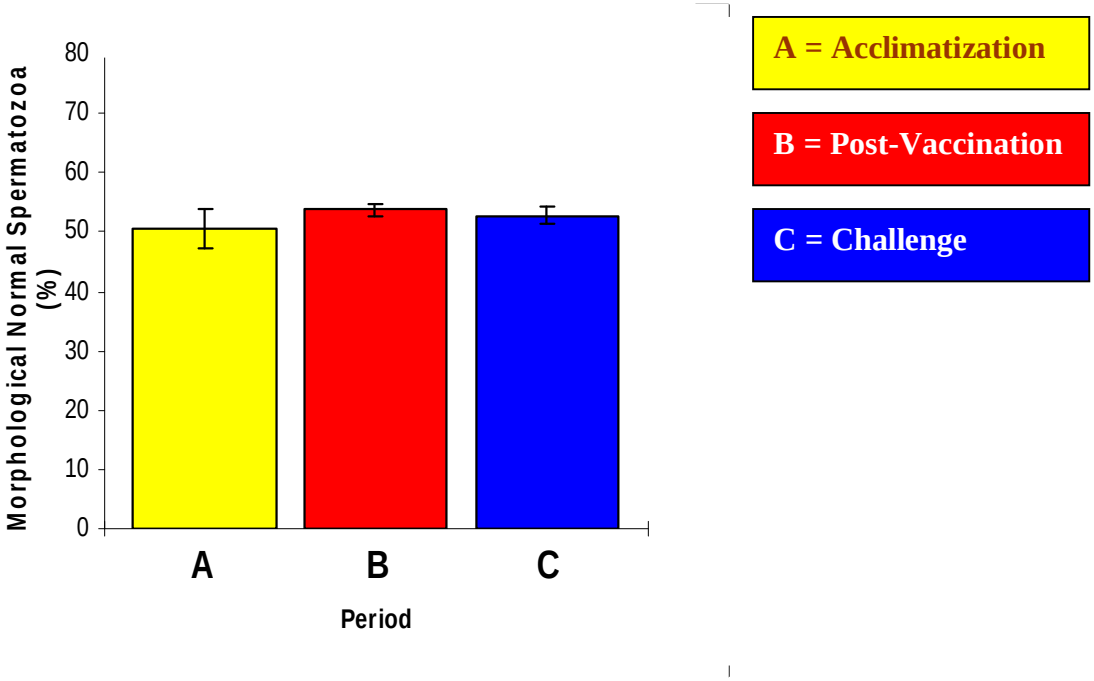


**Figure 16: Mean ± SEM Morphologically normal spermatozoa of bulls (n=6).**

*P >0.05 (A vs B; B vs C)*

The general type of sperm cell abnormalities associated with this study are summarized into the total nuclear, acrosome and flagella defects and are presented in Figure 17, 19 and 21. It is clear that values obtained from the various types of abnormalities varied considerably per bull from one sample collection to the next.

The percentage values of the total nuclear defects of six individual bulls before and after experimental infection are as presented in Figure 17. When values obtained from the total nuclear defects were analysed from the six bulls within various period to establish whether there was any difference from one sample collection to the next. Statistics showed no significant difference observed amongst the six bulls i.e. A (*P = 0.818), B (P = 0.5345), C (P = 0.09343), D (P = 0.2184), E (P = 0.1448) and F (P = 0. 8393)* after experimental infection. A mean percentage value for the nuclear defects from the six bulls was therefore established and analysed (Figure 18). However, there was no statistically significant difference *A vs B = (P = 0.4069)* observed after experimental infection.

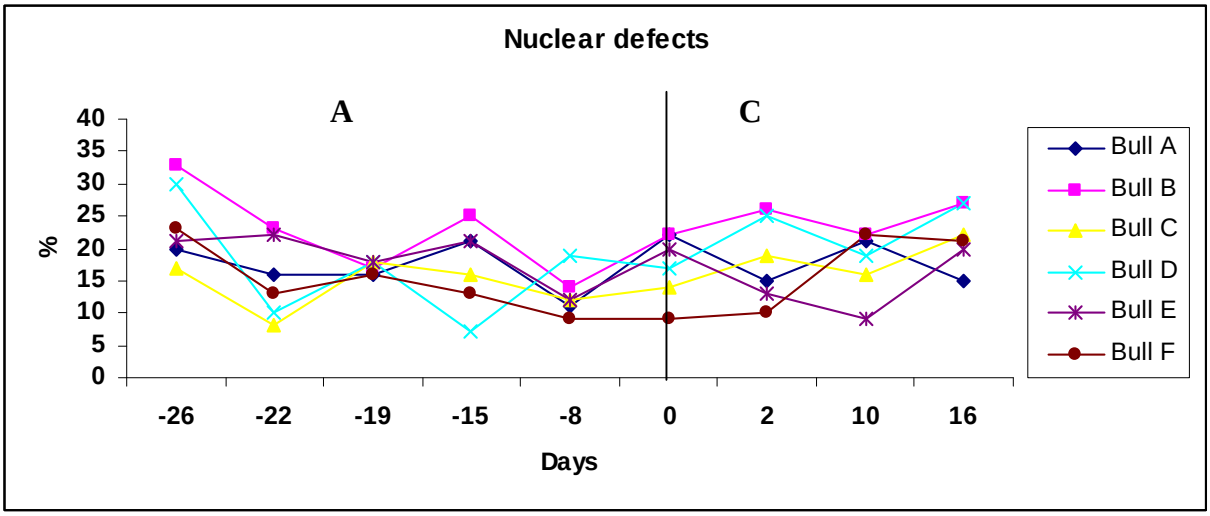


**Figure 17. Frequency of appearance of the total nuclear defects in individual unvaccinated bulls following experimental infection (n =6).**

*A = Acclimatization period; C =Period of challenge*

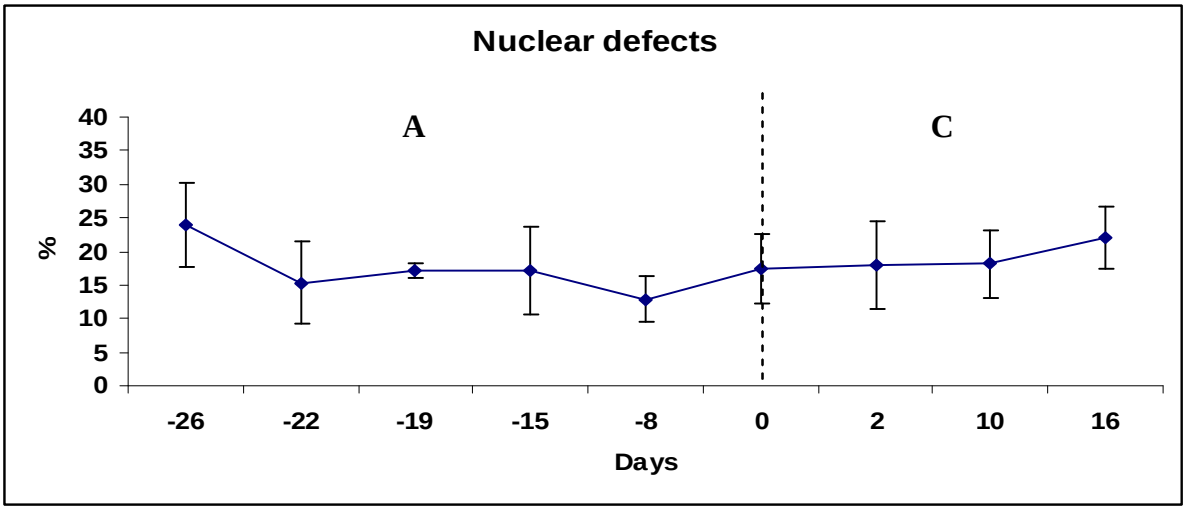


**Figure 18. Mean ± S.D Nuclear defects of unvaccinated bulls (*n*=*6*).**

*P >0.05 (A vs B)*

*A = Acclimatization period*; *C =Period of challenge*

The percentage values of the acrosomal defects of six individual bulls before and after experimental infection are as presented in Figure 19. Values of individual bulls were relatively stable at acclimatization period and remained steady until the trial was terminated in all but two bull (Bulls B and E) (Figure 19). There was no significant difference seen after experimental infection amongst the six bulls' i.e. A (*P = 0.1676), B (P = 0.2231), C (P = 0.4071), D (P = 0.1222), E (P = 0.5312) and F (P = 0. 5785).*

A mean percentage for each day of collection for the six bulls was therefore established and analysed (Figure 20). However, there was no statistically significant difference *A vs B = (P = 0.6988)* observed for the mean percentage value of the acrosomal defects when compared before and after experimental infection.

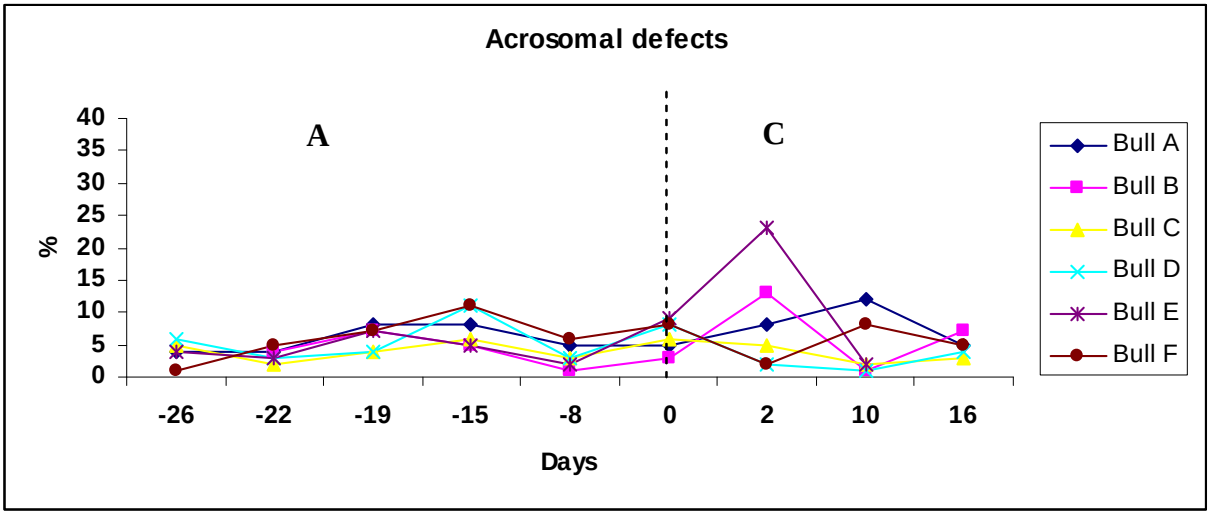


**Figure 19. Frequency of appearance of the total acrosomal defects in individual unvaccinated bulls following experimental infection (n =6).**

*A = Acclimatization period*; *C =Period of challenge*

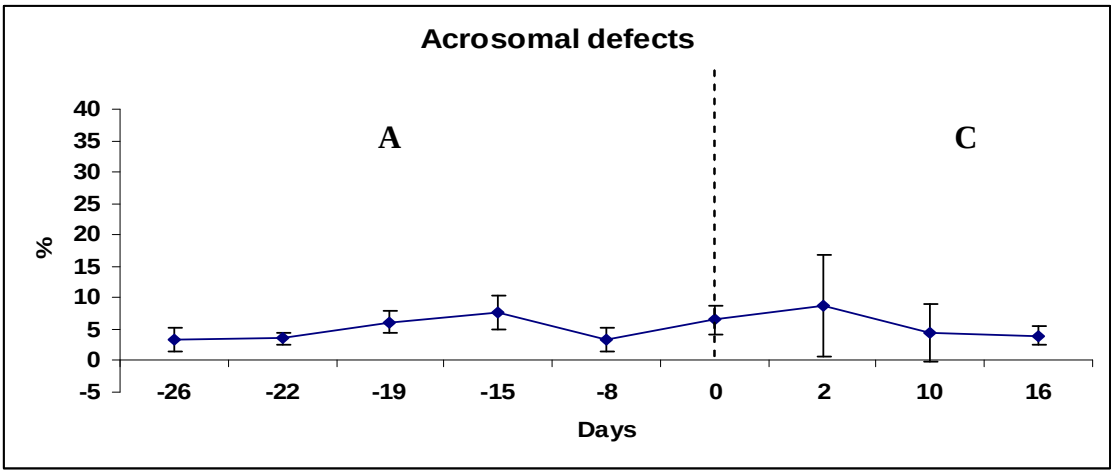


**Figure 20. Mean ± S.D Acrosomal defects of unvaccinated bulls (*n*=6).**

*P >0.05 (A vs B)*

*A = Acclimatization period*; *C =Period of challenge*

The percentage flagella defects was relatively stable in some bulls (A, B, C and F) prior to experimental infection but varied considerably from one sample to the next after experimental infection. Bulls D and E varied dramatically from one sample to the next within the pre-inoculation period and remained stable after experimental

infection (Figure 21). Analysis of values obtained from the six bulls revealed no significant difference in five bulls A (*P = 0.504), B (P = 0.1501), C (P = 0.366), D (P = 0.2542),* E (*P = 0.0647).* There was a significant difference seen in bull *F (P = 0.0135).*

A mean percentage value was established for each day from the six bulls (Figure 22). However, there was no statistically significant difference *A vs B = (P = 0.1173)* observed for the mean percentage value of the flagella defects when compared before and after experimental infection.

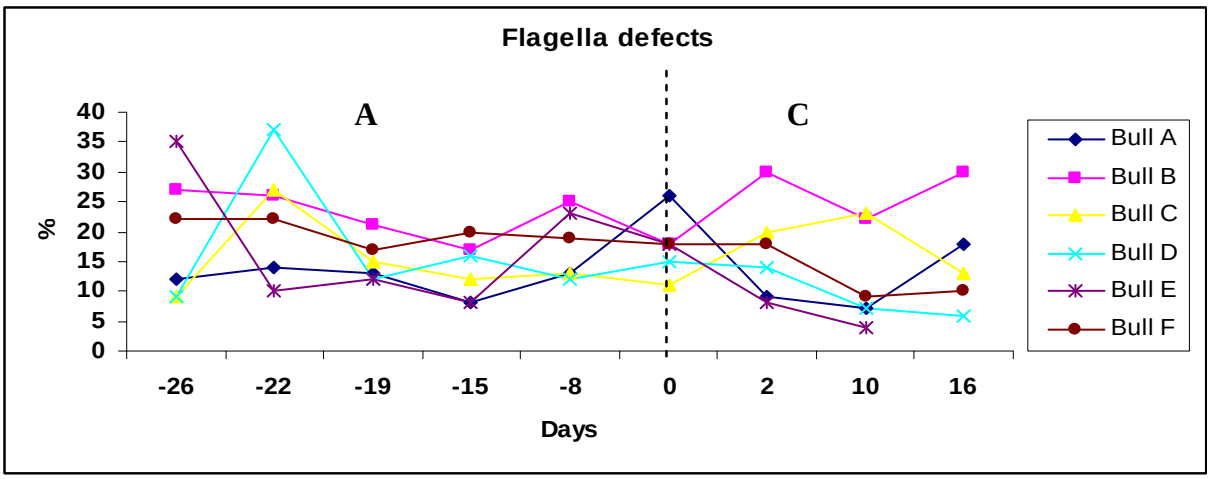


**Figure 21. Frequency of appearance of the total flagella defects in individual unvaccinated bulls following experimental infection (n =6).**

*A = Acclimatization period*; *C =Period of challenge*

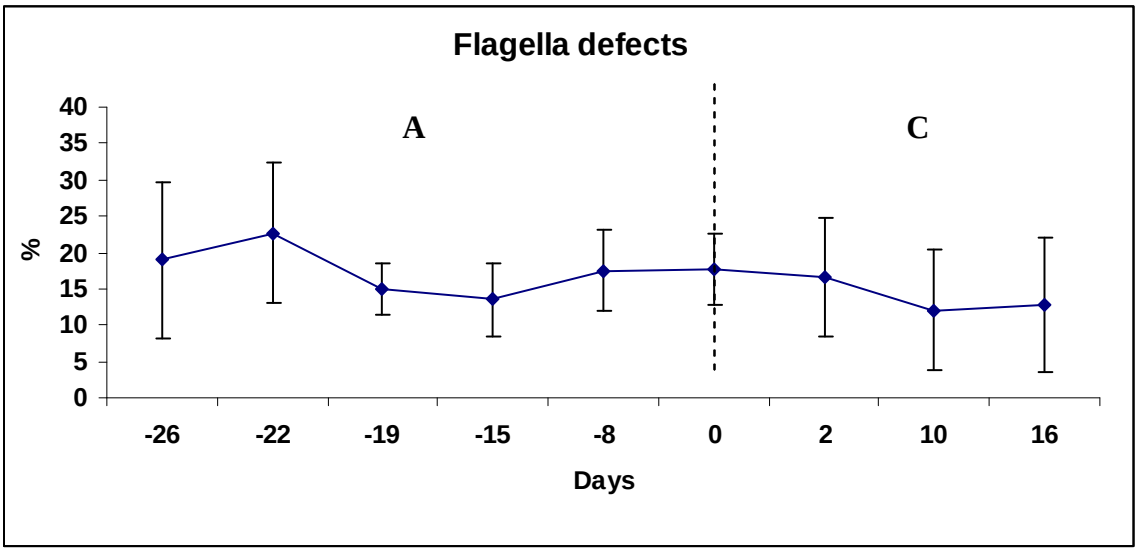


**Figure 22. Mean ± S.D Flagella defects of unvaccinated bull's semen (*n=6*).**

*P >0.05 (A vs B)*

*A = Acclimatization period*; *C =Period of challenge*

***Vaccinated bulls***

The general type of sperm cell abnormalities associated with this study are summarized into the total nuclear, acrosome and flagella defects and are presented in Figure 23, 25 and 27. It is clear that values obtained from the various types of abnormalities varied considerably per bull from one sample collection to the next. Variations appear to be random, and not related in time to either vaccination or experimental infection. However, this tends to decrease over time towards the end of the trial (Figure 23, 25 and 27).

Values obtained from the total nuclear defects was analysed from the six bulls within the various periods to establish whether there was any effect from one sample collection to the next (Figure 23). Statistics showed no significant differences observed between *period A = (P = 0.0586), period B = (P = 0.0511) and period C = (P= 0.8976).*

A mean percentage from the six bulls were therefore established (Figure 24). The mean percentage values for the various sperm cell abnormalities were analysed for the establishment of significance. However, there was no statistically significant

difference (*A vs B = (P = 0.6504); B vs C = (P = 0.1004)* observed for the mean percentage value of the total nuclear defects when compared across periods.

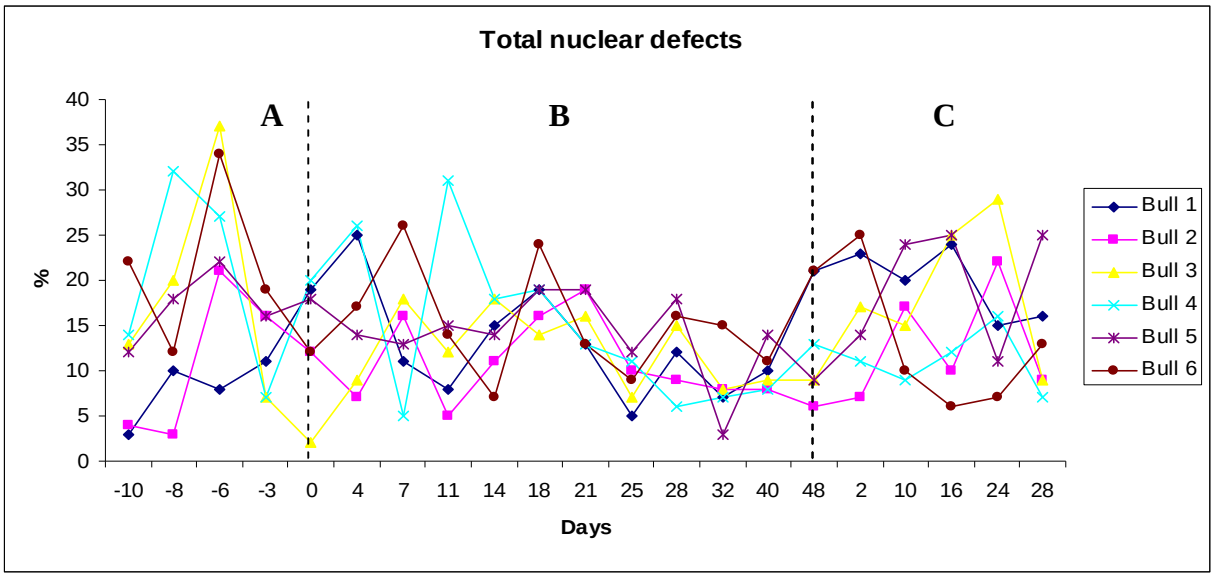


**Figure 23. Frequency of appearance of the total nuclear defects in individual vaccinated bulls following vaccination and experimental infection (n =6).**

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

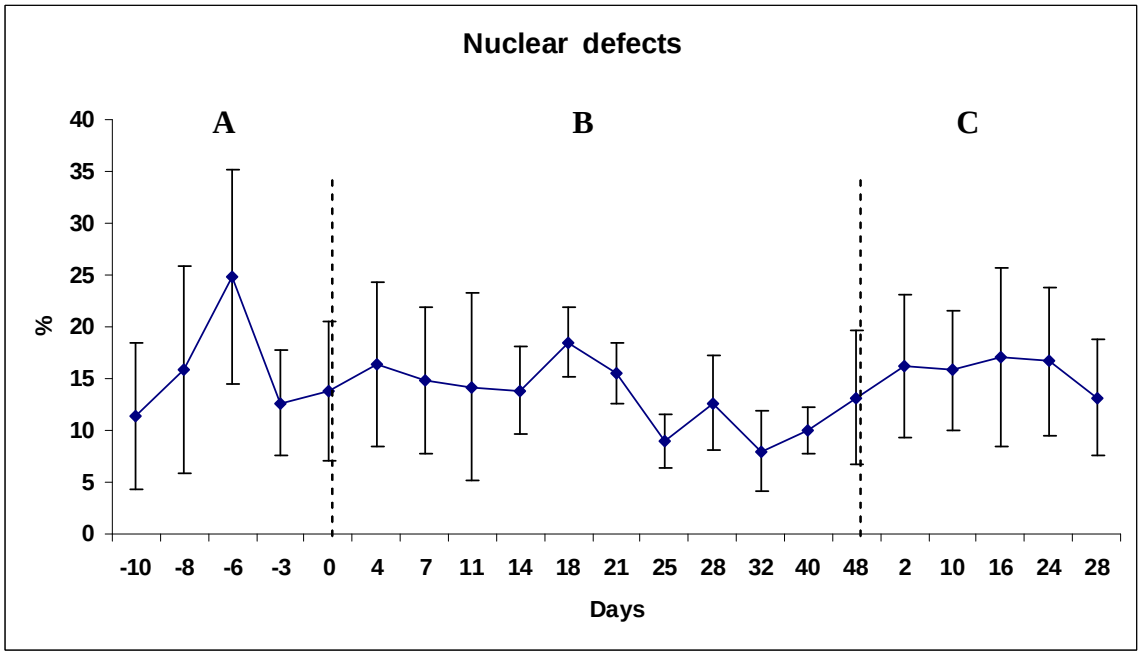


**Figure 24. Mean ± S.D Nuclear defects of bull's semen (*n=6*).**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period*; B = Vaccination period; *C =Period of challenge*

The percentage values of the acrosomal defects of six individual bulls following vaccination and experimental infection are presented in Figure 25. Values of individual bulls varied widely at the pre-vaccination period but became stable from day 21 until the trial was terminated (Figure 25). There was no significant difference seen in the various periods i.e. *period A = (P = 0.1192), period B = (P = 0.0801) and period C = (P= 0.22)* when values for each day of collection for the six bulls were analysed.

A mean percentage for each day of collection for the six bulls was therefore established and analysed (Figure 26). However, there was no statistically significant difference (*A vs B = (P = 0.6504); B vs C = (P = 0.0514)* observed for the mean percentage value of the total acrosomal defects when compared across periods.

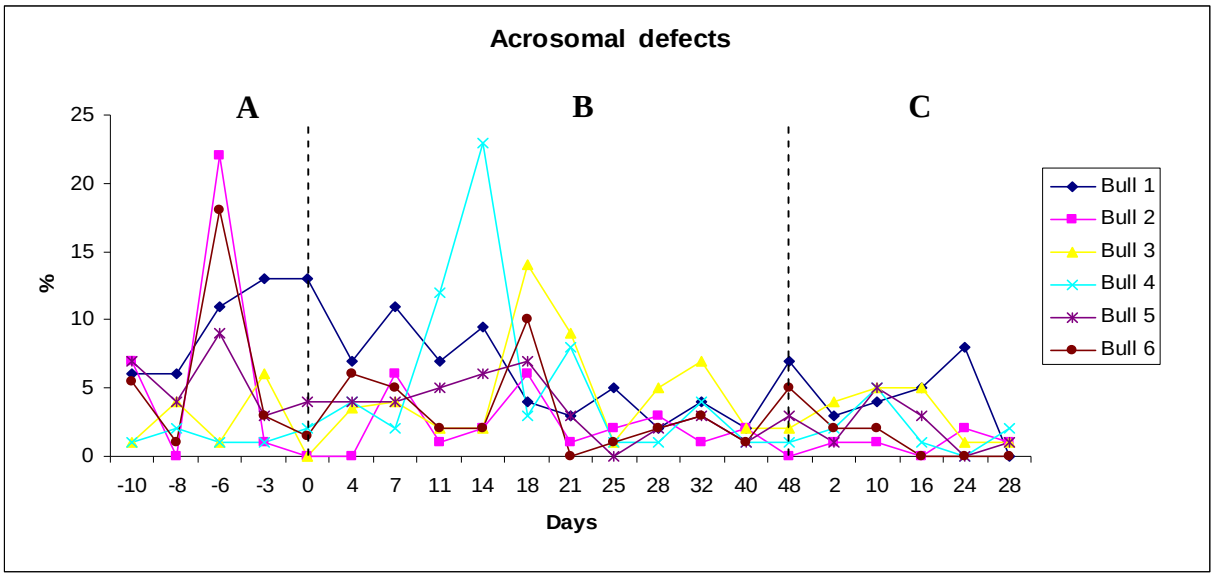


**Figure 25. Frequency of appearance of the total acrosomal defects in individual vaccinated bulls following vaccination and experimental infection (n =6).**

*A = Acclimatization period; B = Vaccination period; C = Period of challenge*

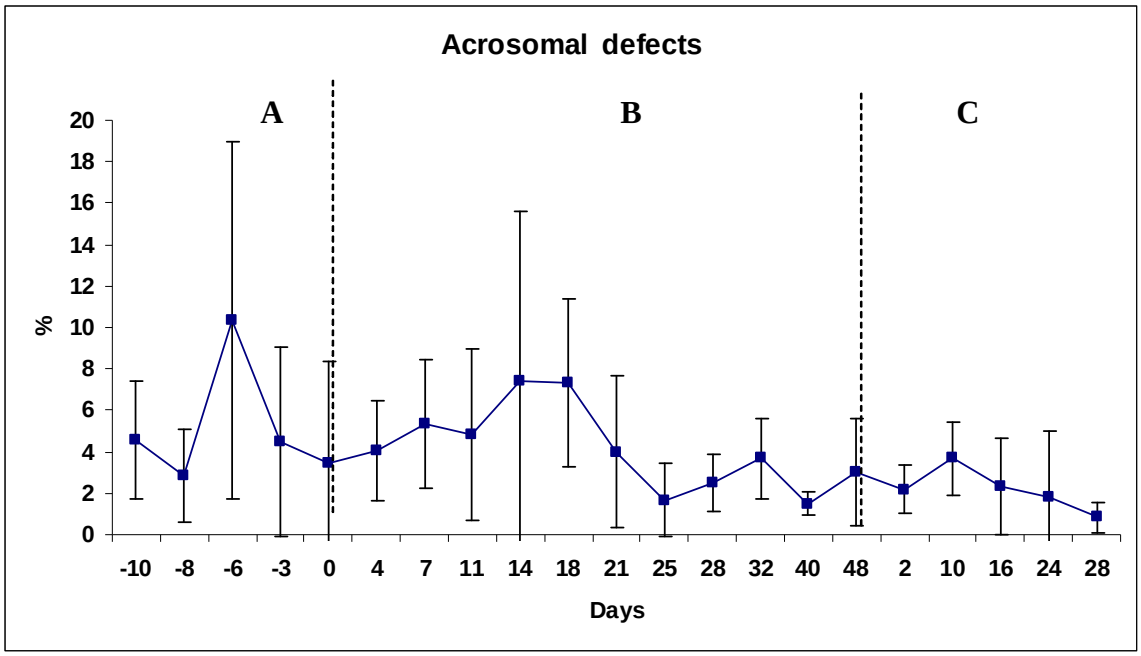


**Figure 26. Mean ± S.D Acrosomal defects of vaccinated bull's semen (*n*=6).**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period; B = Vaccination period; C = Period of challenge*

Although the percentage flagella defects was relatively stable in some bulls (3, 4 and 6), it varied dramatically from one sample to the next in others (1, 2 and 5). The wide variation was only observed within the pre- vaccination period but later remained stable during the period of vaccination and experimental infection (Figure 27). Analysis of values obtained from the six bulls revealed no significant difference in *period A = (P = 0.5847), B = (P = 0.8429 and C = (P = 0.7025).*

A mean percentage value from the six bulls was established for each day (Figure 28). There was also no statistically significant difference; *A vs B = (P = 0.777); B vs C = (P = 0.3648)* between the mean percentage values obtained after vaccination and experimental infection for this parameter (Figure 28).

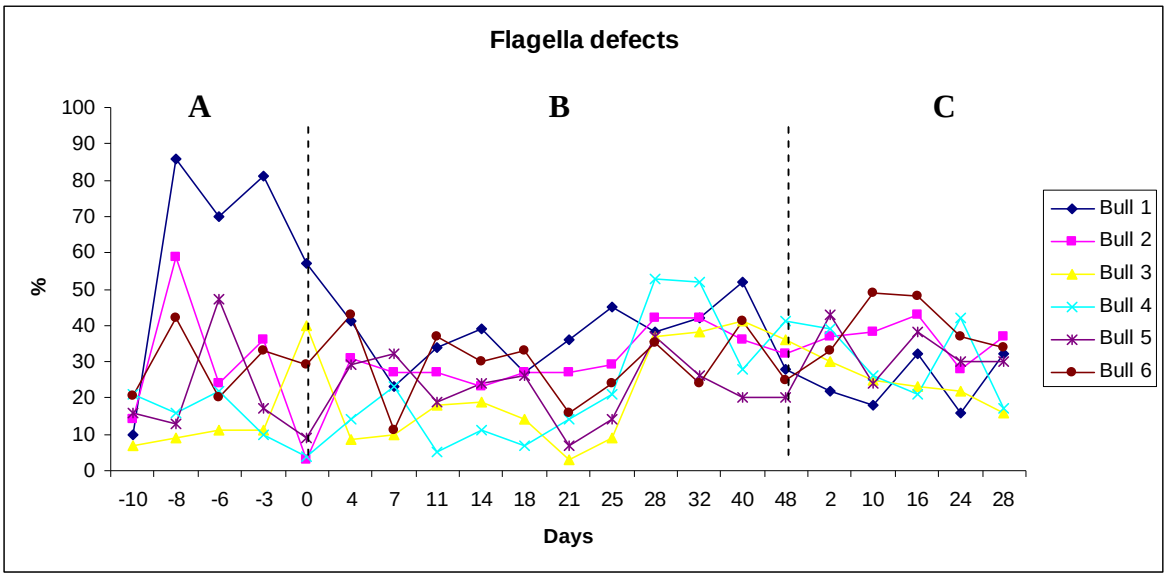


**Figure 27. Frequency of appearance of the total flagella defects in individual vaccinated bulls following vaccination and experimental infection (n =6).**

*A = Acclimatization period; B = Vaccination period; C = Period of challenge*

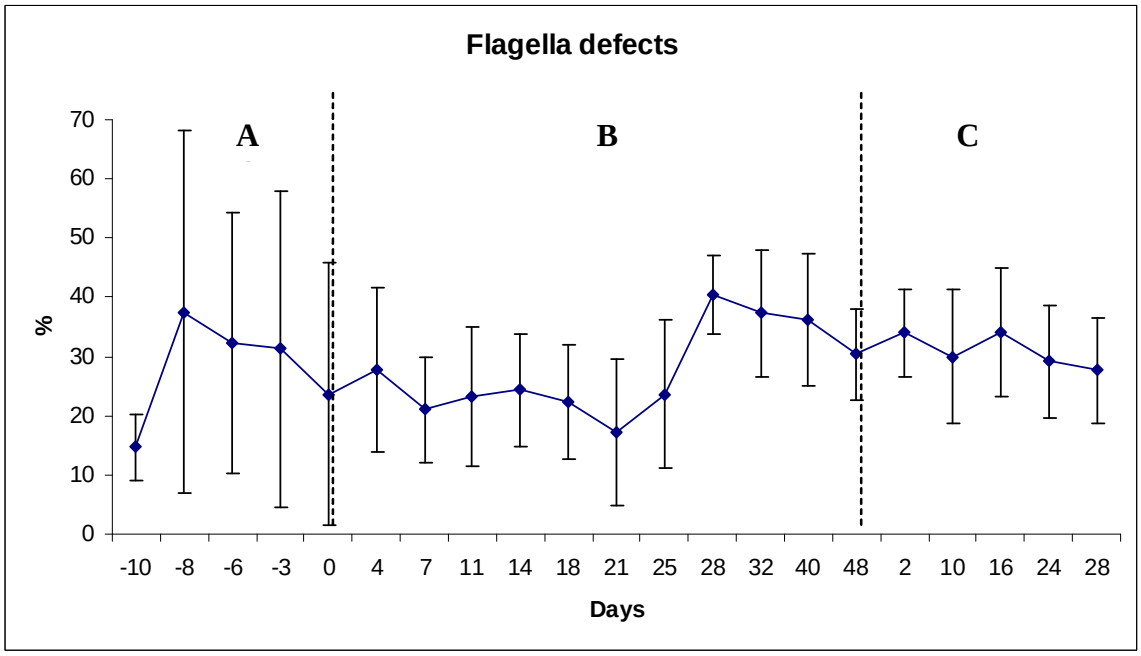


**Figure 28. Mean ± S.D Flagella defects of vaccinated bull's semen (*n*=6).**

*P >0.05 (A vs B; B vs C)*

*A = Acclimatization period; B = Vaccination period; C = Period of challenge*

# CHAPTER 4

# DISCUSSION

## 4.1 Suitability of experimental animals

As illustrated earlier, twelve Dexter bulls were used in this study. A previous study has demonstrated their suitability indicating a similar response to field infection to LSDV (Tuppurainen *et al.*, 2005). Some advantages of the use of this specific breed include the breed's ability to adapt to the housing and management available to this study. Their small size was also taken into consideration due to the limited housing facility provided for the bulls in this study. Since the budget allocated for the study was limited, it was also more cost effective.

Whether the number of animals used or the choice of breed had any influence on the results cannot be ruled out. The preventive abilities of the Neethling strain vaccine in preventing or limiting seminal shedding of LSDV when challenged using a different breed or a larger number of animals should be undertaken.

## 4.2. Clinical Signs

***Unvaccinated bulls (controls)***

In this study, two bulls developed classical signs of LSD, three showed mild symptoms and one were asymptomatic. Results obtained from the six experimentally challenged unvaccinated bulls agrees with previous reports indicating that 10 – 50 % of animals exposed to LSD fail to develop generalised disease in the field or following experimental infection (Capstick *et al.*, 1959; Carn and Kitching, 1995b; Tuppurainen *et al.*, 2005). The incubation period of 7 to 14 days following experimental infection of bulls in this study is consistent with reports from previous studies (Carn and Kitching, 1995b; Capstick *et al.*, 1959; Haig, 1957; Prozesky and Barnard, 1982). Enlargement of the prescapular lymph nodes was only observed in the two bulls showing classical signs of LSD. This result is in agreement with observation by Tuppurainen *et al.*, (2005).

The short duration of fever in this group is in agreement with observations reported by Kitching and Hammond, (1992). The appearance of skin lesions following fever reaction in two bulls on day 8 p.i., one day after the start of the fever reaction, are similar with observations reported by Weiss, (1968) and Brenner *et al.*, (1992) where skin lesions appeared 24 to 48 hrs after the initial rise in temperature. There was a marked variation in the clinical signs observed between various bulls, similar to findings reported by Tuppurainen *et al.*, (2005).

One possible explanation for the variation in clinical signs observed in different bulls is the genetic resistance determined by major histocompatibility complex present on cell surfaces of individual animals described by Amills *et al.* (1998).

***Vaccinated bulls***

Although the number of bulls used in this study was small, no clinical abnormalities were found in the bulls after vaccination. These results are in contrast to previous reports which demonstrated mild responses after vaccination using the attenuated Neethling strain vaccine (Weiss, 1968), and severe clinical signs using a live attenuated Kenya sheep and goat pox strain 0240 vaccine (Yeruham *et al.*, 1995) in cattle. On the other hand, the same vaccine strain (Kenya sheep and goat pox strain 0240) has also been used effectively without any severe clinical signs in sheep and goats (Kitching, 1986).

When the vaccinated bulls were experimentally infected with a virulent strain of LSDV, clinical signs were limited to mild lymph node enlargement in the four bulls. These results are similar observations made by Ngichabe *et al.*, (1997) using a recombinant capripox-rinderpest vaccine against rinderpest and LSD. In a similar experiment performed by Brenner *et al.*, (1992) using six bulls, four bulls were vaccinated with sheep pox vaccine and two bulls were not vaccinated to act as controls. Thereafter, the six animals were challenge with LSDV using an infective dose of $10^4$ $TCID_{50}$ /ml. Two unvaccinated animals showed classical signs of LSD, two vaccinated bulls had mild lymph node enlargement which appeared 24-48 hrs and disappeared 8-10 days p.i. The other two vaccinated bulls had skin lesions as well as lymph node enlargement till the termination of the trial. These results indicated more

severe clinical signs than those seen in this study although the virus was a different virus.

When clinical results from the experimentally infected vaccinated bulls were compared to those obtained from the experimentally non-vaccinated bulls, it is evident that vaccination prevented the clinical disease caused by LSDV in the bulls after experimental infection. These results are compatible with previous reports by Carn, (1993) and Ngichabe *et al.,* (2002).

### 4.3. Viraemia

***Unvaccinated bulls (controls)***

In this study, one bull (bull D) was viraemic from days 9- 21 and another bull (bull E) on 8 occasions from day 9- 23 (Table 2). Fever in bull D started on Day 7 p.i. and lasted for 10 days (Day 18 p.i.). In bull E, fever started on Day 8 p.i. and lasted until the end of sample collection (Day 34 p.i.). The time of viraemia in these bulls are similar to a recent study by Tuppurainen *et al.*, (2005) where virus was isolated a day before, the same day or a day after the onset of fever. The period of viraemia in these bulls is also consistent with observations reported by Carn and Kitching, (1995b).

Virus was isolated from blood samples on 3 occasions in the two bulls that showed no clinical signs (Table 2). Again, results here are consistent with observations reported by Tuppurainen *et al.*, (2005) showing that viraemia in bulls that developed inapparent disease persisted much longer than that in bulls that showed mild clinical disease.

In one bull (bull C), showing mild clinical disease, virus was isolated only once. The number of times virus was isolated in this bull is similar to results obtained from a bull that developed mild infection in the study of Tuppurainen *et al.*, (2005).

Virus was not isolated from blood on tissue culture in one bull (bull B) (Table 1). The inability to isolate virus even though lesions were evident in this bull was noteworthy. A possible explanation for this is a low concentration of virus in the blood, beyond the

limit of detection on cell culture. The susceptibility of the virus to cell culture might also have played a role.

***Vaccinated bulls***

The data in this study showed that no vaccinated bulls were found to be viraemic after challenge with the same strain of LSDV to challenge the unvaccinated bulls. It is therefore evident that vaccination did prevent viraemia.

**4.4. Serology**

***Vaccinated bulls***

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. A further rise in antibody titre was also detected in the four bulls (Figure 4). The absence of a detectable antibody titre in bull 3 at the end of the trial, which had an antibody titre of 1:14 at the end of the vaccinated period, was unexpected (Figure 4). One explanation for this may be due to the very low levels of antibody which is characteristic of the response to LSDV (Carn, 1993). It is important to note that the specific days chosen for the SNT in this study were also in accordance to the peak periods reported in a similar trial by Tuppurainen *et al.*, (2005) where titres were shown to rise on day 13 and peak on day 19 after challenge.

Serological results in this study are in agreement with other reports (Manual of Diagnostic tests and vaccine for Terrestrial Animals, 2004; Tuppurainen, 2005). Tuppurainen *et al.*, (2005) also demonstrated that the level of antibody titres is proportional to the severity of clinical disease observed. Due to the fact that very low levels of neutralising antibody usually develop (Manual of Diagnostic tests and vaccine for Terrestrial Animals, 2004; Kitching and Hammond, 1992), the use of serology is not sensitive enough to identify animals that have had contact with LSDV. It also supports further that immunity to LSD infection is predominantly cell mediated (Carn, 1993; Manual of Diagnostic tests and vaccine for Terrestrial Animals, 2004). This agrees with the low antibody titre found in the vaccinated bulls that showed no clinical signs when challenged. The data suggests that high antibody titres are not

necessary to prevent or reduce the manifestation or severity of the clinical signs of the disease in the bulls when experimentally infected.

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. Although immunity to LSD is cell-mediated, antibodies are a useful measure of response to vaccination, and because there was no antibody detection by day 21, this prompted us to consider the possibility of vaccine failure in this instance, a phenomenon which is well documented (Hunter and Wallace, 2001; Roth, 1999). Many factors can be responsible for vaccine failure ranging from mismanagement of the vaccine or the physiological state of the animals at the time of vaccination. It could also be due to the fact that the immune response to LSDV is predominantly cell mediated hence a very low antibody titre making detection of antibodies very difficult using the SNT (Carn, 1993; Manual of Diagnostic tests and vaccine for Terrestrial Animals, 2004; Kitching and Hammond, 1992).

Since the objective of this trial was to study the seminal shedding of LSDV and semen quality in bulls which had been adequately vaccinated and since the animals had not seroconverted at day 21 p.v., we decided to repeat the vaccination using a different batch of vaccine. This was a deviation from our original study design which included a single vaccination. Whether different results would have been obtained had the vaccination not been repeated cannot be determined.

### 4.5. Presence of LSDV in semen

***Unvaccinated bulls (controls)***

The polymerase chain reaction has been reported to be more sensitive in detecting lower levels of LSD viral nucleic acid in the semen of infected bulls than virus isolation (Tuppurainen *et al.,* 2005). Previous work by Irons *et al.,* (2005) has already demonstrated the excretion of LSDV in semen of experimentally infected un-vaccinated bulls using the PCR. In this study, all six unvaccinated bulls excreted the virus in semen at least at one point during the course of the trial (Table 4).

Furthermore, the bulls in this study that showed inapparent infection excreted the virus only once in their semen at one point during the trial.

Whether the LSD viral nucleic acid detected by PCR in semen is viable and able to cause infection when used for insemination of susceptible cattle is not known. Specific days chosen for viral detection in the present study were in accordance with periods of shedding as demonstrated in a similar study (Irons *et al.*, 2005). Excretion of LSDV was demonstrated on these days in all the unvaccinated animals.

It is important to note that sampling was done every second day. The justification for this sampling frequency is due to the fact that, to induce ejaculation more frequently than we did with the electro-ejaculator could be very stressful to the animals. Furthermore, it takes a minimum of 10-11 days for semen to move through the epididymal duct. For these reasons, it is very likely that any virus that had been present in the semen would have been detected by PCR when sampling every second day.

A very high concentration of the virus is located within the skin nodules (Chihota *et al.*, 2001), and similar nodules and ulcers are seen in the mucous membranes of the reproductive tract (Barnard *et al*. 1994; Thomas and Mare 1945). One may speculate that LSDV may have been excreted from these nodules within the genital tract into the semen on their passage through the testes or epididymal duct of infected bulls. This could be one reason why bulls that showed very mild infection did not excrete the virus in their semen.

***Vaccinated bulls***

In this study, no viral nucleic acid was detected by PCR in the semen of the vaccinated bulls at any point following vaccination or challenge. It is also important to note that specific days chosen for the detection of viral nucleic acid using the PCR correspond with the periods of detection of virus in semen as seen in the control bulls which excreted the virus.

Our findings agree with the observations made by Dewey, (2002) and Chio *et al.*, (2002) on PRRS and Castro *et al.*, (1992) on Pseudorabies, but are different from

those of Swenson *et al.*, (1995) and Thomas *et al.*, (1997) on PRRS indicating presence of vaccine virus in semen after vaccination.

When the results obtained from the experimentally infected vaccinated bulls and those from the non-vaccinated bulls were compared, it showed that vaccination prevented the excretion of LSDV in the semen of experimentally infected vaccinated bulls. One possible explanation may be the fact that vaccination prevented the production of nodules which is characteristic of LSD hence the absence of the virus in the semen of vaccinated bulls. Another explanation could be that the vaccine prevented the generalization of the virus within the system thereby limiting spread and resulting in rapid clearance of the virus. Transient shedding of virus in semen of the vaccinated bulls from days out of the peak periods chosen cannot be excluded but is highly unlikely. This is the first report on the prevention of the excretion of LSDV in semen by vaccination after challenge.

### 4.6. Semen quality

In this study, it was observed that vaccination against LSD had no effect on semen quality. Although the semen volume and sperm concentration varied widely between samples, there was no significant difference in semen volume and the sperm concentration seen after vaccination against LSD and experimental infection. This result agrees with observations on semen volume and sperm concentration in the vaccination of black quarter and FMD vaccination (Murugavel and Veerapandian, 1998) but differs from observations made by Reddy *et al.*, (1991) on FMD showing that semen volume and sperm concentration was affected. Analysis of the volume of the unvaccinated bulls showed that the seminal volume was not affected by the experimental infection.

The findings of an absence of any effect of vaccination and subsequent challenge on linear motility in this study does not agree with observations made when vaccinated against FMD (Singh *et al.*, 2003; Manqurkar *et al.*, 2000) showing that linear motility was affected but agrees with the observations made by Castro *et al.*, (1992) on Pseudorabies disease. Results in this study on the vaccinated animals are in contrast with observations seen in the unvaccinated animals indicating a significant difference

in the linear motility of two bulls. One possible explanation for the difference may be caused by the increase in temperature (Figure 3) observed in the unvaccinated bulls. Increase in temperature has been well documented to affect semen quality (Meyerhoeffer *et al.*, 1985; Vogler *et al.*, 1993; Skinner *et al.*, 1966). It is also important to note that no increase in temperature was seen in the vaccinated bulls in this study (Figure 2).

It was also shown that sperm morphology was not affected either by vaccination or experimental infection. This result does not agree with observation by Reddy *et al.*, (1991) on FMD and Murugavel and Veerapandian, (1998) on blackquarter and FMD. When the results of the un-vaccinated bulls were analyzed, there was a significant difference in the mean percentage morphologically normal sperm cells of three of the bulls. Results here are in contrast with those obtained from the vaccinated bulls but agree with Irons and Gerber, (2004) on the morphology of sperm in LSDV infected unvaccinated bulls. One possible explanation for the difference may be due to the various clinical anomalies which are characteristic of the virus, and which were prevented by vaccination in this study.

It is evident that the adverse effect of LSDV on semen which has already been reported (Irons and Gerber, 2002) was prevented by vaccination in this study. It is likely that LSDV could affect semen quality by the disturbance of the thermoregulation in the testes. This could be due to scrotal skin thickening or by inducing fever or by its direct effects on the testicular tissue.

Budget constraints prevented the inclusion of a simultaneous unvaccinated control group to rule out factors other than vaccination which may have affected semen quality in this study. Therefore, the study design was to compare semen quality between periods (Figure 1). There was no effect on semen quality when individual bulls acted as their own control, thus validating our result. When results obtained from the vaccinated and unvaccinated bulls were compared, it was evident that semen quality was not affected following vaccination and experimental infection and vaccination did prevent any adverse effect on semen quality of vaccinated bulls after experimental infection.

Changes in semen quality following vaccination and experimental infection with LSDV have not been reported. However, these finding should be regarded as a

preliminary report due to the fact that the numbers of animals used was small. Another study on the effect of LSD vaccination as well as its ability to prevent the adverse effects on semen quality when challenged would be required to authenticate this result.

The present study provides preliminary evidence that semen quality is not affected by either LSD vaccination or experimental infection with a virulent LSDV in vaccinated bulls. We consider it likely that should animals experience more severe clinical effects following vaccination as may occur (Weiss, 1968; Yeruham *et al.*, 1995), the possibility of adverse effects on semen quality cannot be ruled out.

In conclusion, this study provides evidence of the absence of LSD vaccine viral nucleic acid following vaccination. It also illustrates further that vaccination against LSD did not have any effect semen quality. This work also provides the first evidence of the ability of LSD vaccination to prevent the excretion of LSD viral particles in semen when vaccinated bulls are experimentally infected. Furthermore, it showed that vaccination against LSD did prevent the adverse effect of experimental infection with virulent virus on semen quality.

The current data supports the inclusion of a LSD vaccination programme using this vaccine as a biosecurity measure with regards to the movement of semen from LSD infected areas. Therefore, the requirement for the importation of bovine semen should include veterinary health certificate indicating that the donor animal has been vaccinated against LSD, and no clinical signs of the disease were seen on the day of collection of the semen.

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Appendix A

| Date | Bull 1 | Bull 2 | Bull 3 | Bull 4 | Bull 5 | Bull 6 |
|---|---|---|---|---|---|---|
| | | | | | | |
| Bull number | | | | | | |
| General health | | | | | | |
| Lymph nodes | | | | | | |
| Temperature | | | | | | |
| Skin lesions | | | | | | |
| Scrotal circumference | | | | | | |
| Testes | | | | | | |
| Semen from sheath | | | | | | |
| Semen from prot.penis | | | | | | |
| Fraction: | <1/2, >1/2 all | <1/2, >1/2 all | <1/2, >1/2 all | <1/2, >1/2 all | <1/2, >1/2 all | <1/2, >1/2 all |

Appendix B

**Lumpy skin disease trial**

| Date: | Bull 1 | Bull 2 | Bull 3 | Bull 4 | Bull 5 | Bull 6 |
|---|---|---|---|---|---|---|
| General health | | | | | | |
| Temperature | | | | | | |
| Skin lesions | | | | | | |
| Lymph nodes | | | | | | |
| Scrotal Circumference | | | | | | |
| Testes | | | | | | |
| Collection start time | | | | | | |
| End time | | | | | | |
| Semen ran from sheath | | | | | | |
| Blood sample to lab | | | | | | |
| Semen sample to lab | | | | | | |
| | | | | | | |
| Volume | | | | | | |
| Colour | | | | | | |
| Consistency | | | | | | |
| pH | | | | | | |
| | | | | | | |
| Mass motility | | | | | | |
| Individual motility | | | | | | |
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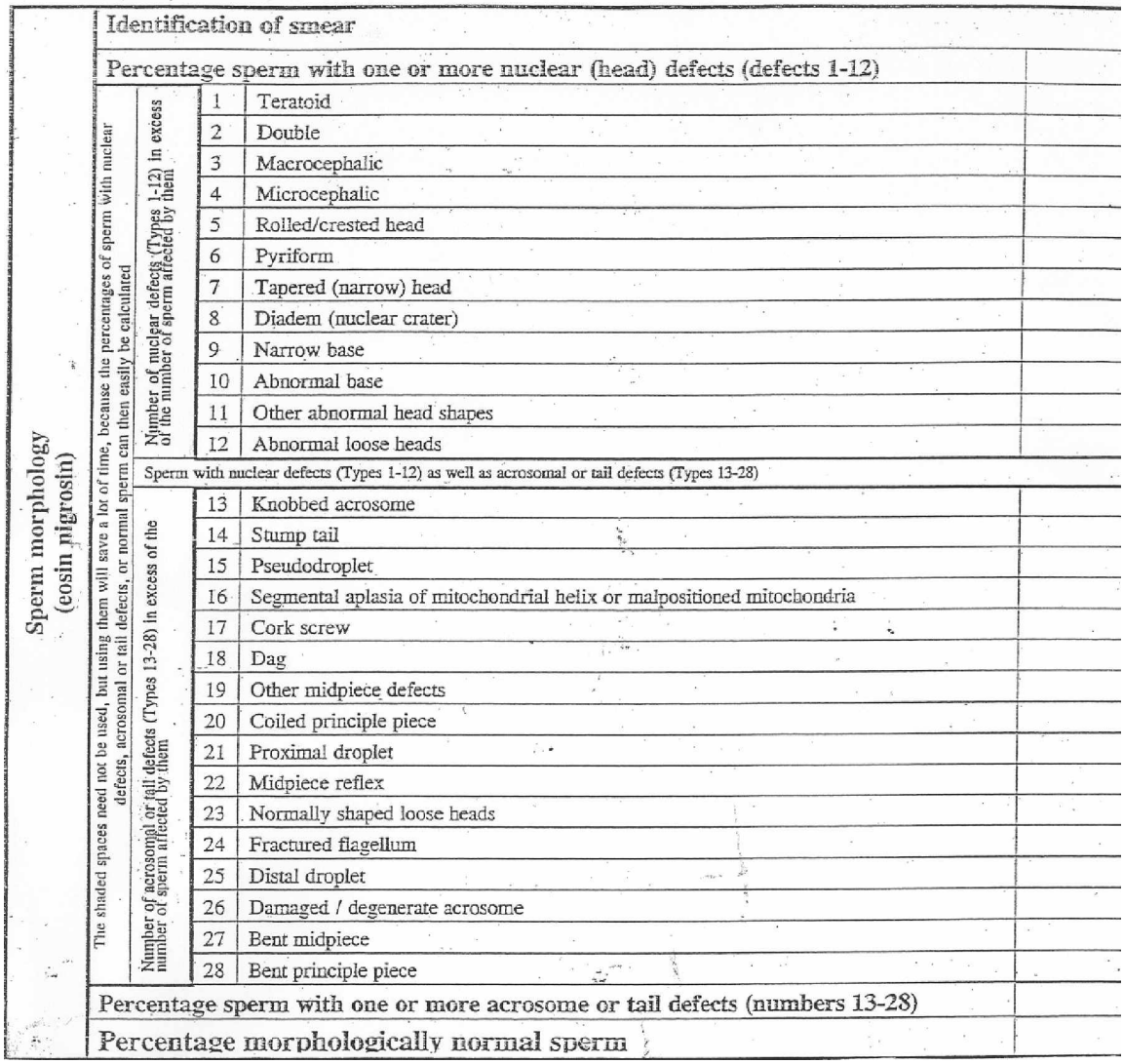
| | | | | | | |
| Average 10 fields | | | | | | |
| Linear motility | | | | | | |
| Aberrant motility | | | | | | |
| Eosin/Nigrosin smear | | | | | | |

Appendix C



Sperm morphology (eosin nigrosin)

Identification of smear

Percentage sperm with one or more nuclear (head) defects (defects 1-12)

The shaded spaces need not be used, but using them will save a lot of time, because the percentages of sperm with nuclear defects, acrosomal or tail defects, or normal sperm can then easily be calculated

Number of nuclear defects (Types 1-12) in excess of the number of sperm affected by them

| | |
|---|---|
| 1 | Teratoid |
| 2 | Double |
| 3 | Macrocephalic |
| 4 | Microcephalic |
| 5 | Rolled/crested head |
| 6 | Pyriform |
| 7 | Tapered (narrow) head |
| 8 | Diadem (nuclear crater) |
| 9 | Narrow base |
| 10 | Abnormal base |
| 11 | Other abnormal head shapes |
| 12 | Abnormal loose heads |

Sperm with nuclear defects (Types 1-12) as well as acrosomal or tail defects (Types 13-28)

Number of acrosomal or tail defects (Types 13-28) in excess of the number of sperm affected by them

| | |
|---|---|
| 13 | Knobbed acrosome |
| 14 | Stump tail |
| 15 | Pseudodroplet |
| 16 | Segmental aplasia of mitochondrial helix or malpositioned mitochondria |
| 17 | Cork screw |
| 18 | Dag |
| 19 | Other midpiece defects |
| 20 | Coiled principle piece |
| 21 | Proximal droplet |
| 22 | Midpiece reflex |
| 23 | Normally shaped loose heads |
| 24 | Fractured flagellum |
| 25 | Distal droplet |
| 26 | Damaged / degenerate acrosome |
| 27 | Bent midpiece |
| 28 | Bent principle piece |

Percentage sperm with one or more acrosome or tail defects (numbers 13-28)

Percentage morphologically normal sperm