FOOT AND MOUTH DISEASE CIRCULATING AND VACCINE-INDUCED ANTIBODIES AND EVALUATION OF COCONUT WATER AS A POTENTIAL EXTENDER ON BULL SEMEN

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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award at any other institution

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DEDICATION

This Thesis is dedicated to all those involved in Livestock production and disease control.

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ABBREVIATIONS AND ACRONYMS

AI	Artificial insemination
APPs	Acute Phase Proteins
CIAS	Central for Artificial Insemination Station
CPE	Cytopathic Effect
DMSO	dimethyl sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
IFN	Interferons
KEVEVAPI	Kenya Veterinary Vaccines Production Institute
KAGRC	Kenya Animal Genetic Resource Centre
NaHCO3	sodium bicarbonate
OIE	Office International des Epizooties
QMS	Quality Management System
SAA	Serum amyloid A
VNT	Virus Neutralization Test
WHO	World Health Organization
WOAH	World Organization for Animal Health

ABSTRACT

Artificial Insemination (AI) is the single most important technique that employs genetic improvement of cattle for producing quality livestock breeds. Bulls farmed for AI in Kenya are vaccinated against the Foot and Mouth Disease (FMD). It is however, not clear if all FMD vaccinated bulls develop immunity against the vaccines. There have been no studies to establish if vaccinated bulls produce semen in parameters within the normal range (as assumed that vaccinated bulls have good semen). In addition, although the FMD in Kenya is known to occur in certain endemic regions while other regions remain nonendemic, there have been no studies carried out to establish if animals in endemic areas develop natural immunity or if animals in the disease- free regions could be seropositive. The present study was aimed at determining the impact of foot and mouth circulating and vaccine-induced antibodies and evaluation of coconut water as a potential extender of bull semen for artificial insemination. The cross-sectional study used bulls specifically farmed for production of semen for AI and therefore, vaccinated against FMD. For a pilot study to establish natural protection against FMD, bulls in both endemic and none endemic regions were used. Antibodies were quantified using virus neutralization test. Semen samples were obtained from FMD vaccinated bulls and analyzed for parameters. Coconut water was evaluated alongside a commercial semen extender to establish its potential for use in semen processing. Data were analyzed using the GraphPad InStat software utilizing one way analysis of variance (ANOVA) and Kruskal-Wallis test with Tukey and Dunn post-tests respectively. In addition, t-test was used to analyze for intergroup differences while Spearman test was used for correlation analysis. A P value < 0.05 was considered statistically significant. Results indicated that in FMD non-endemic region, 23%, 10.3%, 2.6% and 7.7% of the animals had protective levels of antibodies against the FMD virus strains O, A, SAT 1, and SAT 2 respectively with a significantly higher protection conferred by induced by the O strain virus as compared to SAT 1 ($P = 0.0124$). In the FMD endemic area, all sampled animals showed protection levels at 100%, 100%, 100% and 29% for virus strains O, A, SAT 1, and SAT 2 respectively with the antibody titres showing significant differences ($P < 0.05$) for all the intergroup analysis except between strains O vs SAT 1 and A vs SAT 1 ($P > 0.05$). Analysis of semen parameters in FMD vaccinated bulls showed at least two animals from each of the bulls that developed immunity and those that failed to develop immunity to have light creamy or watery semen instead of the normal cream-colored semen. In addition, while most of the semen parameters were within normal values, bulls that failed to develop immunity following vaccination were associated with significantly higher sperm recovery post thawing as compared to animals that seroconverted ($P < 0.0001$). Comparable sperm recovery rate was obtained from semen processed with coconut water and commercial extender post thawing ($P > 0.05$). In conclusion, the present findings point to a possible emergence of FMD in disease non-endemic region while most animals in disease prevalent regions have protective levels of virus specific antibodies. In addition, there appears to be some negating effect of FMDV specific antibodies on recovery of sperms post thawing. Furthermore, coconut water appears to perform similarly to commercial semen extender and shows potential as a medium for semen processing. The study recommends surveillance of FMD in disease non-endemic regions and the confirmation of the present findings using large sample sizes in order to make more informed conclusive decisions

CHAPTER ONE

INTRODUCTION

1.1 Background information

The Foot and mouth disease (FMD) affects animals, particularly the ruminants, and is an acute and highly contagious disease that is caused by the foot and mouth disease virus (FMDV). It infects both domestic and wild ruminants (Arzt *et al.*, 2011). It negatively impacts on livestock production leading to huge losses through death and disruption in trade of affected animals and their products. A feasible strategy for the prevention of the disease is the use of FMD vaccines in farm animals (OIE, 2018) to keep our animals in health for productivity. However, while the use of the FMD vaccine to prevent the viral disease is seen as a strategy to increase productivity in livestock, the effects of vaccination especially in bulls reared as breeds for production of semen for use in AI, has not been evaluated, yet the vaccine- immune-system induced products might have negative impact on the quality of semen in these animals. As a result, scientific studies aimed at research and product development with a goal of increasing livestock productivity are paramount and are urgently needed especially in the face of increasing cost of products including the commercial semen extenders (volume extenders). In addition, studies aimed at evaluating the effects of vaccination on reproductive capacity of farm animals and may help shed light on any negative consequences associated with the specific vaccines.

There are seven documented serotypes of FMDV including O, A, C, SAT1, SAT2, SAT3 and Asia-1 (WOAH, 2023). Infection with any one virus serotype does not induce protection against another strain (KEVEVAPI, 2021). This becomes a challenge to vaccine production underscoring the need for development of a cocktail of a vaccine against several viral strains. In Kenya, the possible circulating viruses associated with FMD are strains O, A, Sat 1, and Sat 2. FOTIVAX TM is an inactivated FMD vaccine developed for use in cattle, sheep, pigs, and goats for the prevention and control of FMD associated with infections by the virus serotypes A, O, SAT 1 and SAT2. Vaccination against FMD in cattle in Kenya is restricted to only the endemic areas such as the Central, South West and Western regions. The Eastern part of the Country including south Eastern regions are not considered endemic for the disease. However, there has not been any documented reports on the natural protection status in animals farmed in the disease endemic or the disease-free regions of Kenya to establish the current status of infection based on seroprevalence. Thus, a pilot study can inform decisions on future vaccination programmes - if animals in disease endemic regions are naturally protected against FMD, then it would be cheaper not to vaccinate them during disease outbreaks. The objective of the current study was to evaluate the coconut water as a cheap locally available alternative medium of use in place of the more expensive commercial extenders in semen processing for artificial insemination. In addition, the study aimed at establishing the impact of the FMD vaccination on the quality of semen in bulls bred for production of semen used in commercial artificial insemination. Furthermore, the study also sought to assess the natural protection status of cattle farmed in a disease endemic and non-endemic region in Kenya.

Artificial insemination (AI) is one of the effective tools available to dairy cattle producers to improve herds productivity and profitability (Borges *et al.*, 2016). Semen in the early days of artificial insemination was delivered every day to the technicians by a bull stud, as semen was not frozen at this time until semen commercial extenders (skimmed milk) and glycerol came into use for semen freezing we cannot freeze semen without using semen volume extenders. Semen freezing process was discovered by a man known as Polge (1949). These commercial extenders are imported (component of semen processing) and are very expensive impacting on the cost of production of livestock that are bred through AI. Although there has been improved productivity in cattle produced through AI, the cost of production has also increased proportionately. With more research and product discovery, an alternative medium for use as semen extender whose availability is cheap and can significantly lower the cost of livestock production can be easily developed.

1.2 Statement of the problem

Foot and mouth disease (FMD) is great threat to dairy industry and bulls kept for semen production should be vaccinated with FMD vaccine (OIE, 2017a) especially the imported animals. These vaccines may be mistakenly assumed to improve quality of bull semen and may instead negatively affect the quality of good semen meant for artificial insemination (AI). In addition, processing of semen for AI requires the use of commercial imported extenders. However, these commercial extenders are expensive, and not readily available. Locally available coconut water that may be developed cheaply for use as an alternative to the expensive commercial semen extenders has not been evaluated for its potential application. While vaccination of cattle in Kenya against the FMD is mainly concentrated in disease endemic regions leaving out other areas which are considered as non-endemic, it is likely that animals in these areas are naturally protected against disease; hence vaccination only leads to vaccine waste. In addition, there could be unforeseen cases of infections of cattle farmed in presumed disease non-endemic regions in Kenya, there has not been any studies carried out to assess the extent, if any, of the proportion of cattle naturally protected against the FMD.

1.3 Justification for the study

While it is important to vaccinate cattle against the foot and mouth disease ensures increased livestock productivity, it is equally important to evaluate the impact of the FMD vaccine-induced antibodies on semen quality in bulls that are bred for production of sperm for AI. This is because the use of vaccines may negatively affect the reproductive ability of vaccinated bulls by affecting semen quality and this argument forms the rationale of the present study.

The production of livestock should follow a system that ensures maximum productivity while minimizing costs. However, in the science and application of AI, the processing of semen requires the use of expensive commercial semen extenders and this increases, significantly, the cost of producing farm livestock. Development of an alternative medium as semen extender to replace the more expensive commercial extenders can largely lower the cost of artificial insemination. Coconut water constitutes of rich in nutrients, vitamins, mineral salts and phytohormones and may be a potential substitute for expensive commercial extender. However, this has not been fully investigated. Therefore, the need to develop the coconut water as a medium for use in semen processing as in the present study is urgent, being locally available and hence more economical than imported commercial extender. Furthermore, the reproduction and stocking of cattle for commercial exploitation starts with ensuring good breeds and this is initially defined by use of quality semen and therefore the need to evaluate semen quality in the present study.

Effective disease surveillance and preparedness for outbreaks in livestock requires documentation of the proportions of livestock encountering disease pathogens and their inherent response to infection. A pilot study to assess the protection level of cattle farmed in FMD endemic region and a disease non-endemic region can inform decisions on future vaccination programmes, in addition to providing important information on the genetic or inherent level of disease susceptibility or resistance. Thus, it becomes important to establish the level of natural protection in cattle farmed in an area of no disease and a disease burden area. Such regions can only be purposely selected as study choices due to their natural disease prevalence status.

1.4 Research questions

i. What the protection status in bulls from a foot and mouth disease-endemic and nonendemic regions in Kenya?

ii. What are the serology titres produced against virus strains in bulls from a FMD endemic or non-endemic region of Kenya?

iii. What is the semen parameter levels in bulls vaccinated against FMD?

iv. What is the sperm recovery rate from cryopreserved semen samples processed using coconut water?

1.5 Hypotheses

i. There is no significant difference in protection status between bulls from a foot and mouth disease-endemic and those from non-endemic regions in Kenya.

ii. The serology titres produced against current vaccine strains in bulls from a FMD endemic or non-endemic region in Kenya are comparable.

iii. The semen parameters in bulls vaccinated against FMD are within normal values.

iv. Sperm recovery rate from semen processed using coconut water is the same as the recovery from duplicate samples processed using Opticell commercial extender after cryopreservation.

1.6 Objectives

1.6.1 **General objectives**

The general objective of the study is to determine the impact of the food and mouth disease vaccine-induced antibodies on quality of semen and to evaluate coconut water as a potential extender on bull semen for Artificial insemination.

1.6.2 **Specific objectives**

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Commented [M2R1]:

i. To establish the protection status in bulls from a foot and mouth disease-endemic and non-endemic regions in Kenya.

ii. To determine serology titres levels produced against virus strains in bulls from a FMD endemic or non-endemic region in Kenya.

iii. To analyze the semen parameters in bulls vaccinated against FMD.

iv. To determine the recovery rate of sperms from semen samples processed using coconut water after cryopreservation.

1.7 Significance of the study

The research study examines the impact of FMD vaccine on semen quality and this will provide information that will guide on strategies to remedy the negative effects of animal vaccines for increased productivity. In addition, development of coconut water as cheap alternative semen extenders in this research study will greatly reduce the cost of artificial insemination. Information on the extent of natural immunity development against the FMD in a region of disease burden and a non-endemic area will be important in guiding future disease vaccination programmes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Artificial insemination

Artificial Insemination (AI) is the introduction of bull spermatozoa into a female's cervix or uterine cavity for the purpose of achieving a pregnancy through in vivo fertilization without using sexual means (Greyling *et al*, 2022). Artificial insemination is the single most important technique used for the genetic upgrading in animals. High quality semen selected from superior bull can produce enough spermatozoa to inseminate thousands of cows per year (Tadesse *et al*., 2022). The breeding of cows meant for dairy use has seen much improvement when artificial insemination is used (Mercadante, 2016).

The number of sperm cells in an ejaculate of semen must be of high quality to ensure acceptable pregnancy rates. In the livestock industry efficiency of reproduction of the herd is mainly achieved by use of technologies in AI. Artificial insemination significantly reduces incidences of sexually transmitted disease in cattle while increasing utilization of sires that are genetically superior and this is associated with improvement of performance of the herd (Vishwanath and Shannon, 2000). In addition to ensuring up take of high-quality semen of AI, it is important that the cost of the AI services be low and affordable by the farmers. Reduced cost can be determined by reducing the cost of processing of semen for AI.

In AI when sample of semen is obtained it is analyzed for its physical appearance, quantity or volume, and motility, sperm concentration and morphological features, by macroscopy.

The semen sample is diluted in order to improve its quantity and the shelf life of the sperm cells during storage under very low temperatures with the possibility for application in AI in the future. The viability of AI is dependent upon deposition of high volumes of high quality and viable sperm cells in the proper anatomical site and at the right timeframe relative to the receptive cow's ovulation while ensuring use of a sterile technique (Mulu *et al*., 2018). As AI is considered an economically important technology in livestock production, it is necessary that the technique be applied widely in order to increase the reproduction efficiency.

2.2. Semen collection

Semen for AI is can be collected from bulls through several methods including using an artificially made vagina, use of electric-induced ejaculation technology and by massage through the rectum (Mengistu, 2019). The female or dummy should be held in position through appropriate structures. The artificially made vagina is held at 45° in relation to the direction of the penis. Usually, the artificial vagina is held using the left hand by a right-handed person, and when the bull mounts the cow, the sheath of the bull will be grasped by the operator using his right hand who then directs the penis into the artificially made vagina, into where the bull ejaculates (Chenoweth, 2004).

2.3 Laboratory semen quality analysis

Comparison between pre-vasectomy and post-vasectomy volumes of semen indicates that approximately ninety percent of semen is composed of fluids coming from accessory tissues including the seminal vesicles and prostate and with minimal contributions from the epididymis and the Cowper's glands. Semen has two main attributes that are quantifiable.

These include the total spermatozoa number which reflects production of sperms from the testes and post-testicular ducts; and the total volume of fluids contributed a number of accessory glands.

Results of laboratory parameters of semen quality depends on several factors including whether a complete sample is collected (Björndahl and Kvist, 2003); the accessory sex glands activity; the time of collection since the last sexual activity (Jonge *et al*., 2004); and the penultimate period of abstinence since one ejaculation cannot completely empty the epididymitis (Cooper *et al*., 1993); and the size of the testis, which may influence the total spermatozoa number per ejaculate (Behre et al., 2000; Andersen et *al.*, 2000). Common semen parameters measured in the laboratory include, total perm count, ejaculate volume, sperm concentration, total motility, progressive motility and sperm morphology. The normal range for these parameters should conform to the World Health Organization reference range (WHO, 2010).

2.3.1 Physical appearance of semen

The first quality measure of semen is described by the gross physical appearance of freshly collected sample from a bull. This fleshly collected sample described as neat (unaltered) semen appears normally as a thick slightly yellowish to whitish fluid whose quality consistency is commonly determined by the concentration of the spermatozoa that it contains (Narud *et a*l., 2022). Bull semen will have very little normal odor. The microscopic normal appearance of semen from bulls varies between bull ejaculates and among individual bulls, the animal breed, as well as age (Mulu *et al*., 2018). High quality bull's semen is generally white or yellowish creamy in color, most often referred to as cream in color.

2.3.2 Ejaculate volume

The semen volume of a bull's ejaculate can readily be measured at the point of collection by collecting the semen sample directly into a vial that is graduated vial (Garner, 1991). Alternatively, the volume of ejaculates can be measured by weighing the vials after semen collection is using a top-loading balance, and then converting the weight reading into milliliters using computer programs. The latter methods have been indicated to reduce errors that may be associated with the former method of visual reading of ejaculate tubes particularly when small volume is obtained or bubbles are present (Bearden and Fuquary, 2000). When younger bulls are used or when there has been frequent ejaculation or failed or incomplete ejaculation or in situations where there is bilateral seminal vesiculities, the volumes will be low.

The average semen volume of an ejaculate can be 8.7 ml (Filipcik *et al.*, 2023). The volume of a bull's semen can vary between ejaculates, individual bulls, the animal breed, and age. However, a bull which gives less than 2ml of volume of semen per ejaculate is usually not acceptable (Zewdie *et al*., 2005). According to KAGRC standard operating procedures normal range is 1ml above, concentration 0.200. 60% initial dilution and 40% post thawing) Differences between bulls' semen volumes have reportedly attributed to variations in the age, animal breed, nutritional status, and geographic locations as well as seasons during the year of study, method used to collect semen and the manner in which bulls are handled during sample collection, the procedure used and the frequency of semen collection (Andrabi *et al*., 2002).

2.3.3 Spermatozoa motility

Spermatozoa motility is the percentage estimation of sperm cells that are active or motile in a semen sample. Progressive motility of sperm cells has been described as those spermatozoa that are naturally moving or progressing actively from given point to another in a more or less straight line. Sperm motility can easily be assessed by visual observation of bulk motility of sperm cells from a concentrated semen sample. Motility can be calculated by placing a single semen drop on a glass slide, and examining the movement of sperms in a bright field microscope under low magnification of 200X.

Semen motility is usually scored as very good (80-100% sperms are in motion), or good (60-80% sperms are motile), fair (40- 60% sperms in motion), or poor (20- 40% perms are cells), and very poor (10- 20% sperms are motile). Good quality sample of semen is indicated to have at least 30% of vigorously mobile sperms when the sample is diluted and examined under a microscope. Surrounding temperature, presence of shock as well as other factors have the potential to greatly interfere with sperm motility scores. In order to be acceptable bull semen should have at least 70% and 40% motility respectively at the time of collection and after freezing (Zewdie *et al*., 2005).

2.3.4 Sperm concentration

Concentration of sperm cells is described as the total quantity of sperms per milliliter of semen and the quantity must be recorded for each ejaculation. Sperm particle counter, spectrophotometer or Haemocytometer (Bearden and Fuquay, 2000).

2.3.5 Sperm cell morphology

The morphology of a sperm cell describes the physical appearance of the sperm cell as visualized under a microscope. A normal sperm cell is having a tail piece and a head and is divided into three-piece areas including the end-, mid- and the main piece. Morphologically, assessment of the sperm cell is routinely carried out with the aid of a phase microscope. The theriogenology society (SFT) encourages the use of nigrosin-eosin stain to increase resolution (Gacem *et al.,* 2021). Sperm cells that are abnormal can be categorized into abnormal head (micro, asymmetrical, pyriform, and tapering), or abnormal tail (longer, bent, filiform, broken, truncated and double pieces), and having droplets in the cytoplasm from the neck of the sperm cells during spermatogenesis (Noakes *et al*., 2009).

The semen samples that pass the initial screening are further extended or diluted before being cooled and packaged into straws for freeze preservation. After freezing, usually a representative semen sample is thawed and evaluated for quality status using various laboratory tests. These semen post-thaw evaluation procedures not only document the ability of the samples to withstand the conditions of used in the semen processing but they also inform on the level of fertility of the semen. Assessing the progressive semen motility is considered the most important post-thaw viability evaluation.

2.4 Processing of bull semen for artificial insemination

Semen extenders are used in the processing of bull sperms for AI and their role is to protect and preserve sperm from harmful factors, including freeze and osmotic shock, ice crystals-induced cell injury, and oxidative stress (Bustani and Baiee, 2021). Semen extenders can preserve sperm quality by stabilizing their properties such as sperm motility, morphology, viability, as well as acrosomal, membrane and DNA integrity. Other conditions involved in semen processing include organization and sensitization of the technical staff to improve the repeatability and reproducibility of the test results, customizing information system to capture details of semen collection, collection crate, bull handlers, time and temperature at each stage of processing (Tadesse *et a*l., 2022).

2.5 Semen extenders and sperm cryopreservation

Artificial insemination relies on sperms in semen that has been cryopreserved. During storage of semen under low temperatures, Semen extenders are used. Following their discovery semen extenders were developed for their use to protect sperms from harmful factors, including oxidative stress, freeze and osmotic shock, and cell injury that may be caused by ice crystals (Bustani and Baiee, 2021). Semen extenders usually preserve sperms by stabilizing their properties, including motility, morphology, viability, and membrane, acrosomal, and DNA integrity. It is therefore necessary that semen extenders provide favorable pH, antioxidant activity, adenosine triphosphate, anti-freeze shock, and anticooling activity to improve and preserve semen quality for fertilization (Bustani and Baiee, 2021).

There are two basic methods used in semen storage and these include chilling and cryopreservation. In the chilling technique, the semen is preserved at 4-5°C for 3 days duration for maximum and best results. Cryopreservation of semen involves freezing the sample preparation for 3 h at 4°C. The semen is then filled into 0.25 mL straws before finally preserving and storing them in liquid nitrogen for long duration often lasting years (Baiee *et* *al*., 2017). The critical conditions for long-term semen preservation to maintain its quality are: cooling for 2-3 h, adding a cryoprotectant, and then freezing in liquid nitrogen (Baiee *et al.*, 2018). The main anti-freezing semen cryoprotectants include dimethyl sulfoxide (DMSO) and propylene glycol. For better results 5-15% cryoprotectant should be maintained during freezing and thawing of samples (Bhattacharya, 2018).

Semen extenders are important in maintaining sperm cell survival during the process of cooling and freezing (Bustani and Baiee, 2021). Extenders are used to protect sperms, and to conserve sperm motility, and fertility during storage and processing by stabilizing the plasmalemma. Therefore, sperms can have increased fertilization rate if high-quality extenders are used during chilling and cryopreservation. Semen extenders also preserve and maintain sperm cell metabolic processes, they control the pH of the media during and also after post-thawing, they control bacterial transmission as well as contamination, and also reduce cryogenic damage (Raheja *et al*., 2018). Extenders also maintain pH at 6.8-7.2 and provide needed energy and antioxidant that can reduce oxidative stress.

Commercial Semen extenders are made from different materials including both animal sources such as, skimmed milk and egg yolk, and plant sources such as soybean lecithin (Layek *et al*., 2016). Current semen extenders are associated with diverse problems depending on the type of the extender and the source species. Soybean lecithin as a sperm extender has been reported to have a more hygienic nature when compared with egg yolk extenders (Chaudhari *et al*., 2015). These extenders are very expensive with egg yolk extenders being more extensively used both in the laboratory and in field techniques due to their reasonable lower price coupled with satisfactory results (Layek *et al*., 2016). Due to the problems associated with the current semen extenders including high cost for the plant lecithin-based extenders and the unhygienic nature of egg yolk-based extenders, there is need to evaluate other plant-based products with potential for use as semen extenders. An alternative to the current semen extenders is coconut water which can be very cheap and readily available and can therefore significantly lower the cost of artificial insemination.

2.6 Coconut water and its potential use as semen extender

Coconut water is considered a nutritionally rich and refreshing natural drink with thirst quenching properties and is rich in vitamins, minerals and important phytohormones (Naik *et al*., 2022). It is considered to be a natural isotonic drink because of its salt composition. Therapeutically, coconut water has been used to inhibit and or alleviate numerous human health-associated conditions and disorders such as diarrhoea, kidney-related issues, dehydration, urinary tract infections, myocardial infarction, fatigue, and digestion problems, as well as antiaging issues (Gayán *et al*., 2020).

When compared with normal drinking water, it has been observed that consumption of coconut water high volume causes less nausea, and fullness with no stomach upset (Wang *et al*., 2008). It is likely that the lack of any adverse effects in the body following consumption of coconut water could be due to its comparable physiological properties with body fluids. Apart from being important for human consumption, coconut water is also suitable as a growth-promoting medium that can support viability of many types of beneficial microorganisms. Therefore, coconut water has currently gained a special attention in biotechnological research. In recent years, research scientists have focused their interest in the numerous health and medicinal properties associated with consumption of coconut water and Sugars, free amino acids, vitamins proteins, minerals, and growth promoting factors have been documented to be among the most important biologically essential components found in coconut water (Tuyekar *et al*., 2021).

A recent study evaluating the protective ability of coconut water against intraperitoneally infused carbon tetrachloride-induced toxicity in rats concluded that there were increased antioxidants in treated rats and that treatment did not alter the biochemical parameters in study subjects (Emmanuel *et al*., 2022). Coconut water phytohormones components including auxin, iron, cytokinin's, copper, minerals for electrolyte balance like potassium, sodium, magnesium, phosphorus and calcium and other components like vitamins, sugars, and chlorides among others elements (Tuyekar *et al*., 2021) make this plant product very important with potential for use as a semen extender. Furthermore, coconut water is composed of 95.5 % water, vitamin B complex, cytokines lipids, and enzymes.

2.7 Foot and Mouth Disease (FMD)

Foot and mouth disease (FMD) is among the most devastating and severe diseases of cloven-hoofed livestock (Lalzampuia *et al*., 2023). The disease is caused by the FMD virus (FMDV), which belongs to the genus Apthovirus of the family Picornaviridae. Seven serotypes of FMD virus are documented and these include, O, A, C, SAT1, SAT2, SAT3 and Asia 1 (Figure 2.1). It has been established that Infection with any one viral serotype does not induce immunity against another. Foot and mouth disease is a highly contagious and severe viral disease of affecting livestock and has a significant impact on productivity.

Intensively reared animals have been shown to be more highly susceptible to the viral disease than traditional breeds (WOAH, 2022).

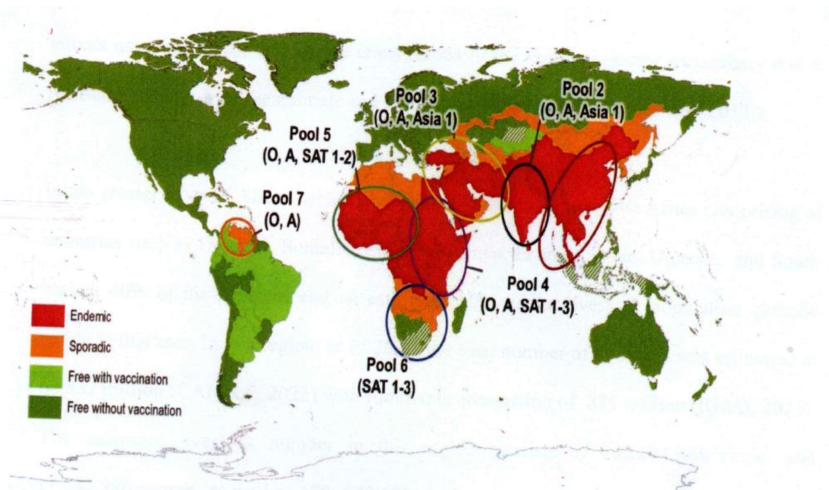


Figure 2.1: Map showing WOAH FMD status and historic endemic Pools of foot-and mouth disease. Some countries may have WOAH-free status with or without vaccination, for example Russia. Striped coloring indicates areas that currently have FMD-free status suspended (WOAH, 2023).

The foot and mouth disease presents with acute fever and accompanied by blister-like sores affecting the tongue and lips, and the mouth, teats and the areas between the hooves. Morbidity due to FMD is usually high, and although mortality is low, animals recovering from the disease usually become carriers that subsequently shed viruses (Artz *et a*l., 2018).

Carrier state can last as long a year in cattle. Young animals show higher mortality rate associated with myocarditis. Although generally FMD has low fatality rates, the economic impact associated with the disease is enormous in the livestock sector particularly due to restrictions on trade of the animals and animal products (Jamal and Belsham, 2013).

In the greater Horn of Africa, which may also include the greater East Africa comprising of countries such as Djibouti, Somalia, Ethiopia, Kenya, Eritrea, Sudan, Uganda, and South Sudan, 40% of the Africans and an estimated 10% of the livestock population globally reside in this area. In this region, as of 2022, the total number of livestock was estimated to be 532 million (CAFRLF, 2022) with ruminants comprising of 375 million (IGAD, 2022).

The estimated livestock number in this region consists of 102,104,688 cattle, and 11,742,390 camels, as well as 179, 579,520 small ruminants. The FMD is among the most important livestock contagious diseases with a great economic impact globally. The FMDV infects over 70 domestics (cattle, goats, sheep, and swine) as well as wild (African buffalo, gazelle) cloven-hoofed animals (Alexandersen and Mowat, 2005) and the virus exists in 7 serotypes (O, A, C, Asia1, SAT1, SAT2, and SAT3)

The FMDV serotypes O and A in order of prevalence are found in the Horn of Africa. Serotypes SAT1 and SAT2 have been reported to be prevalent in Ethiopia, Kenya, and Uganda (Tekleghiorghis *et al*., 2016). In Uganda, SAT1 and SAT2 and have been isolated in the African Buffalo within Queen Elizabeth National Park (Ayebazibwe *et a*l., 2010), while SAT3 was documented in Uganda in 2013. Despite serological detection of Serotype C, this strain has never been isolated from this region and there are no clinical cases of FMD caused by serotype C have been reported or detected in the last 15 years (Paton *et al*., 2021).

The FMD is reported to circulate in 77% of the livestock population, and it is estimated that, the cost burden incurred due to this disease in Africa is 50% of the total cost of livestock diseases in the continent (WOAH, 2022). Foot and mouth disease is endemic in Kenya and causes significant lose in affected livestock (Chepkwony *et al*., 2021). In Kenya FMD is associated with sporadic outbreaks which occur throughout the year. The foot and mouth disease are reported to be endemic in the central part of the country as well as Western, Rift valley. Other areas including North Eastern, and Eastern are considered non-endemic for the FMD.

2.8 Immunology of Foot and Mouth Disease

In cattle, following natural infection with the foot and mouth disease virus (FMDV), the nasopharyngeal mucosa becomes the primary site of replication of the pathogen, and this is followed by dissemination to the lungs and subsequently by viraemia lasting about 3 – 5 days (Arzt *et al*., 2011). The main mechanism involved in controlling the FMDV infection involves development and secretion of neutralizing antibodies, which can be within 4 days post infection and peaking at 14 days and these antibodies are maintained in the animal for long periods of time lasting years (Grubman and Baxt, 2004). Humoral immune responses that develop after infection or following vaccination can protect the animals against FMD but may not completely prevent viral replication within the nasopharynx nor do the prevent establishment of persistent infectious or carrier status (Stenfeldt *et al*., 2016).

Innate immune response characterized by type I and III interferons (IFN) has been documented to play a role in early protective immune response against the viral disease in pigs and cattle (Perez-Martin *et al*., 2014). Although the FMDV has evolved mechanisms to antagonize the interferon response in vitro *(*Medina *et al.,* 2018) type I and III interferons are readily detectable in the serum following FMDV experimental infection in cattle, African buffalo, pigs, and mice (Maree *et al.,* 2016). Significant production of acute-phase proteins (APPs), and other substances including haptoglobin and serum amyloid A (SAA) have been described in cattle serum during acute disease with (Stenfeldt *et a*l., 2011).

2.9 Vaccination against Foot and Mouth Disease (FMD)

Vaccination against diseases is an important strategy for reducing the impacts of food and mouth disease in cattle and helps to block circulation and recirculation of the causative virus in livestock (WOAH, 2022). Effective preventive measures against FMD can be achieved through vaccination mainly with inactivated vaccines. Inactivated vaccines, unfortunately, only offer short-term protection and require cold-chain and high-containment facilities adding to the challenges associated with vaccines against the FMD (Kame *et al*., 2019). Currently, major research and pursuit of advances in hot topics in vaccine development including vectorology are ongoing, involving DNA vaccines, peptide vaccines, and live attenuated vector vaccines. Vaccination against FMD induces both type 1 and type 2 acquired immunity with strong IgG response. In Kenya, vaccination against the FMD is achieved by use of the FOTIVAX TM vaccine which is produced by the Kenya Veterinary Vaccines Production Institute (KEVEVAPI). This vaccine confers protection against the four FMD viral strains circulating in the country including strains O, A, SAT 1, and SAT 2 (KEVEVAPI, 2021

It is a vaccine produced for use in cattle, goats, pigs and sheep to protect the animals from disease caused by any of the four virus strains. Protection against disease is mediated by antibodies produced against the viruses. Since infection with any single viral serotype does not protect against any other virus type, an effective immunity in vaccinated animals should induced antibodies against the four viral types including strains O, A, SAT 1, and SAT 2. Thus, complete protection against the FMD involves production of specific antibody tires above 1.36 against each type of the strains O, A, SAT 1, and SAT 2 viruses (Jo *et a*l., 2019).

Maternal antibodies in young calves have been reported to maintain strong protection against disease for at least two months with antibody levels decreasing significantly three months after vaccination (Lalzampuia *et al*., 2023).   Bulls bred as stock for production of sperm for AI should be vaccinated   against the foot and mouth disease. However, although vaccination against the FMD may be associated with high economic value, the effect of FMD vaccine-induced antibody responses in bulls bred for AI on semen quality is unknown and hence the need for investigation to establish this missing link. Protection against the FMD is mediated by virus- specific antibodies. Immunoglobulin G (IgG) is associated with protection against disease in vaccinated cattle (Brito *et al*., 2015). Immunoglobulin M (IgM) mediates protection against disease in naïveinfected cattle (Pega *et a*l., 2013). Maintaining high levels of anti-FMDV total antibodies is important in order to prevent disease outbreaks.

2.10 Future perspectives on vaccination against Foot and Mouth Disease and economics of Bull semen processing for artificial insemination

Foot and Mouth disease is an extremely contagious disease among cloven mammals – its mortality rate can be very high (100%) in native cattle placing economic constraints on the international livestock trade and can be reintroduced into the disease-free areas unless strict precautions are in place. While this disease can be prevented through vaccination against the causative virus, it is not clear if all vaccinated animals develop protective levels of antibodies. In addition, it is not clear if induced FMDV-specific antibodies have any effect on semen production in bulls bred for artificial insemination

Imported semen extenders are required for processing of semen used for artificial insemination but these commercial extenders are expensive and often are not readily available making the cost of artificial insemination very expensive to the farmers. For this reason, it is necessary to find alternatives that are cheap and easily obtainable. Development of coconut water as an alternative to the commercial semen extenders can offer cheap and readily available semen processing extenders and this can significantly reduce the cost of artificial insemination. For effective disease surveillance and preparedness for possible outbreaks, it is important to document real-time and reliable reports on the natural disease protection status in the affected animal. Thus, reports on the proportions of naturally protected animals farmed in FMD endemic and a non-endemic region in Kenya can be a valuable tool for guiding in future vaccination and disease control programmes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was conducted at the Kenya Animal Genetic Resource Centre (KGRC is a quarantine station) located in Lower Kabete which is 16 kilometers west of Nairobi city center. The current study used bulls of the Friesian breed for collection of semen and blood samples for laboratory analysis, Friesian bulls are less stressful and best for collecting semen. FMD analysis blood samples were collected from non-endemic and endemic regions of Friesian breed, four villages were identified for purpose of collecting the blood serum sample, ten bulls from each village. Makueni a non – endemic region and Kiambu endemic region were purposely chosen. The Centre was established in 1946 as a station for producing bull semen for the Kenya white's highlands farmers (Kenya Gazette notice Number 557 of 19th June 1946) and was then named the Centre for Artificial Insemination Station (CIAS) with the objective of controlling venereal diseases and improving the genetics of exotic dairy cattle.

The Centre occupies 358 acres of Land and hosts various breeds of bulls including- Ayrshires, Friesians, Guernsey, Jerseys, Boran, and Sahiwal. The mandate of the Centre is to produce, preserve and conserve animal genetic materials and rear breeding bulls for provision of high-quality disease-free semen for artificial insemination locally and for export. It is a profound achievement that the center has attained ISO 9001: 2015 QMS certification in management standards on the world best practices (www.kagrc.ac.ke). Semen in the center is produced according to internationally Accepted (OIE regulations) standards and all semen straws are labeled and examined for viability before being distributed.

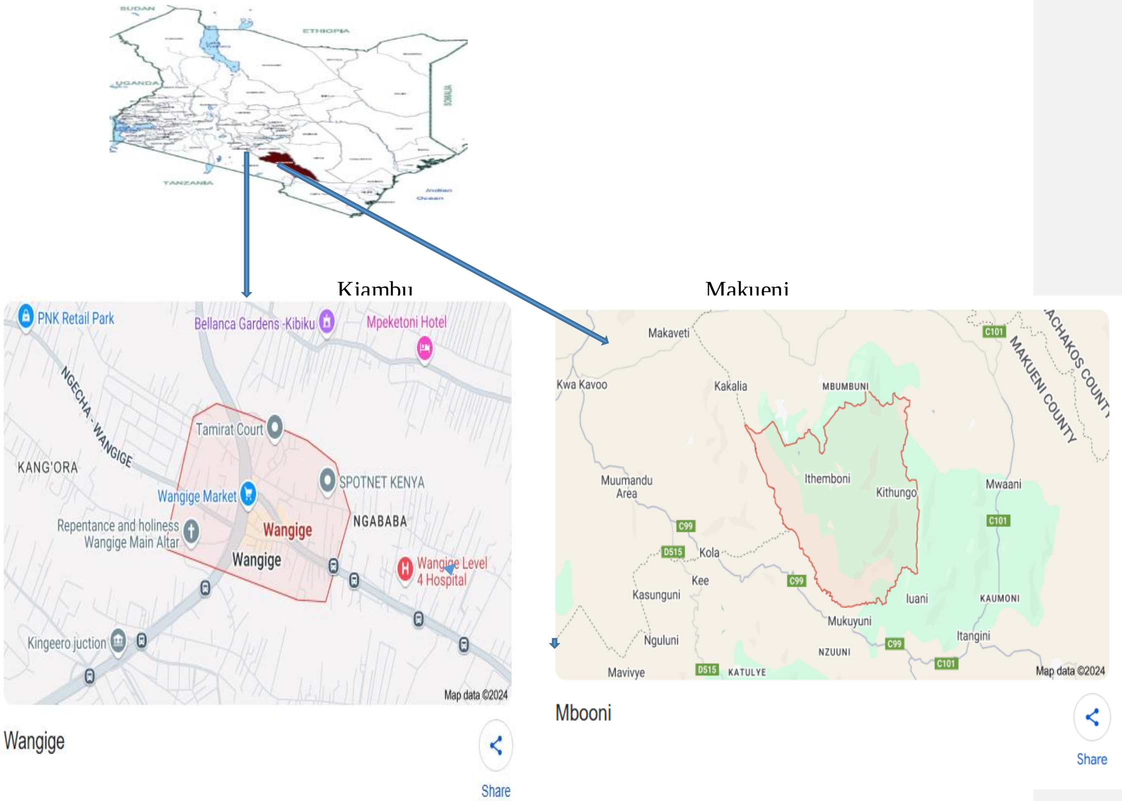


Figure 3.1: Map of Kenya showing study site of FMD endemic and non-endemic regions

3.2 Study animals and samples

These animals included only those bulls vaccinated against the FMD and bred specifically for semen production for AI. Semen sample collection was done by laboratory technologist using prescribed artificial vagina for collecting semen for AI. All FMD vaccinated animals including seroconverting and non-seroconverting were included in the study. In addition, blood samples were collected from the same bulls for analysis of FMD vaccine IgG antibodies. Collected samples were taken to the laboratory for analysis with the help of a veterinary doctor. For animals included in the assessment of natural protection against the FMD, bulls sampled within an area of disease endemicity as well as in a FMD non-endemic area. Blood samples were obtained from these animals for quantification of circulating antibodies against each of the four viral strains including, O, A, SAT 1, and SAT2.

3.3Preparation of coconut water for development of alternative semen extender.

Coconuts were purchased from the city market located at the Nairobi central business district. The coconuts were taken to the Kenya Animal Genetic Resource Centre laboratory for extraction of coconut water. The nuts were washed with clean tap water and soap before rinsing with plenty of tap water. The coconuts were then washed again with distilled water and dried in a biosafety hood. Working in a biosafety hood, the coconuts were opened using a sterile bottle cork opener through the soft stalk area and the coconut water was sucked by use of automated sterile serological pipette into sterile 50 ml centrifuge tubes which were stored briefly at 4°C until used for processing semen samples as test extenders.

3.4 Study design

A total of 24 Friesian bulls aged 18 months (mature age to harvest semen) and above were used for semen sample collection, this is 30% of Friesians bulls that were at the age of collecting semen at the station. All the animals selected for this analysis included bulls vaccinated for FMD. Twelve bulls were in the category of FMD vaccinated animals which developed immunity while the other 12 bulls were vaccinated bulls which failed to develop immunity against the circulating viruses. Bulls selected for semen collection were assembled, washed and kept for 20 minutes to dry up. The bull teaser was used where bulls mounted during semen collection. A laboratory technologist and laboratory assistants helped in collecting semen sample form the bulls. The collecting personnel used artificial vagina with collecting labeled test-tube attached to the artificial vagina. The laboratory technologist recorded the volume of semen sample he has collected and hand over the samples to the veterinary doctor for further processing.

Semen samples from vaccinated animal groups were analyzed for all parameters of semen quality including ejaculate color, volume, sperm concentration, motility, absorbance and post thaw recovery rate. For development of coconut water as semen extender, semen samples in duplicate were processed using commercial semen extenders while another set of semen samples also in duplicate were processed using coconut water before analysis of semen samples for quality parameters. Blood samples of 2mls were also collected from the same bulls for quantification of FMD-vaccine-induced IgG antibodies. For analysis of the relationship between the levels of IgG antibodies and semen quantifiable parameters, semen processing was carried out with commercial extenders. For assessment of acquisition of natural immunity, at least seven bulls were sampled from a FMD endemic (Wangige in Kiambu County) and non-endemic (Mbooni in Makueni county- 39 bull) regions in Kenya in a pilot study. All antibody quantification was carried out using virus neutralization Enzyme Linked Immunosorbent Assay (ELISA) based methodology.

3.5 Quantification of serum Antibody titres from blood samples using ELISA

Fifty µL of Eagles (MM1) media were added using a pipette into all the wells of a microtiter plate but excluding wells in row A before adding 100 µL of a 1/4 dilutions of the control sera and test sera samples in row A. Fifty 50 µL of this 1/4 dilution were transferred from the row A of microtiter plate to row B and the hundred µL contents were carefully mixed several times using a pipette, while ensuring no bubbles are introduced (this resulted in a doubling dilution). Fifty µL were transferred from microtiter plate row B to C and mixing was repeated. From row C to row D, 50 µL were transferred, mixed carefully and this step was repeated down to row H. Fifty µL of the dilution were discarded from row H leaving a final volume of fifty µL (this resulted in 1/4 to 1/512 dilutions in fifty µL volumes). This step was repeated with all test samples and duplicate wells were performed both for the test and control sera. The virus antigen dilutions were then added at this stage:

Fifty µL of the 100TCID50 virus dilution were added to all the wells of the test and control plates resulting in dilutions from 1/8 to 1/1024 before incubation microtiter plates for 1 hour at 37˚C. The cell suspension was added to the microtiter wells at this stage: Fifty µL /well of the cell suspension were added at the prepared concentrations of 0.4-1.0 x 10^6 cells/ml of LFBK, BHK-21 or IB-RS 2 cells in Eagles (MM1) media. The microtiter plates were sealed and incubated at 37˚C for upto72 hours before reading results: The microtiter plates were viewed under an inverted microscope for cytopathic effect (CPE) after 48 hours of incubation. Wells that did not show CPE were recorded as positive while those that showed CPE were regarded as negative. After 72 hours of incubation, the plates were stained with naphthalene blue black dye. Wells staining blue black were considered positive and those appearing colourless were considered negative. For quality control, standard antiserum of known titre, a control cell, a media control and a virus titration are included in the test in every test and used to calculate the actual virus titre. The virus titre was then calculated and results interpreted. The virus titre was considered the dilution where fifty percent of the cells showed positive CPE which upon staining appeared colorless. The endpoint was reported as where there was no CPE and the cell monolayer stained blue black, the color of the stain. This was carried out following the procedure by KEVEVAPI standard operating procedures and Kärber (1931):

The microtitre wells number showing hundred percent CPE divided by the wells number per dilution before subtracting 0.5 (correction factor) then multiplied dilution interval of log. The highest step of dilution with hundred percent CPE was added to all the microtitre wells. The serum Antibody titre was then calculated: With each virus neutralization test (VNT), titration of virus was added, so that the exact titre of virus and doses of virus could be determined (Kevevapi standard operating procedures). For every dose of virus, the corresponding titre of serum was established. The titres of serum were expressed as the reciprocal of log10 dilution which showed fifty percent protection of cultures against infection by that virus dose. The endpoint titre of the sera was expressed reciprocal of the log dilution which recorded protection levels of fifty percent in cultures against 100TCID50 of virus. This was carried out by using plots of doses of the virus ranging $10^{1.5}$ to $10^{2.5}$ versus the corresponding titres of serum and extrapolating the final titre of serum at $100TCID_{50}$. Antibody titres of Log10 ≥ 1.36 were considered protective as per the established laboratory protocols (Appendix I).

3.6 Semen quality analysis

3.6.1 Semen quality analysis for freshly collected samples

Semen samples were collected from bulls (Figure 3.6) through KAGRC laboratory established protocol (Appendix II). Semen samples were analyzed in the laboratory for various quality parameters including sperm count and ejaculate volume, sperm concentration, total motility, progressive motility and sperm morphology. Results from the analysis of these parameters were compared with reference ranges as per protocols established at the Kenya Animal Genetic Resource Centre (KAGRC standard operating procedures) laboratories as well as literature data as described by *Morrell et al.* (2018).

Figure 3.6: Semen collection from a bull at the Kenya Animal Genetic Resource Centre.

3.6.1.1 Determination of ejaculate volume

The ejaculate volume was measured by weighing the semen sample in the same vessel in which it is collected. Samples were collected in a pre-weighed, disposable clean container. The vessel was weighed with the semen in it and the weight of the container was subtracted from the total weight. The semen volume was calculated from the sample weight assuming the density of the semen to be 1 g/ml (*Cooper et al.,* 2007). All semen samples of volumes from at least 1 ml are considered to be of normal volume and were selected for further analysis.

3.6.1.2 Quantification of total sperm count and concentration in ejaculate

Sperm numbers in ejaculate were scored by examining a well-mixed and undiluted sample preparation from liquefied semen on a glass slide under a coverslip. This process was used to determine the appropriate dilution level and the appropriate counting chamber to use. Semen was then mixed and prepared with dilutions with fixative (made by dissolving 50 g of sodium chloride and 10 ml of 35% (v/v) formalin in 1000 ml of purified water) before loading to the Neubauer haemocytometer and allowing the spermatozoa to settle in a humid chamber for 10 minutes. At least 200 spermatozoa were counted per replicate and replicates were compared to see if they are close enough for an acceptable level after which the spermatozoa number was calculated. The concentration of spermatozoa was calculated per ml before the total number of sperm cells per ejaculate was calculated (WOHA 2023, WHO, 2010). Semen samples with normal estimated concentration as counted using the Neubauer haemocytometer were analyzed further for more accurate concentration value using Bovine Accucell Photometer (IMV Technologies Group, France) which is a tool specifically developed for accurate measurement of ejaculate concentration. The sperm concentration was expressed as number of sperms per ml x 10^6.

3.6.1.3 Analysis of progressive sperm motility

Sperm motility was evaluated using a phase-contrast optics microscope and stage warmed at 37^0c. Semen samples were diluted with warmed (37°C) non-fat skim milk-glucose extender to a concentration of 25 to 30 million perms per ml in order to improve the accuracy and repeatability of the sperm motility evaluation. Skim milk-glucose extender supports motility of sperm without interfering with the microscopic visualization of the cells. Assessment of sperm motility included total sperm motility (% of sperm exhibiting motility of any form), progressive sperm motility (% of sperm exhibiting rapid, linear movement), and sperm velocity (on an arbitrary scale of 0 (immotile) to 4 (rapid motile). Highly motile sperm are defined as those with forward progression of at least 25 µm per second. Low sperm motility was recorded as asthenospermia or asthenozoospermia and was categorized as either slow or sluggish progressive motility, non-progressive motility which is a movement less than 5 µm per second, or having no mobility at all. Semen samples with these sperm's features were discarded. If less than 40% of the sperm sample were motile, the sample was considered to have low motility (KAGRC standard operating procedures).

3.6.1.4 Examination of sperm color and morphology

Thin semen smears were prepared and heated up to 36°C to dry them before fixing in 96% ethanol. Briefly, smears were rinsed in running water and stained with 10% blue eosin dye solution for 30 seconds. After rinsing the slides in water again, smears were stained again with gentian pigment (Sigma-Aldrich, USA) for 3 minutes. The slides were then rinsed in water and dried, before microscopic examination to evaluate the spermatozoa morphology. Parameters for normal morphology included smooth rimmed and oval shaped head measuring 2.5 to 3.5 µM wide and 5 to 6 µM long; acrosome that is between 40 to 70% of the head; head that is free of large vacuoles and have not more than two small vacuoles that take up less than 20% of total head; mid-piece of sperm should be much slimmer but about the same length as the head; free from head or tail abnormalities and; uncoiled, 45 µM – long tail that is thinner than sperm head and mid-piece (WHO, 2010). Only sperms with the normal morphological features were selected. Sperms were examined for their appearance including colors such as watery, cream, light cream among others. Cream color was considered the best for normal semen samples as per the established laboratory procedures (KAGRC standard operating procedures).

3.6.2 Assessment of sperm recovery in post-thaw semen samples

After freezing of the samples, one straw of semen sample was picked randomly for evaluation. The sample after freezing was stored in liquid nitrogen container so that one straw for evaluation would pick from there and transferred to another container containing liquid nitrogen. The sample was put in a test tube standing in a water bath heated at 37^0C for 30 seconds to thaw (Jasmer *et al*., 2018). The thawed straw semen vial was dried using dry towel. During thawing both sides of the semen storage vials were cut, and the semen in the straw was poured in thawing test tube standing in water bath heated at 37^0C. After thawing, a drop of the sample was transferred to a glass slide and covered with a cover slip, then put on stage of a warmed phase contrast microscope for examination for motility at x250 magnification. On the standard microscope examination, the sample was assessed for its percentage progression and recorded in a scale interval of 80-100% (if almost all spermatozoa are moving); 60-80%, 40-60%, 20-40% and 1-20% forward progression depending on the proportion of spermatozoa observed. Morphological changes were also observed and recorded on the same scale intervals. Parameters of semen quality were compared between samples processed with commercial extenders and those processed using the coconut water test medium. Likewise, semen quality parameters from samples collected from FMD vaccinated bulls that developed immunity were compared with parameters of semen from bulls that failed to develop immunity following vaccination against the FMD.

3.8 Data analysis

Mean values of data on parameters on variables between the experimental and control animal groups were analyzed by use of GraphPad InStat software of statistical data analysis: Data on antibody levels between FMD vaccinated and non-vaccinated bulls, mean values of parameters of semen qualities between samples treated with commercial extenders and coconut water test medium, as well as mean values of parameters involving quality of semen between vaccinated and non-vaccinated animals all treated with commercial semen extenders were analyzed using student-t-test statistics. Differences between more than two groups of treatments were analyzed using both parametric one-way analysis of variance (ANOVA) and the Kruskal-Wallis test with Tukey and Dunn test as post-tests respectively. Discontinuous data values involving sperm morphology for samples obtained between the two animal study groups were analyzed through descriptive statics while correlations analysis between antibody levels and continuous semen parameters were carried out using Spearman rank correlation analysis test. All data cleaning and normality tests were carried out on each data set by the analysis software.

Significance level was set at P < or > than 0.05. Results were presented in the form Text, Tables, Graphs as well as proportions.

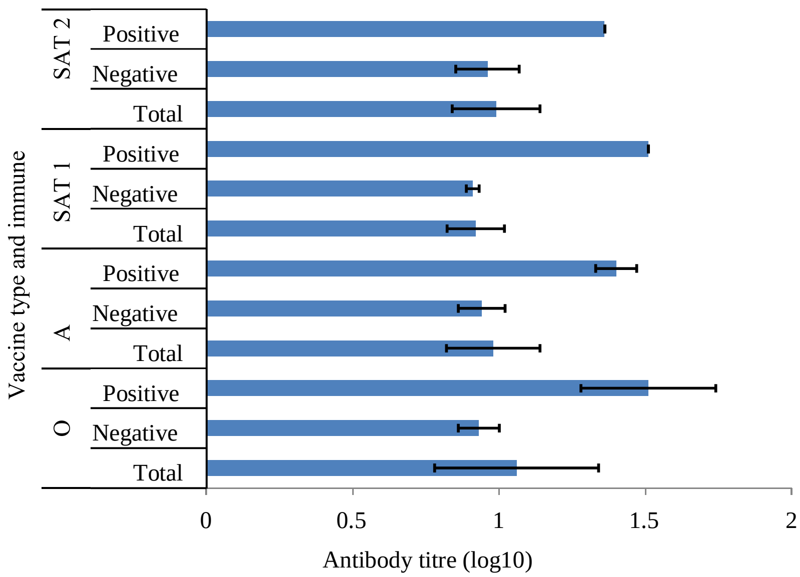
CHAPTER FOUR

RESULTS

4.1 Immune status in bulls from a foot and mouth disease endemic and non- endemic region in Kenya.

4.1.1 Immune status in bulls from FMD non-endemic region

For the animals sampled (n=39) from Makueni County, a region considered non- endemic for the Foot and Mouth Disease, the natural protection status against the four virus strains including O, A, SAT 1, and SAT 2 indicated that, the seroprevalence for the FMD positive were 23%, 10.3%, 2.6%, and 7.7% with antibody titre levels of 1.51±0.24, 1.4±0.07, 1.51±0 and 1.36.±0 All other FMD negative animals had antibody titre levels below 0.96 while the mean value for the total number of bulls in each category of the virus strain was below 0.99 (Figure 4.1). Although on average, all antibody levels against each of the virus strain were below the protective value of 1.36 log10 (animal antibody titre level > 1.36 $\log_{10}$ is protected, animal antibody titre < 1.36 $\log_{10}$ is unprotected), there was a significant difference between tire levels against virus strain O and the SAT 1 strain with antibodies against strain O, being slightly higher ($F = 3.745$; $q = 4.728$; $P = 0.0124$). Antibody levels compared between any other two groups were not different ($P > 0.05$).

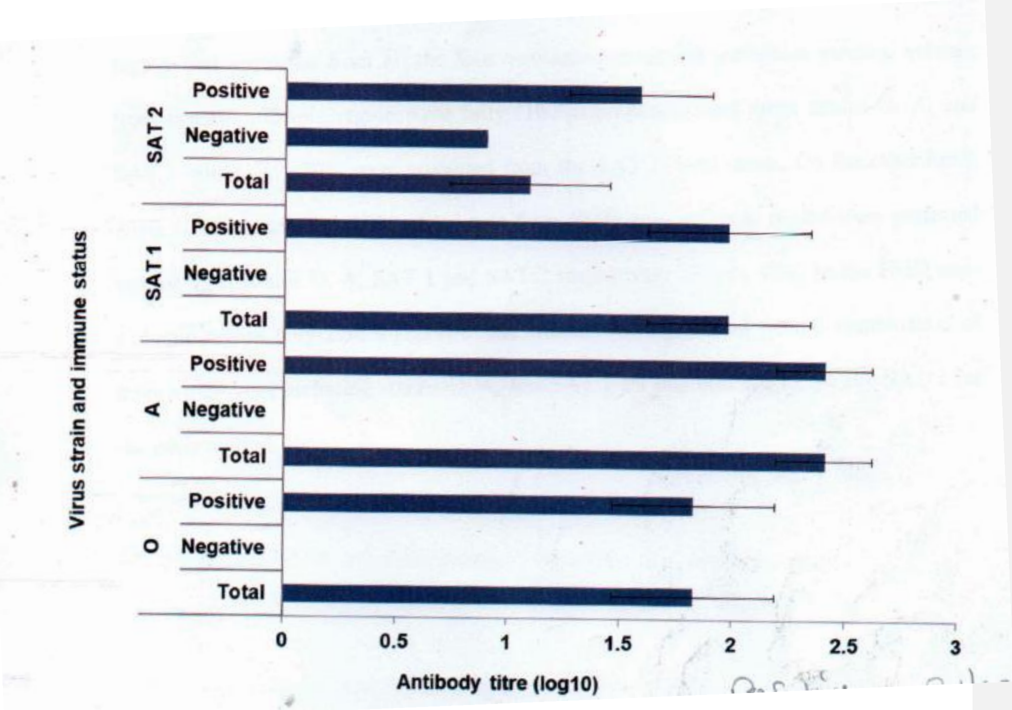


Positive denotes – protection status
Negative denotes- unprotected status

Figure 4.1: Natural protection status of cattle from FMD non-endemic region. Blood samples were obtained from study animals and quantified for antibody levels against virus strains O, A, SAT 1 and SAT 2 by ELISA to establish the level of naturally acquired immunity against disease. Data are presented as mean±SD (standard deviation). Animals with antibody titre levels > 1.36 are considered protected against the FMDV.

4.1.2 Immune status in bulls from FMD endemic region

In Wangige, Kiambu County, an FMD endemic region, antibody titres for the four viruses including strain O, A, SAT 1 and Sat 2 ranged from 1.83±0.36 to 2.41±0.21 for the seropositive animals with the highest level being associated with the A strain virus. All sampled animals were sero-positive for viral strains O, A, and SAT 1 while 61% of the study subjects were sero-negative for the viral strain SAT 2. On average the total number of animals recorded antibody levels of 1.83±0.36, 2.41±0.21, 1.98±0.36 and 1.09±0.36 against viral strains O, A, SAT 1 and SAT 2 respectively (Figure 4.2). Comparing the mean values of the antibody titre against the four virus strains, there was a significant difference ($F = 19.216$; $q > 3.901$; $P < 0.0001$) indicating varying immune statuses. Only Antibody titre levels against virus strain O vs SAT 1 and strain A vs SAT 1 were comparable ($P > 0.05$) while comparison antibody levels between any other two groups concluded a significant difference ($P < 0.05$).



Positive denotes – protective status

Negative denotes- Un-protective status

Figure 4.2: Natural protection status in bulls from FMD endemic region. Blood samples were obtained from study animals and quantified for antibody levels against virus strains O, A, SAT 1 and SAT 2 by ELISA to establish the level of naturally acquired immunity against disease. Data are presented as mean±SD (standard deviation). Animals with antibody titre levels > 1.36 log10 are considered protected against the FMDV.

4.1.3 Proportions of bulls from an endemic and non-endemic region that have acquired natural protection against the FMDV infection

Comparing the proportions of bulls from FMD endemic and non-endemic regions that had acquired natural protection through possible infections with one or more of the circulating viruses' strains, results indicated that overall, 29% of the bulls from FMD endemic region were protected from all four virus strains while none (0%) of the bulls from non-endemic region was protected from all the four viruses. Considering individual viruses, animals from disease endemic region were fully (100%) protected from virus strains O, A, and

SAT 1 while only 29% were protected from the SAT 2 viral strain. On the other hand, 23%, 10.3%, 2.6% and 7.7% of animals from FMD non- endemic region were protected against viral strains O, A, SAT 1 and SAT 2 respectively (Figure 4.3). In the FMD nonendemic region only 2.56% (1/39) of the animals were protected from a combination of three virus types including strains O, A, and SAT 1 for one bull and O, A, and SAT 2 for the other animal.

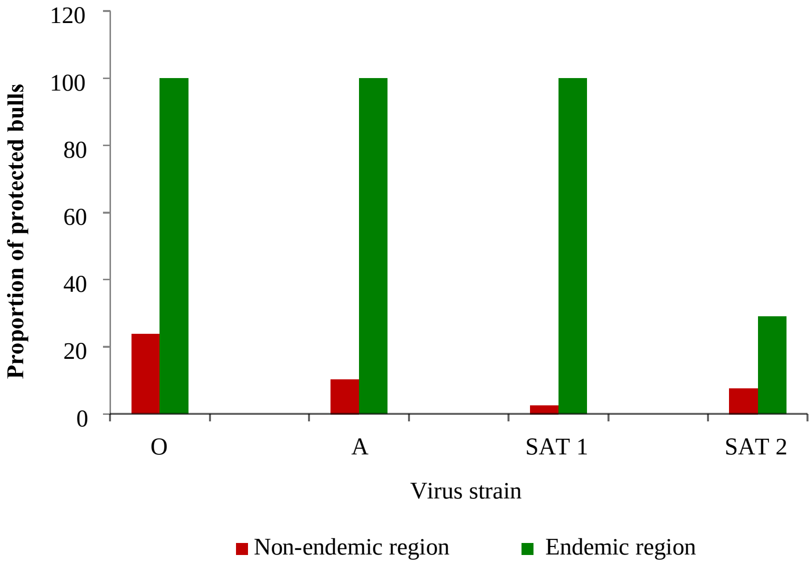


Figure 4.3: Proportions of naturally protected bulls against FMD in both disease endemic and non-endemic regions. Blood samples were obtained from study animals and antibody levels against FMDV were quantified using ELISA to determine to percentages of bulls that were protected against viral strains O, A, SAT 1, and SAT 2 by having antibody titres greater than 1.36. The graph represents proportions of bulls that are protected against FMD associated with the four virus strains.

4.2 Serology titre levels against FMD virus strains

Following vaccination of a group of bulls with vaccines against FMD viruses' strains O, A, SAT1, and SAT 2, and assessment of protection status, results indicated that, out of 12 animals only one (8.33%) bull did not develop any protection against any of the viruses. Two other animals did not develop protection against the strain O of the virus's antibody titres of 1.2 (Figure 4.4). For the vaccine-induced protection, the antibody titres ranged from 1.36 against viral strain O to 2.85 for the A strain vaccination.

The protection achieved 91% level for each of the virus strains A, SAT 1, and SAT 2 while the viral strain O achieved a protection level of 75% among the vaccinated animals. Among the various vaccine categories, antibody titres were significantly different ($F = 4.889$; $q > 3.78$; $P = 0.0051$). Significantly higher antibody titres were recorded for vaccines for virus strain A and SAT 2 as compared to strain O ($P < 0.01$ and $P < 0.05$). There were, however, no significant difference when antibody titres between any other two vaccine categories were compared ($P > 0.05$).

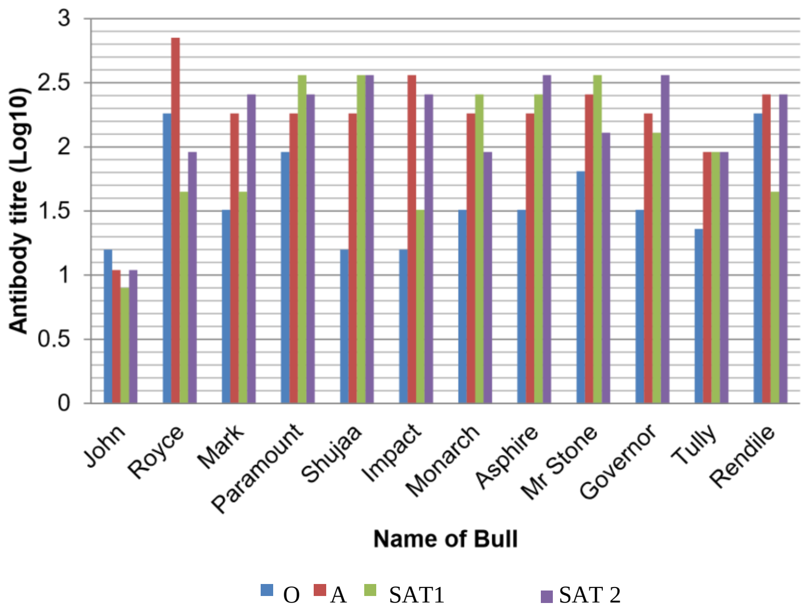


Figure 4.4: Antibody titres in bulls vaccinated against FMD viral strains O, A, SAT 1 and SAT 2. A group of bulls were vaccinated against four FMD viruses and antibody levels were quantified using ELISA. The Graph represents vaccinated bulls and the mean of duplicate antibody titres for each of the vaccines against viral strains O, A, SAT 1, and SAT 2.

4.3 Semen parameters in bulls vaccinated against FMD

4.3.1 Semen parameters in bulls that develop immune against FMD following vaccination

Vaccinated bulls that developed immunity against the FMD showed semen volumes ranging from 4.3 to 8.0 ml and absorbance ranged between 0.3005 and 0.86 nm (Figure 4.5). Sperm concentrations ranged between 302 and 1345 sperms per ml of semen (Figure 4.6). Motility for diluted semen ranged between 55 and 90% while recovery post thawing was in the range of 25 to 70% (Figure 4.7). Two bulls had light cream semen while the rest had the normal cream-colored semen. One of the bulls which had light cream colored semen had the lowest sperm concentration of 302 sperms per ml despite having above average volume of 6.5 ml. Sperm motility in the diluted semen was 60%. However, this bull was associated with very low sperm recovery of 25% post thawing. The other bull whose semen was light cream in color had a volume of 8.0 ml with the second lowest concentration of 364 perms per ml of semen and 0.339 absorbance level. Motility of diluted semen was 55% and the recovery post thawing was also relatively low at 30%.

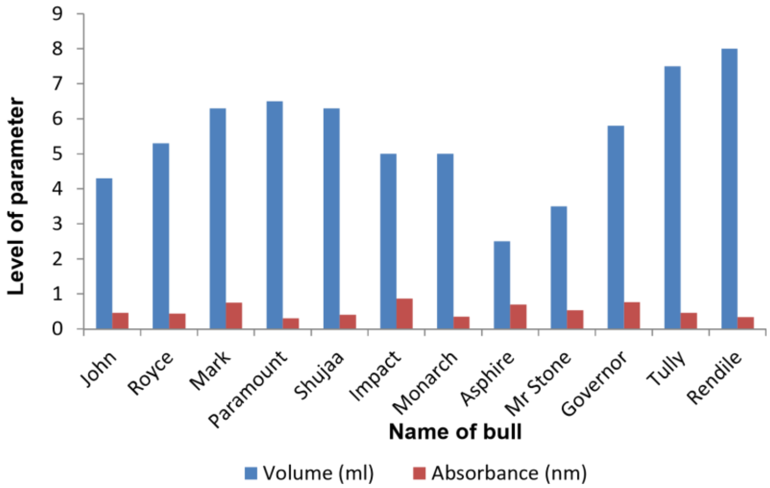


Figure 4.5: Volume and absorbance of sperms from semen obtained from bulls which developed immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples were obtained from these bulls and analyzed for their volume and absorbance. The Graph represents the levels of these parameters in the various sampled bulls.

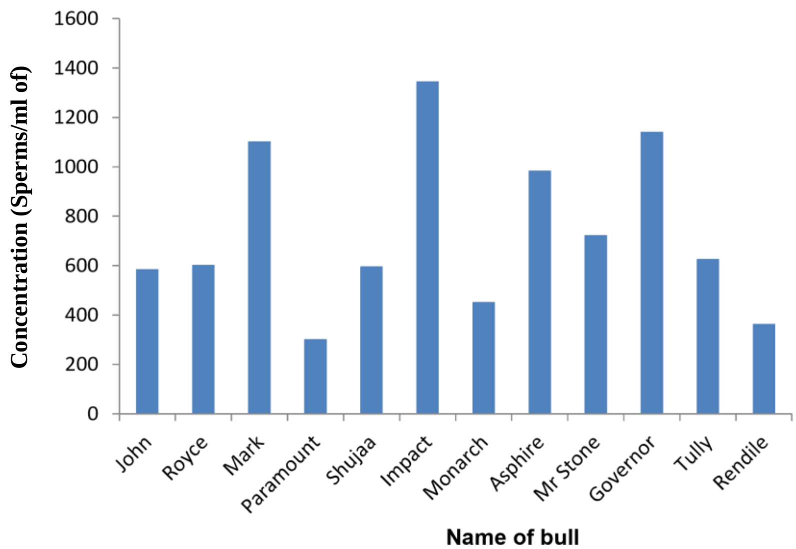


Figure 4.6: Sperm concentration per ml of semen obtained from bulls which developed immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples were obtained from these bulls and analyzed for their concentration. The Graph represents the concentration of sperms per ml of semen obtained from various sampled bulls.

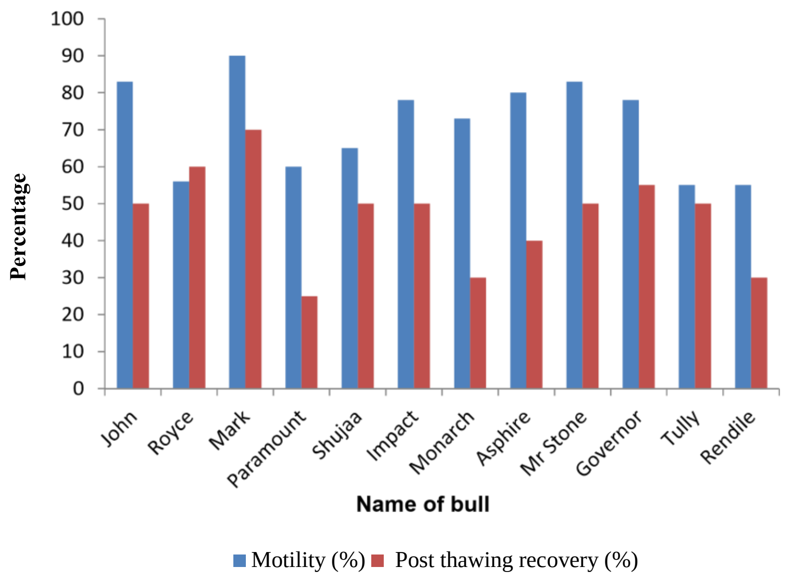


Figure 4.7: Percentage motility and post-thawing recovery of sperms in semen from bulls which developed immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples were obtained from these bulls and analyzed for their motility and post thawing recovery. The Graph represents the percentage of these parameters in the various sampled bulls.

4.3.2 Semen parameters in bulls that failed to develop immunity against FMD following vaccination

Semen parameter analysis carried out in bulls which failed to develop immunity against the FMD following vaccination with current viral vaccines, indicated that although most of all the parameters where within the normal range, 25% of the semen were either light cream or watery instead of having the normal cream color. Semen concentration showed a wide variation ranging from 301 to 2270 spermatozoa per ml of semen (Figure 4.8). Semen volume ranged from 2.3 to 8.3 ml per ejaculation while absorbance ranged was between 0.2 and 1.18 nm (Figure 4.9).

Motility of the diluted semen samples ranged between 70 and 88% with post thawing recovery ranging from 35 to 65% (Figure 4.10). One bull with light cream-colored semen had the lowest concentration of 301 sperms per ml and an absorbance of 0.27. However, the volume of semen for this bull was 5 ml which was above average. Another bull which produced the lowest semen volume of 3.3 ml had watery semen and the concentration and absorbance were 488 sperms per ml and 0.396 respectively. These values were relatively low and this bull also had the lowest motility percentage of 70% for the diluted semen. There was also another bull whose semen was watery but all the other parameters were good. Post thawing recovery of the spermatozoa was recorded from between 35 to 65%. Thus, no association between vaccination status and

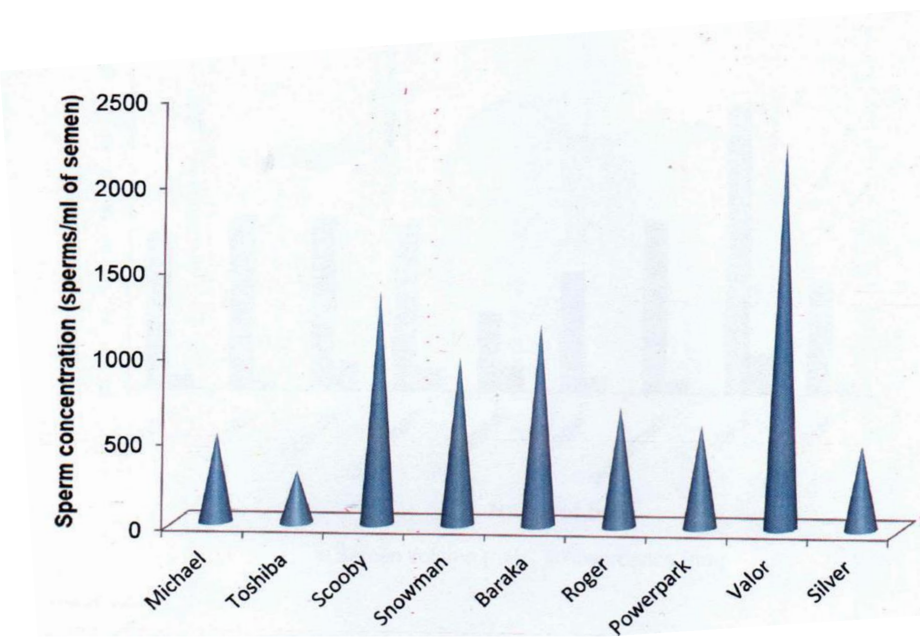


Figure 4.8: Sperm concentration per ml of semen obtained from bulls which failed to develop immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples

were obtained from these bulls and analyzed for their concentration. The Graph represents the concentration of sperms per ml of semen obtained from various sampled bulls.

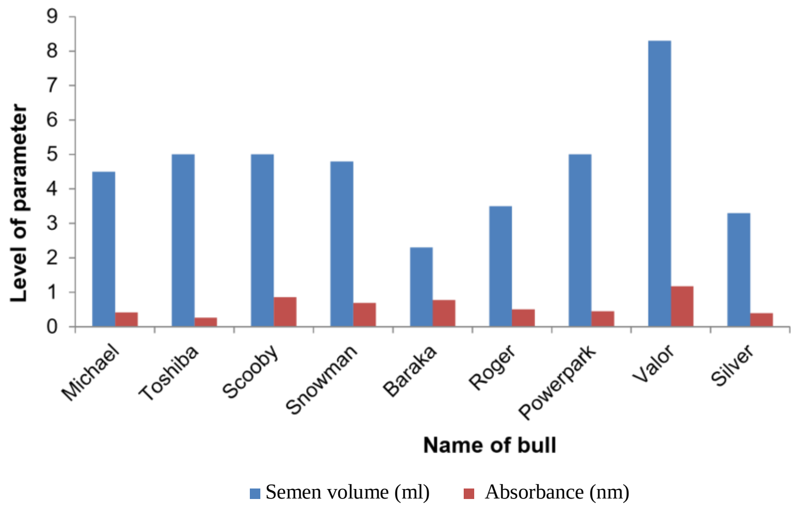


Figure 4.9: Volume and absorbance of sperms from semen obtained from bulls which failed to develop immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples were obtained from these bulls and analyzed for their volume and absorbance. The Graph represents the levels of these parameters in the various sampled bulls.

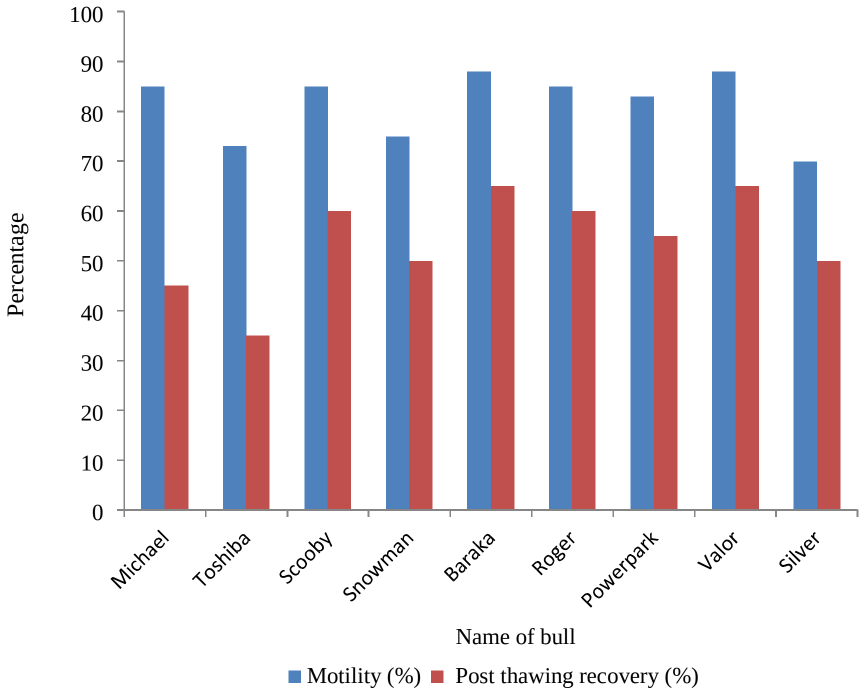


Figure 4.10: Percentage motility and post-thawing recovery of sperms in semen from bulls which failed to develop immunity against FMD following vaccination. A group of bulls were vaccinated for all the four FMDV strains including strain O, A, SAT 1, and SAT 2- and four-months later semen samples were obtained from these bulls and analyzed for their motility and post thawing recovery. The Graph represents the percentage of these parameters in the various sampled bulls.

4.3.3 Comparison of semen parameters between FMD immune and non-immune bulls following vaccination

Figure 4.11 represents compared bulls' post vaccination semen parameters data. The main difference in semen color between the bulls that failed to develop immunity and those that developed antibodies against vaccination, was a watery semen sample in a single animal in the vaccine negative group. Semen volumes ranged from 2.3 to 8.3 ml and 2.5 to 8.0 ml in the vaccine negative and vaccine positive animals respectively. Mean difference between the mean semen volumes of the vaccine positive minus the mean semen volume in the vaccine negative bulls was 0.8667 ml. The two-tailed P value is 0.2405, considered not significant ($t = 1.212$; $df = 19$). Sperm concentration in the semen samples ranged from 301 to 2270 and 302 to 1345 per ml for the vaccine negative and vaccine positive bulls respectively. There was no significant difference in the sperm concentrations between the two animal groups ($t = 0.9481$, $df = 19$; $P = 0.3550$).

Semen absorbance for the vaccine negative bulls ranged from 0.27 to 1.18 while in the vaccine positive animals the absorbance was between 0.3005 and 0.8615. Analysis of results indicated that the level of absorbance in the two animal groups were comparable ($t = 0.8398$, $df = 19$; $P = 0.4115$). The mean percentage of the sperm motility in the diluted semen was 81 ± 7 and 71 ± 13 in the vaccine negative and vaccine positive bulls respectively. The percentage sperm motility in the vaccine negative bulls was significantly higher than the motility in the vaccine positive bulls ($t = 35.878$, $df = 8$; $P < 0.0001$). Sperm recovery post thawing in the vaccine negative group ranged from 35 to 65% while in the vaccine positive bulls the range was 25 to 70%. The post thaw sperm recovery in the vaccine negative bulls was significantly higher than the recovery in the vaccine positive bulls ($t = 16.280$, $df = 8$; $P < 0.0001$).

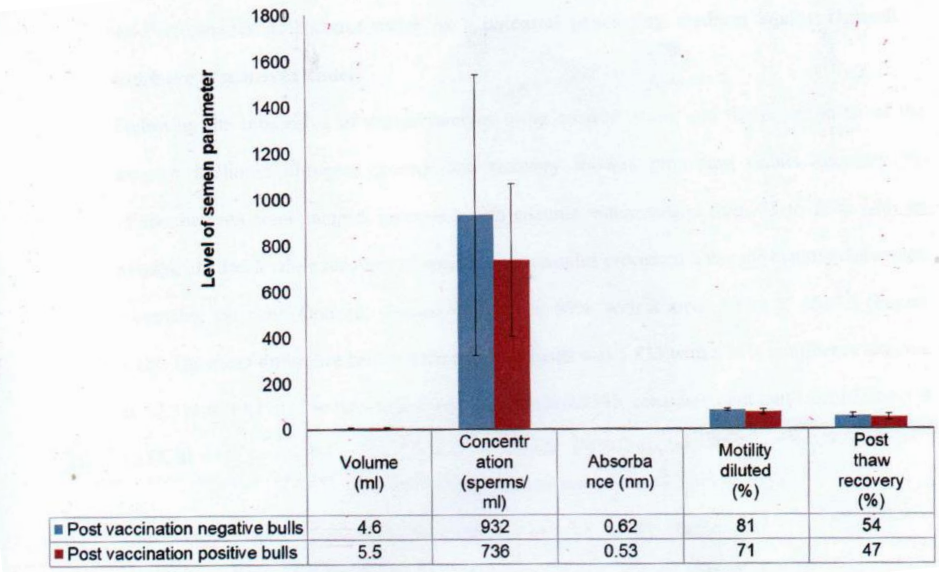


Figure 4.11: Relationship between semen parameters in bulls post vaccination. Bulls were vaccinated against the FMD and their semen samples were analyzed and semen parameters were compared both for the animals that developed immunity and the bulls that failed to develop immunity against the vaccine.

4.4 Performance of coconut water as a potential processing medium against Opticell commercial semen extender

Following the processing of semen samples using coconut water and the preservation of the samples in liquid nitrogen, spermatozoa recovery showed promising results. Recovery (%) of spermatozoa from samples processed with coconut water ranged from 35 to 50% with an average of 43±5.2 while recovery of sperms from samples processed using the commercial semen processing extender, Opticell, ranged from 45 to 60% with a mean value of 48±7.5 (Figure 4.12). The mean difference between the two treatments was 5.833 with a 95% confidence interval of -

2.511 to 14.178. The two- tailed t-test P value is 0.0595, considered not quite significant (t = 1.557, df = 10).

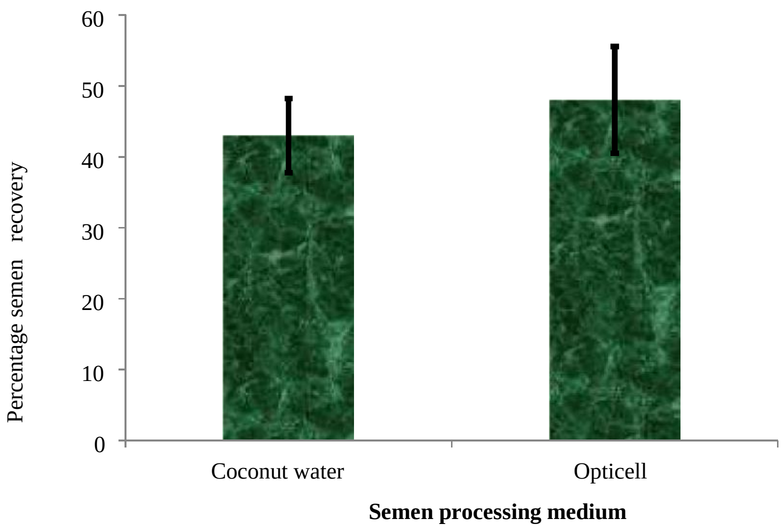


Figure 4.12: Percentage spermatozoa recovery from semen processed using coconut water and Opticell before preservation and recovery from liquid nitrogen. Duplicate semen samples from six bulls were processed using coconut water and the commercial semen extender, Opticell before preservation in liquid nitrogen. Samples were recovered from liquid nitrogen and the percentage motility of sperms was analyzed. The Graphs represent the mean±SD of the percentage recovery of sperms from cryopreserved semen samples.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Protection status among bulls from a foot and mouth disease endemic and non-endemic region in Kenya

In nature pathogens infect animals and while some of the infected animals can develop severe or fatal disease, others may develop mild or no symptoms at all. Some animals are naturally resistant to some diseases, and others become asymptomatic carriers. The foot-and-mouth disease is a highly contagious infection (Kim *et al*., 2023) and is associated with huge loses in affected livestock (WOAH, 2023). In a disease non-endemic area, it is not common to find animals that have developed antibodies against a pathogen that is not available unless the animal was transferred from a distant disease endemic region. It is clear that if some animals in an area without disease are protected against the specific disease pathogen, then there could be an emerging disease.

The findings that in the present study, varying numbers of cases of bulls having above minimum levels of antibody titres specific for the four FMD virus strains, indicates that the animals had been recently exposed to virus replication, implying infection. The emergence of a new FMD virus strain in a region where there is no reported prior disease occurrence or vaccination coverage is considered a great concern (WOAH, 2023). In the present study, the presence of circulating FMD virus strains in the disease non-endemic study area cannot be ruled out. This may call for further investigations to unravel the extent of disease emergence which could arise due to climate changes. The absence of reports of disease outbreaks in the FMD non-endemic region is obviously not due to natural resistance in the animals since only a small percentage of the bulls bore protective antibody titre levels. In addition, virus strain O may be the most common but with all strains represented by at least one or more cases of seroprevalence, disease monitoring and surveillance should be initiated. On the other hand, with a protection level of 100% in all three other FMD virus strains apart from SAT 2 which had a protection level of 29%, it is clear that most animals in the disease endemic region have been naturally infected and able to develop immunity against infection as indicated by the high antibody titres found in the sampled bulls. A recent study carried out on sero-epidemiology of FMD in Northern Pakistan reported a prevalence of 67% (Ullah *et al*., 2023) indicating a high natural protection level (high is 1.85- 2.41 log10, low > 1.36 log10). Thus, the development of natural protection can be high in a disease endemic area. Based on the findings in the present study, it would be important to carry out extensive studies to establish the extent of seroprevalence in a large cattle sample size because achievement of herd immunity to high levels may signify that vaccination is not necessary in disease endemic regions.

5.1.2 Serology titre levels produced against FMD virus strains

Vaccination against the FMD using a combined vaccine for the viral strains O, A, SAT 1, and SAT 2 is expected to protect animals against each of the four viral strains. There is no cross protection between the various FMD viral strains infection (KEVEVAPI, 2021, WOAH, 2023, Kim *et al*., 2023). It therefore, poses a challenge if the combined vaccine for FMD does not induce immunity against each of the viral strains. In the present study, the FOTIVAX TM (preparation containing chemically inactivated tissue culture derived FMD virus strains) combined vaccine appeared to achieve good protection status due to its ability to fully induced antibody titre levels above the minimum required threshold in at least 75% of the total vaccinated bulls. However, it was not immediately clear why one vaccinated bull completely failed to produce antibodies against any of the vaccine viral antigens. This remained a puzzle because it is not a common occurrence. It is likely that some animals may fail to respond to vaccination probably due to genetic factors or other aspects that are yet to be investigated such as issues of vaccine delivery. The failure of 2 (16.6%) vaccinated bulls to produce protective antibody levels against the virus strain O while being fully protected against all the other strains calls for further investigation in order to establish if there is a need to repackage the vaccine antigens aimed at improving the composition contributed by the FMD virus strain O. This will ensure achievement of complete protection antibody levels for all the virus strains. There have been significant interests towards developing improved FMD vaccines and progress has been made by use of different approaches (Belsham, 2020). Because the current inactivated vaccines provide protection for only 6 months, a different approach in vaccine development such as recombinant vector technology can probably achieve better longer duration and correlates of protection.

5.1.3 Semen parameters in bulls vaccinated against FMD

Vaccination against FMD is an important strategy to control disease spread and it has been documented that vaccination of cattle is enough to prevent disease transmission to other animals within a given stock (Bravo DE Rueda *et a*l., 2015). In the production of semen for AI, quality standards are practiced and one strategy is to ensure that all bulls bred for sperm donation are vaccinated against the FMD. In Kenya a combined vaccine aimed to protect bulls reared for production of semen for AI against the four FMD circulating viruses including strain O, A. SAT 1, and SAT 2 is used (WOAH, 2023). The use of this vaccine is not expected to have any negative effect on semen parameters. In the present study, it was not very clear why a large number (44%) of the vaccinated bulls that failed to develop immunity following vaccinated produced watery or light creamy semen and the observation of 15% similar findings in the animals that seroconverted upon vaccination calls for more investigation on this aspect especially in larger number of samples.

While largely all the semen parameters for the two groups of vaccinated animals including the seroconverting and the non-seroconverting bulls following vaccination were within normal values, the observation that 25% of the vaccine positive compared to 11% of the vaccine negative bulls failed to achieve a normal sperm recovery post thawing, is worrying. The difference between the vaccine positive and negative bulls was the presence and absence of anti-vaccine antibodies respectively. Therefore, whether the presence of antibodies in a bull can have an effect that can influence sperms quality at any stage of semen processing, is a subject for further investigation. Furthermore, there are not available previous similar studies that can provide reference on this finding.

5.1.4 Effect of coconut water as a potential semen processing medium

The cost of artificial insemination services is largely dependent on the cost of semen processing. Semen extenders are important media that preserve sperms by stabilizing their properties, including sperm motility, morphology, and viability and DNA, membrane, and acrosomal integrity (Bustani and Baiee, 2021). Commercial semen extenders can be very expensive and this can slow down the uptake of AI services due to increased cost (Khatimah *et al*., 2021). Semen extenders are important component in semen processing.

The comparable sperm recovery rate from semen processed using Opticell commercial extender and coconut water in the present study is an indication that coconut water can be potentially used as an alternative semen processing extender in place of the current expensive commercial extenders. Furthermore, semen extenders should provide adenosine triphosphate, anti-cooling and anti-freezing shock, favorable pH, and antioxidant activity in order to preserve the quality of semen for effective fertilization (Bustani and Baiee, 2021). The main goal in semen processing and preservation is to achieve a high percentage of sperm recovery post thawing and indeed if semen samples processed with coconut water in the present study could achieve a recovery rate with high progressive motility, then it is appropriate to conclude that coconut water has the potential to be used as an alternative cheap semen extender in place of the current expensive commercial extenders. The research study used semen from quarantine bull's station.

5.2 Conclusions

i. In the FMD non-endemic region 23.8%, 10.3%, 2.56% and 7.69% of the bulls were protected against virus strains O, A, SAT 1, and SAT 2 respectively while in the FMD endemic region, All the sampled bulls were fully protected against strains O, A, and SAT 1 and 29% of the animals were protected against the SAT 2 strain of the viruses.

ii. The serological antibody titre levels produced against the FMD viruses ranged from 1.36 to 1.51 at Log_{10} among the bulls farmed in the non-endemic region while in the disease endemic area the Log_{10} of the titre levels ranged from 1.85 to 2.41.

iii. Among the FMD vaccinated bulls both seroconverting and non-seroconverting bulls showed 16.7% of the animals having light cream or watery semen. Both groups of bulls had most of the semen parameters falling within normal range (Normal above 1ml- KAGRC operating producer). However, the non- seroconverting bulls recorded significantly higher rate of semen motility and recovery as compared to the bulls which developed immunity against the vaccine.

iv. Sperm recovery rates in semen processed with coconut water and in samples processed with the Opticell commercial extender were comparable.

5.3 Recommendations

i.There should be monitoring and surveillance for FMD emergence in non-endemic regions.

ii.. Animals farmed in FMD endemic regions may already have developed natural immunity and as such do not require vaccination.

iii. Base on the study and other related studies, Coconut water should be considered as a cheap and locally available medium for semen processing in place of the expensive commercial extender.

5.4 Suggestions for further studies

i Further studies should be carried out to establish if vaccine-induced antibodies against FMD vaccination have negative effects on sperms

ii Studies using large sample sizes of animals in FMD endemic regions should be carried out to establish the extent of acquisition of herd immunity

iii Investigations on differences in sperm quality should be carried out in FMD vaccinated animals particularly comparing parameters between seroconverting and non-seroconverting bulls

iv Investigations on nutrition and management practices should be carried out since can influence semen quality, quantity and concentration

5.5 Limitations of the study

The study used blood serum samples for FMD virus test using 7 animals (small size) from endemic region due to financial constrain.

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APPENDICES

Appendix I: Virus neutralization assays and antibody titre determination protocols

MOALFC/LTPS/FMD/SE/01 Version: 00

REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE LIVESTOCK FISHERIES AND COOPERATIVES
STATE DEPARTMENT FOR LIVESTOCK
DIRECTORATE OF VETERINARY SERVICES

NATIONAL VETERINARY LABORATORIES(NVL)	**DOCUMENT NO: MOALFC/LTPS/FMD/SE/01**	**VERSION: 00**	**COPY NO:**
TITLE:PROCEDURE FOR THE DETECTION OF FOOT AND MOUTH DISEASE ANTIBODIES USING VIRUS NEUTRALIZATION TEST (VNT)			

Approval and Document Control
Implementation Date:

	Name	Signature	Date
Prepared by	Joseph Kabugi		15/06/2021
Technical Review by	William Birgen		15/06/2021
QA review by	Dr Kenneth Ketter		15/06/2021
Approved by	Dr. Abraham Sangula		15/06/2021

Note: This is a controlled document and shall not be amended or revised without authorization.

1 Introduction

Foot and mouth disease (FMD) is a highly infectious disease caused by a virus of the genus Aphthovirus, family Picornaviridae. It is the most economically important disease of livestock due to the large numbers of animals affected causing great production losses and being a constraint to international trade. There are seven serotypes of FMDV (Foot and mouth disease virus) namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia1 that affect clovenhoofed animals, including domestic and wild bovids, ovines, caprines and porcines. Serotypes O, A, SAT 1 and SAT 2 are the common ones occurring in Kenya. FMD is characterized by fever and blister-like sores on the tongue and lips, in the mouth, on the teats and between the hooves.

Serological tests for FMD are performed for various purposes: to certify individual animals prior to import or export (i.e. for trade); to confirm suspected cases of FMD; to substantiate absence of infection and to demonstrate the efficacy of vaccination. Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

Virus neutralization test (VNT) is one of the FMDV serotype-specific antibody detection tests. The test is highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain circulating in the field. The VNT requires cell culture facilities, the use of live virus and takes 2–3 days to provide results.

2 Purpose

To provide a procedure for sera and/ blood sample receiving, processing, detecting and quantifying FMDV serotype specific antibodies by VNT, and reporting of results to the client.

3 Scope

This SOP applies to all serological samples received from the field and other laboratory sections. It is applicable to all staff involved in the sera receiving, processing, testing, analysis and reporting of results to the client and to testing of serum samples from all species of cloven –hoofed animals for FMDV serotype specific antibodies.

4 Abbreviations and definition of terms

4.1 Abbreviations

4.1.1	DVS	Director of Veterinary Services
4.1.2	NVL	National Veterinary Laboratories
4.1.3	SOP	Standard Operating Procedure
4.1.4	QM	Quality Manager
4.1.5	OIE	World organization for Animal health
4.1.6	CPE	Cytopathic effect
4.1.7	FMDV	Foot-and-mouth disease virus
4.1.8	BHK-21	Baby Hamster Kidney (cell line) clone 21
4.1.9	IBRS-2	Swine kidney cell line
4.1.10	LFBK	Line of foetal bovine kidney
4.1.11	BCS	Bovine Convalescent Sera
4.1.12	BSC	Biosafety cabinet
4.1.13	NSP	Non-Structural Proteins
4.1.14	SP	Structural Proteins
4.1.15	O	FMDV serotype O
4.1.16	A	FMDV serotype A
4.1.17	C	FMDV serotype C
4.1.18	SAT 1	FMDV serotype South Africa Territory Type 1
4.1.19	SAT 2	FMDV serotype South Africa Territory Type 2
4.1.20	TVD	Test Virus Dose
4.1.21	VNT	Virus Neutralization Test
4.1.22	FCS	Foetal Calf Serum
4.1.23	TCID50	Tissue culture infective dose of virus that kills 50% of cells

4.2 Definition of terminologies

4.2.1 Serological sample – Sera samples from any species of FMD susceptible animals.

4.2.2 Bivids- comprising the hollow-horned ruminants, as oxen, buffaloes, antelopes, sheep, and goats

4.2.3 Porcines-of, relating to, to swine

4.2.4 Caprine-of, relating to, or characteristic of a goat

4.2.5 Ovine - of, relating to, or resembling a sheep.

5 Principle of the test

Virus neutralization test is an antigen-antibody based immunological test. Serial dilutions of heat inactivated test serum are incubated with known viral suspension of infectious FMDV. If antibodies to the virus are present, they bind to the virus, preventing its attachment to and subsequent infection of cells. Following this incubation, virus susceptible cells are added to the virus-serum mixture, and the final virus/serum/cell combination is incubated for a period of 2-3 days. After this incubation period the test is read by examining each well of the plate for the presence of viral infection by direct microscopic examination or staining of the test wells of the plate for evidence of viral cytopathic effect (CPE). Sera which contain antibodies to the virus in question are able to neutralize the aliquot of virus used in the test, thus preventing infection of the cells when they are added to the plate. Where high concentrations of antibody to the virus in question are present in the serum sample, virus neutralization will occur even at high serum dilutions. Conversely, where little or no antibody to the virus is present in the test sample, it will be unable to neutralize the aliquot of infectious virus at the first dilution used in the test. The result of the test is the point at which the serum sample has been diluted such that it is no longer able to neutralize all the virus in the test. This dilution, or its log equivalent, is reported as the titre of the serum tested.

6 Performance Characteristics

Antibody positive wells (where the virus has been neutralized and the cells remain intact) are seen to contain blue-stained cells sheets; antibody negative wells (where virus has not been neutralized) are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected. The test is considered to be valid when the amount of virus used per well is in the range log10 1.5–2.5 TCID50, and the positive standard serum is within twofold of its expected titre.

7 Responsibility

7.1 Director of Veterinary Services

7.1.1 Not applicable

7.2 Head of division

7.2.1 Not applicable

7.3 DVS Quality Management team

7.3.1 Identifies training needs related to this procedure.

7.4 Head of Foot and Mouth Disease Laboratory

7.4.1 Approves and authorizes this SOP

7.4.2 Ensure this procedure is available at the point of use and is adhered to.

7.4.3 Ensure only competent staff are authorized to carry out this procedure.

7.4.4 Authorize staff to carry out the procedure.

7.5 Quality Manager (QM) at Foot and Mouth Disease Laboratory

7.5.1 Conducts quality reviews on this procedure.

7.5.2 Update the document master list as required.

7.5.3 Avail and ensures that only the latest version of this procedure is in use in their respective laboratory section.

7.5.4 Ensure that this procedure is adhered to.

7.6 Head of the Lab/Technical Head

7.6.1 Avail and ensures that only the latest version of this procedure is in use at the point of use.

7.6.2 Ensure that this procedure is adhered to.

7.6.3 Initiate technical reviews on this procedure.

7.6.4 Ensure only competent personnel carry out this procedure.

7.7 Quality Champion

7.7.1 Avails and ensures that only the latest version of this procedure is in use at the point of use.

7.7.2 Ensure that this procedure is adhered to.

7.7.3 Initiates reviews on this procedure.

7.8 Technical Staff

7.8.1 Prepares and/or reviews this procedure.
7.8.2 Adhere to this procedure.
7.8.3 Implements this procedure.

8 Required Equipment and Reagents

8.1 Equipment

8.1.1 Pipettes (5-20ml)
8.1.2 Reagent Troughs
8.1.3 Water Purification System
8.1.4 Refrigerator at +4°C
8.1.5 Freezer at -20°C and -80°C
8.1.6 Incubator/Hot room at 37°C
8.1.7 Microscope
8.1.8 Water bath at 56°C
8.1.9 Staining troughs
8.1.10 pH Meter
8.1.11 Glassware/Plastic ware
8.1.12 Cryopreservation Vials
8.1.13 Vortex Mixer
8.1.14 Weighing Balance
8.1.15 Timer
8.1.16 Tissue culture plates cover lids
8.1.17 Haemocytometer
8.1.18 Cell counter
8.1.19 Bijoux bottles (5ml) and universal bottles (20ml)
8.1.20 Inverted microscope
8.1.21 BSC
8.1.22 Multichannel pipette.

8.2 Consumables

8.2.1 0.2% (w/v) Citric Acid (for decontamination)
8.2.2 Reagent reservoirs
8.2.3 Sterile tips for multi-channel pipette
8.2.4 Sterile tips for multi pipette
8.2.5 Absorbent paper/ cloth/ towels, lint free, and disposable
8.2.6 Tissue culture plates, flat bottom, 96 well microplates
8.2.7 pH indicator strips - pH indicator (pH 5-10)

8.2.8 Marker pens (water proof) and adhesive labels

8.2.9 Phenol Red - pH indicator (pH 6.4-8.2)

8.2.10 Sterile water

8.2.11 Naphthalene black stain.

8.2.12 Gloves

8.2.13 FCS (Foetal calf serum)

8.2.14 Eagles (MM1) medium with antibiotics (neomycin, streptomycin and penicillin)

8.2.15 LFBK, IB-RS 2 or BHK cell suspension in Eagles (MM1) media 0.4-1.0 x 10^6 cells/ml

8.2.16 Positive control sera: Cattle sera collected 2 weeks post second vaccination with FMDV vaccines prepared against the homologous virus used in the VNT

- 8.2.16.1 SAT1 -t155/71
- 8.2.16.2 SAT2- k52/84
- 8.2.16.3 Type A- k5/80
- 8.2.16.4 Type O- k77/78
- 8.2.16.5 Type C- k267/67

8.2.17 Vaccine virus strains

- 8.2.17.1 SAT1 -T155/71
- 8.2.17.2 SAT2 -K52/84
- 8.2.17.3 Type A -A K5/80
- 8.2.17.4 Type O -OK77/78
- 8.2.17.5 Type C -CK267/67.

9 Calibration Procedure

9.1 Calibrator

9.1.1 A contracted agent shall be used.

9.2 Calibration

9.2.1 All equipment shall be calibrated annually and internal checks done on a regular basis.

9.2.2 All records shall be filed and kept by the QM.

10 Environmental and Safety Controls

10.1 Safety and precautions

10.1.1 All the work with live virus must be carried out in a cubicle that is fitted with a BSC.

10.1.2 Materials that were in contact with virus must be deposited in a container with
0.2% citric acid solution.

10.1.3 Afterwards the working area of the cabinet must be cleaned with 0.2% citric acid followed by 70% ethanol, and left for 20 minutes before another virus can be handled in the same cabinet.

10.1.4 Aseptic techniques should be used in all steps.

11 Types of Samples, sample container and sampling plan

11.1 Samples

11.1.1 Whole Blood in plain sterile tubes

11.1.2 Serum in sterile vials.

11.2 Sample container

11.2.1 Vacutainer tubes, vacutainer needles, vacutainer holders, cool box with ice packs, cryovials.

11.3 Sampling plans

11.3.1 Where applicable, samples are collected in accordance to procedure for sample collection and handling of test items MOALFC/ LQMS /QMP /21.

11.3.2 Collect whole blood from which serum will be separated.

12 Procedure

12.1 Sample reception and preparation

12.1.1 Enter relevant details in the Laboratory sample reception form LQMS/FM/015 and designate a reference number to each sample.

12.1.2 Inactivate complement in the sample at 56°C for 30 minutes

12.1.3 Store the samples at +4°C if it is going to be worked on soon or at -20°C if is for long term storage.

12.1.4 Dispose the waste material in 0.2% citric acid.

12.2 Prepare disinfectant Solution (0.2% (w/v) citric acid)

12.2.1 Dissolve 200 gm of citric acid per 1 litre of locally produced distilled/deionized water.

12.2.2 Label and store at room temperature.

12.3 **Titration of virus and choosing virus dilutions for the VNT**

12.3.1 Prepare virus stocks and keep at -70°C.

12.3.2 Titrate each new batch beforehand in microtitre plates to determine its titre as follows:

12.3.3 Add 50 µl of Eagles (MM1) media to all wells of columns 2-12.

12.3.4 Add 100 µl of virus suspension into column 1 wells and make a twofold serial dilution of the virus from column 1-11 of the microtitre plate by transferring 50 µl across the columns and discarding 50 µl from column 11 with column 12 being the cell control.

12.3.5 Add (overlay) 50 µl of Eagles (MM1) media to all the wells of the microtitre plate to a total volume to 100 µl.

12.3.6 Add 50 µl of LFBK, BHK-21 or IBRS-2 cells at a concentration of 0.4-1.0 x 10^6 /ml.

12.3.7 Incubate the plate for 72 hours at 37°C and determine the titre as in 15.2.

12.3.8 Determine the dilution/s of virus to be used in the VNT thus: first subtract 2.0 logs from the virus titre to determine a dilution that will give 2 logs of virus. Then determine four values that are 0.5 logs apart from this value.

12.3.8.1 **Example**: For a virus titre of $10^{5.5}$/50 µl, subtract 2.0, this gives a value of

$10^{3.5}$. Diluting the virus $10^{3.5}$ will give 2.0 logs of challenge virus in the VNT. Test the positive control sera against virus dilutions of log: 3.0, 3.5, 4.0 and 4.5. The positive sera samples are tested against the four virus dilutions.

12.3.9 Mark the plates with the corresponding virus dilutions.

12.4 **Preparation of the microtitre plates**

12.4.1 Add 50µl Eagles (MM1) media (with a multi-channel pipette) to all the wells except the first row (A).

12.5 **Dilution and titration of the test and control sera**

12.5.1 Pipette 100µl of a 1/4 dilution of the control sera and test sera in row A

12.5.2 Transfer 50 µl of this 1/4 dilution from row A to row B and carefully mix the 100 µl contents in the well by filling and emptying the multi-channel pipette tips several times, taking care not to introduce air bubbles. (This will result in a doubling dilution).

12.5.3 Transfer 50 µl from row B to row C and repeat the mixing procedure. Transfer 50 µl from row C to row D mix carefully and repeat this step down to row H.

12.5.4 Discard 50 µl of the dilution from row H to leave a final volume of 50 µl. (This will result in a test sample dilution series from 1/4 to 1/512 in 50 µl volumes).

12.5.5 Repeat the above step with all the test samples and perform both test and control sera in duplicate wells.

12.6 **Addition of the virus antigen dilutions to the test sera**

12.6.1 Add 50µl of 100TCID$_{50}$ virus dilution to all wells of the test and control plates resulting in dilutions from 1/8 to 1/1024.

12.6.2 Plates are incubated for one hour at 37°C.

12.7 **Addition of the cell suspension to the microtitre plates**

12.7.1 Add 50 µl /well of the cell suspension 0.4-`\\\\1.0 x 10^6 cells/ml of LFBK or BHK-21 or IB-RS 2 cells in Eagles (MM1) media. See appendix 3 for cell counting)

12.7.2 Plates are sealed and incubated for upto 72 hours at 37°C.

12.8 **Reading of plates**

12.8.1 View the microtitre plates after 48 hours directly under an inverted microscope for CPE.

12.8.2 Wells that do not show CPE are regarded as positive and those that show CPE are regarded as negative.

12.8.3 Stain plates using naphthalene blue black after 72 hours (See appendix 2)

12.8.3.1 Check wells for blue black staining (positive) or colourless (negative) Appendix 1

12.8.4 Calculate virus titre as indicated in 15.1

13 Quality Control

13.1 Include a standard antiserum of known titre, a cell control, a medium control, and a virus titration used to calculate the actual virus titre used in the test in every test

13.2 The test is considered to be valid when the amount of virus used per well is in the range log10 1.5–2.5 TCID$_{50}$ (32-316 TCID $_{50}$)

13.3 Tissue culture input for virus titration and for neutralization by antibodies should be between 0.4-1.0 x 10^6 /ml.

13.4 The Quality Manager will do random checks to ensure that this SOP is implemented as written.

13.5 All the deviations or violations against this SOP will be documented in the Quality Managers Random Check Log. In such as cases Corrective and Preventive shall be initiated, implemented, and closed out. Documentation will be done in the Non-conformance Corrective and Preventive Maintenance.

14 Principle for calculating results

Not applicable

15 Results and report interpretation

15.1 Calculation of virus titre

15.1.1 The titre of the virus is calculated as that dilution where 50% of the cells will show positive CPE which upon staining, they appear colourless. Endpoint is where there is no CPE and the cell monolayer will take the colour of the stain. This is done according to the method of Kärber (1931) as follows:

15.1.2 Total number of wells exhibiting 100% CPE divided by number of wells per dilutions subtract 0.5 (correction factor) multiplied by the log dilution interval.

Add the highest dilution step with 100% CPE in all the wells.

15.2 Calculation of serum titre

15.2.1 With each VNT, a virus titration is included, so that the actual virus titre and virus doses for that experiment can be determined. For each dose of virus, the corresponding serum titre is determined. Serum titres are expressed as the $\log_{10}$ reciprocal of the dilution which protected 50% of cultures from that dose of virus. The final endpoint titre of the serum is determined as the log reciprocal of the dilution which protected 50% of cultures from $100TCID_{50}$ of virus (Kärber, 1931). This is done by plotting the virus doses between $10^{1.5}$ and $10^{2.5}$ against their corresponding serum titre and calculating the final serum titre at $100TCID_{50}$. In practice, this is done by using the analysis presented for the determination of a regression line on a pocket calculator.

16 Biological reference intervals or clinical decision values

Antibody titres of $\text{Log}_{10} \geq 1.36$are considered protective.

17 Interferences

Environmental conditions, quality of tissue culture cells, seeding rate of cells, Virus inputs (TVD), technician pipetting, Eagles Media reagents.

18 Reference

18.1 ISO/IEC 17025:2017, General requirements for the competence of testing and calibration laboratories, Third edition

18.2 AOAC International: Guidelines for Laboratories Performing Microbiological and Chemical Analysis of Food, Dietary Supplements, and Pharmaceuticals – An Aid to Interpretation of ISO/IEC 17025:2017; August 2018

18.3 Karber, G., 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Archiv fur experimentellePathologie und Pharmakologie, 162, 480-483

18.4 Golding, S.M., Hedger, R.S., Talbot, P. & Watson, J. (1976). Radial immunodiffusions and serum neutralization techniques for the assay of antibodies to swine vesicular disease. Res. Vet. Sci., 20, 142–147.

18.5 OIE manual, 2017 Edition chapter 2.1.5.

19 Document Revision History

Version	Effective Date	Description of changes	Reason for changes	Approved by
N/A				

20 Forms and related documents

Title	Form/Chart Numbers
Document master list	LQMS/FM/001
Document distribution list	LQMS/FM/002
SOP Training Documentation Log	LQMS/FM/003
Sample submission form	FMD/LTPS/FM/001
Hold & Blend FMD Cattle Potency Test Form	FMD/LTPS/FM/002
Sample reception form	LQMS/FM/015
VNT Potency results report form	FMD/LTPS/SE/FM/016
VNT results report form	FMD/LTPS/SE/FM/015
Procedure for document development	MOALFC/LQMS/QMP/01
Corrective Action and Preventive Action Log	LQMS/FM/013
Procedure for Sample reception	MOALCF/LQMS/QMP/20
Work instruction for cell counting	FMD/LTPS/SE/FM/004
Work instruction for VNT plate staining	FMD/LTPS/SE/FM/005
Procedure for sample collection and handling of test items	MOALFC/LQMS/QMP/21
Training of laboratory personnel form	LQMS/FM/039

21 Appendices

21.1 Appendix 1

NEUTRALISATION WELLS CONVERSION TO LOG TITRES

1. Each serum dilution step has a maximum of two wells where the cells can form into a monolayer. If the dilution step contains sufficient antibody levels, infectious virus particles will be neutralised and a cell monolayer will form. Antibody titre is calculated by the presence of these cell sheets and all wells are counted (count all wells with NBBst (stain).
2. In order to calculate this, the antibody titre for each virus dilution must be obtained. Again, the titre is at the point where 50% of the cell monolayers remain. If the example from above is used, the initial dilution was 1/2 (or 0.3 log.) after the addition of neat sera. However, this was again diluted 1/2 by the addition of 50µl of the virus dilution, making the actual serum dilution 1/4 (or 0.6 log.) See Table below.

Table

Complete well(s)	1/2 serum log titre	1/4 serum log titre	1/8 serum log titre	1/16 serum log titre
1	0.60	0.90	1.2	1.51
2	0.78	1.04	1.36	1.65
3	0.90	1.2	1.51	1.81
4	1.04	1.36	1.65	1.96
5	1.2	1.51	1.81	2.11
6	1.36	1.65	1.96	2.26
7	1.51	1.81	2.11	2.41
8	1.65	1.96	2.26	2.56
9	1.81	2.11	2.41	2.71
10	1.96	2.26	2.56	2.86
11	2.11	2.41	2.71	3.01
12	2.26	2.56	2.86	3.16
13	2.41	2.71	3.01	3.31
14	2.56	2.86	3.16	3.46
15	2.71	3.01	3.31	3.61
16	2.86	3.16	3.46	3.76

21.2 **Appendix 2**

WORK INSTRUCTION FOR VNT PLATES STAINNING: FMD/LTPS/SE/FM/005

1. Stain plates:

1.1. Prepare a suitably sized wash container with ≥0.8% citric acid in unsterile PBS. Also prepare a discard container with ≥0.2% citric acid concentration/water/detergent disinfectant, and place in cabinet.

1.2. Treating each plate in turn, remove the plate sealer and place into the discard container. Submerge the plate in the citric acid/PBS wash and agitate gently, ensuring the whole plate is disinfected. Once all plates have been submerged, leave to disinfect for a minimum of 30 minutes.

1.3. Remove the plate(s) from the PBS container and discard the disinfectant from the plate back into the container, tap each plate on an absorbent paper to expel any excess solution.

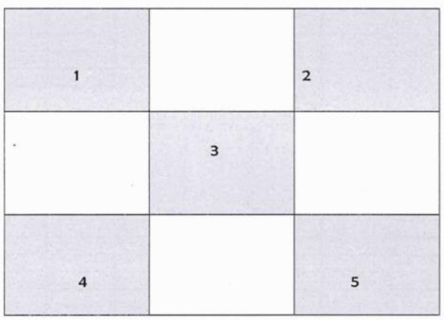
1.4. Fill the stain reservoir with Napthalein Blue Black stain (NBBSt) and dispense 50µl of stain into every well on all test plates. Dispense excess stain back into the stain bottle. Leave the plates for at least 30 minutes - up to 1 hour (after the last plate has been stained) at room temperature (22 ± 3 ºC).

Note: In case of bubbles forming in some of the plate wells when adding NBBst, dispense another 50µl of stain into every well in that column.
Transfer the stained plates to the sink and wash.

21.3 **Appendix 3**

WORK INSTRUCTION FOR CELL COUNTING: FMD/LTPS/SE/FM/004

1. Order monolayer cells from tissue culture laboratory
2. Gently mix the cells and make the following dilutions using Eagles media 1/10, 1/100 and the last dilution 1/1000 using Trypan blue
3. Put on the microscope
4. Charge the Neauber chamber (NC)
5. Using a capillary tube pick well mixed cells from 1/1000 dilution and load into the two chambers of NC.
6. Load the NC onto the microscope and focus using x10 power objective
7. Count live cells in the 5 squares of each chamber



8. Get the average of cells and there **should not** be much deviation
9. Determine the number of cells per ml viz:

   No. of cells counted x Dilution Factor x Conversion Factor.

21.4 **Appendix 4**

VNT POTENCY RESULTS REPORT FORM: FMD/LTPS/SE/FM/016

VNT SEROLOGY TITRES VS CURRENT VACCINE STRAIN	
POTENCY SERA	
Date Received by the Lab:	Origin : KEVEVAPI
Date Test Completed:	FMD LAB REF No. :

V.N.T PARTICULARS		VACCINE PARTICULARS		
VIRUS TYPE		EXP. No.		
VIRUS PASSAGE		VACCINE TYPE		
POS. CONTROL SERUM		VACCINE LOT		
FINAL P.C.S.		PD50	CATTLE	
FINAL TVD INPUT			M.I.T.	

CONTROLS

CELL		VIRUS		SERUM		
DATE		DATE		DATE		
37500		TVD				MAIN
75000		-1				
150000		-2				
MEDIUM		-3				
		LOG10				
		TITRE				

VACCINE	CATTLE No.	ANIMAL BREED	DAY 0	DAY 5	DAY 14	DAY 21	DAY 10 P.C.	DAY 5 POST CHALLENGE FEET AND TONGUE REACTION	DAY 10 POST CHALLENGE FEET REACTION
DILUTION	DATE								
1/1.0									
1/4.5									
1/20.25									
CONTROLS									
TITRATION									

85

21.5 Appendix 5

FMD SAMPLE SUBMISSION FORM: FMD/LTPS/FM/001

MINISTRY OF AGRICULTURE, LIVESTOCK, FISHERIES AND CO-OPERATIVES.

Telegrams: "NVQCL", Nairobi,
Telephone: 553633, 651595, 0203540071, 536018, !

Fax: 537744 E-mail: fmdkenya@yahoo.com

State Department of Livestock
Veterinary Services Division
National Veterinary Quality Control/ Foot-and-Mouth Disease Laboratories: Embakasi
P.O. Box 18021-00500
ENTERPRISE ROAD
NAIROBI - KENYA

FOOT AND MOUTH DISEASE SAMPLE SUBMISSION FORM

Name of submitting officer .... Title....................................
Address/Telephone...
Date collected .... Date dispatched...
Owners name /farm..
Locality of the farm (S/location/division) ....
Address...
Species of the animal (s) affected...
Material submitted...
Packaging...

History of the outbreak

Number of the animal...........................,,,,,,Affected...............................Dead...
Age/ breed affected...
Symptoms...
Other animal species on the farm: affected /not affected...
Duration of the outbreak...
Previous outbreak...
Last date of the vaccination...
Vaccination regimen...
Quarantine noticed date...
Recent introduction of the animals...
Addition information e.g. common watering/dipping/vaccine handling..
Report to be sent to ....
Officer signature: ....
Duplicated to C.D.V.S
Triplicate to D.V.S

NB: Samples suitable for FMD virus diagnosis are fresh epithelium approximately 1 gram preserved in 50% glycerol and PBS (v/v), vesicular fluid. For antibody determination is 4 ml serum. All specimens are stored under negative 20°C during storage / transportation.

Appendix 6

VNT RESULTS REPORT FORM: FMD/LTPS/SE/FM/017

SOURCE:

DATE RECEIVED BY THE LAB: FMD LAB REF NO:

DATE TEST COMPLET :

V.N.T PARTICULARS	
VIRUS TYPE	
VIRUS PASSAGE	
POS. CONTROL SERUM	
FINAL P.C.S.	
FINAL TVD INPUT	

VACCINE PARTICULARS		
EXP. No.		
VACCINE TYPE		
VACCINE LOT		
PD50	CATTLE	
	M.I.T.	

CONTROLS

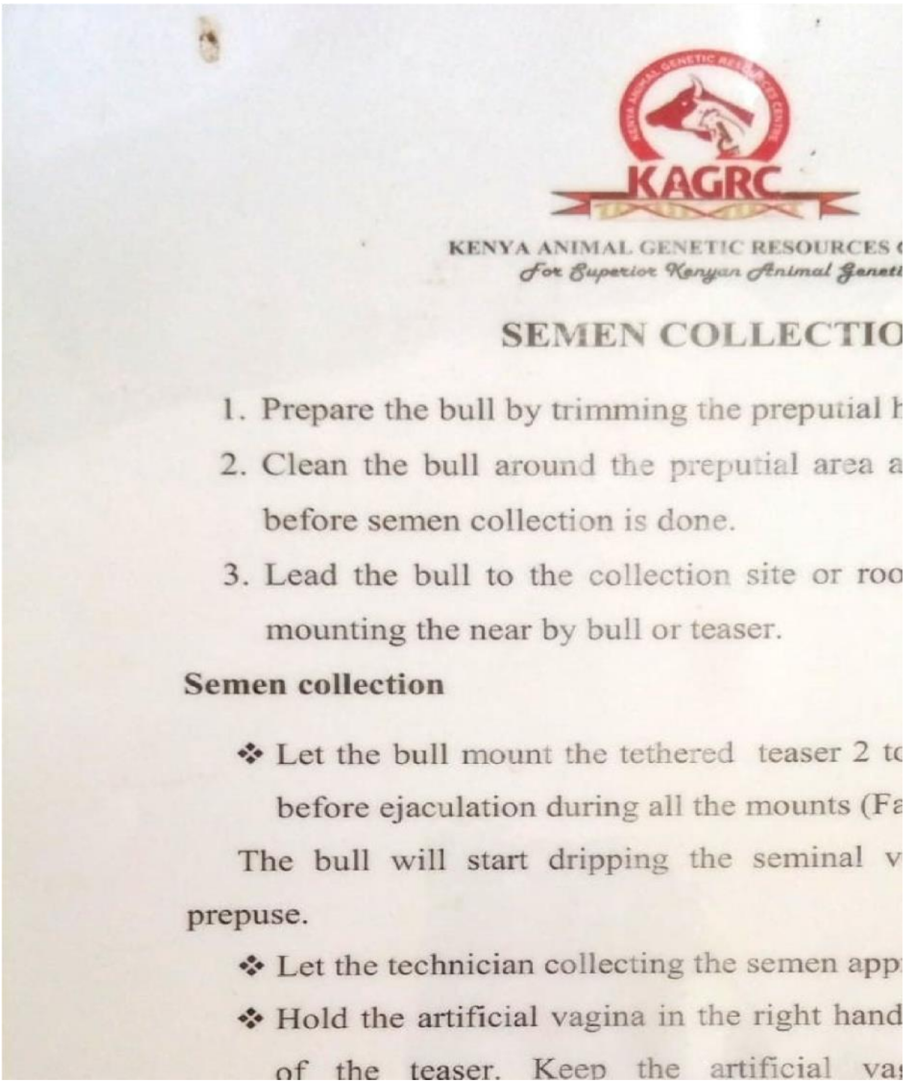
CELL	
DATE	
37500	
75000	
150000	
MEDIUM	

DATE	
TVD	
-1	
-2	
-3	
LOG10	
TITRE	

VIRUS		
DATE		
		MAIN

LAB NO.	VIRUS STRAIN	O K 77/78	A K5/80	SAT 1 T155/71	SAT 2 K52/84
	FINAL PCS				
	FINAL TVD				
	ANIMAL NO.				
1					
2					
3					
4					
5					
6					
7					
8					

Appendix II: Semen collection protocol



KAGRC

KENYA ANIMAL GENETIC RESOURCES C

For Superior Kenyan Animal Geneti

SEMEN COLLECTIO

1. Prepare the bull by trimming the preputial h
2. Clean the bull around the preputial area a
   before semen collection is done.
3. Lead the bull to the collection site or rooc
   mounting the near by bull or teaser.

Semen collection

- Let the bull mount the tethered teaser 2 tc
  before ejaculation during all the mounts (Fa

The bull will start dripping the seminal v
prepuse.

- Let the technician collecting the semen app
- Hold the artificial vagina in the right hand
  of the teaser. Keep the artificial vas

- Move with the bull and let it ejaculate into
- The semen is collected into the attached tub
- Remove the Av from the penis.
- Drain the water from the AV and take the laboratory for evaluation.Take care not to with water.

Prepared by : **Appr**

Lilian Kuchikhi
Chief Laboratory Technologist

Appendix III: Authorization for research

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P/No. 169
KAGRC

31st August 2021

The Managing Director,
KAGRC

Thro'

The Manager Germplasms and Quality Assurance,
KAGRC

Dear Sir,

RE: PERMISSION TO CARRY OUT MY RESEARCH IN KAGRC LAB

I am a M.Sc. degree finalist at Kenyatta University pursuing Immunology currently carrying out research on effects of FMD vaccination in bulls and coconut water extender on semen quality and room temperature frozen semen.

I hereby request for permission to carry on my research proposal in KAGRC Laboratory.

The sample herd size will be 8 bulls (Ayrshires, Friesian, Guernsey and Jerseys) two for each breed. One bull from the sample size will be used as a control. The semen volume from each bull will be halved.

Looking forward to your positive response.

Yours Sincerely,

Michael Mutune
Lab Technologist

KENYATTA UNIVERSITY
OFFICE OF THE EXECUTIVE DEAN GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Executive Dean, Graduate School **DATE:** 17th April 2023

TO: Michael Mutune Kavuso
C/O Department of Zoological Sciences **REF:** I56/38019/2017

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

==

This is to inform you that Graduate School Board, at its meeting on **15th March 2023**, approved your Research Proposal for the M.Sc. Degree entitled, ***"Impact of Foot and Mouth Disease Vaccine Antibodies and Evaluation of Coconut Water as Alternative Extender on Bull Semen"***.

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation and Ethics Review Committee, Kenyatta University.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking and Progress Report Forms per semester. The Forms are available at the University's Website under Graduate School webpage downloads.

Also, please ensure that you publish article(s) from your thesis before submitting it to Graduate School for examination as per the Commission for University Education and Kenyatta University guidelines.

Thank you.

JULIA GITU
FOR: EXECUTIVE DEAN, GRADUATE SCHOOL

cc. Chairman, Department of Biochemistry, Microbiology and Biotechnology

Supervisors:

1. Prof. Michael Gicheru
c/o Department of Zoological Sciences
Kenyatta University

2. Dr. Jemimah Simbauni
c/o Department of Zoological Sciences
Kenyatta University

KENYATTA UNIVERSITY
OFFICE OF THE EXECUTIVE DEAN GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: I56/38019/2017 **DATE:** 17th April 2023

Director General,
National Commission for Science, Technology and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR MR. MICHAEL MUTUNE KAVUSO – REG. NO. I56/38019/2017

I write to introduce Mr. Michael Mutune Kavuso who is a Postgraduate Student of this University. He is registered for M.Sc. degree programme in the **Department of Zoological Sciences.**

Mr. Michael Mutune Kavuso intends to conduct research for a M.Sc. Thesis Proposal entitled, ***"Impact of Foot and Mouth Disease Vaccine Antibodies and Evaluation of Coconut Water as Alternative Extender on Bull Semen".***

Any assistance given will be highly appreciated.

Yours faithfully,

PROF. ELISHIBA KIMANI
EXECUTIVE DEAN, GRADUATE SCHOOL

OFFICIAL RECEIPT

69817

Serial No. C 134209

Receipt No. G-198206

Kenyatta University
P.O. Box 43844-00100, Nairobi Tel: 810901-19

Date: 26-Jan-24

Name: MICHAEL MUTUNE K.

Reg. No: ETHICS REVIE

12:27:34 pm

CODE	DESCRIPTION	AMOUNT
A40014	Miscelleneous Income	2,000.00
	Received	
	TOTAL	2,000.00

KENYATTA UNIVERSITY
OFFICE OF HEAD TREASURY
26 JAN 2024
CASH OFFICE
1
FINANCE DEPARTMENT

Amount in Words: *TWO THOUSAND AND ZERO CENTS ONLY *********************************

Mode of Payment: Deposit

Nbk Payments A/C No. 01003059002400

Fees Balance

Served By: PF6909

26-Jan-24

Appendix IV: Publication

Asian Journal of Advances in Agricultural Research

Volume 24, Issue 8, Page 1-9, 2024; Article no.AJAAR.119739
ISSN: 2456-8864

Foot and Mouth Disease Virus Strain-specific Antibody Titres in Naturally Infected or Vaccinated Bulls in Kenya

Jemimah Simbauni [a*], Michael M. Kavuso [a,b], Joshua M. Mutiso [a], Rebeccah M. Ayako [a] and Michael M. Gicheru [a]

[a] *Department of Zoological Sciences, Kenyatta University, Nairobi, Kenya.*
[b] *Kenya Animal Genetic Resource Centre (KAGRC), Nairobi, Kenya.*

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Appendix v

CURRICULUM VITAE

NAME: MICHAEL MUTUNE M KAVUSO

CELL: 0700742003 / 0738126884

2018 -2024: MSc (immunology) Kenyatta university

2013-2016: BSc (Appl. Biology) Technical University of Kenya

2004-2006: Diploma (Appl. Biology) Kenya Polytechnic

1992- Date: `Kenya Animal Genetic Resources Centre

1982 -1984: KCE Masii Secondary School

1973 -1979: CPE Kyevaluki primary school