SCREENING FOR FOOT AND MOUTH DISEASE VIRUS IN BUFFALOES AND CATTLE IN SELECTED LIVESTOCK-WILDLIFE INTERFACE AREAS OF TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA

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ABSTRACT

Rapid and accurate diagnosis is paramount in understanding the infection status of foot and-mouth disease (FMD) virus (FMDV) in animals. In this study, the singleplex real time RT-PCR (qRT-PCR) assay employing the Callahan 3DF-2, 3DF-R primers and Callahan 3DP-1 probe were used in screening for FMDV genome on esophageal–pharyngeal (OP) fluids. The OP samples were collected from cattle and African buffaloes in livestock-wildlife interface areas of Mikumi, Mkomazi and Ruaha National Parks in Tanzania in 2011, which included National Parks and surrounding areas. The detection rates of FMDV genome were 5.88% (n = 3), 19.44% (n = 7) and 41.18% (n = 21) in Mkomazi, Ruaha and Mikumi National Parks, respectively. FMDV detection rates in Mkomazi and Mikumi were significantly higher in the African buffaloes ($p < 0.05$) compared to that in cattle. There was no correlation of FMDV detection with either age or sex of the animals in the three National Parks. These findings indicate that cattle and buffaloes in Mikumi, Ruaha and Mkomazi were naturally infected with FMDV. Furthermore, the higher FMDV detection rates in buffaloes suggest that buffaloes could potentially act as reservoirs for FMDV and possibly play a significant role in transmission of the virus to other in-contact susceptible animals. Further studies, including serotyping, virus isolation, experimental infection and sequencing of the viruses, are required to elucidate the complex epidemiology of FMD in cattle and buffaloes in the livestock-wildlife interface areas in Tanzania.

DECLARATION

I, Emma Peter, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

....

Emma Peter **Date**

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The above declaration is confirmed

....

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DEDICATION

This work is dedicated to my young sister, Gloria Peter, who is currently in her third year, studying hard to become a veterinary doctor. I hope the gaps left by this study would open her mind wider and show more opportunities in future.

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LIST OF ABREVIATIONS AND SYMBOLS

%	percentage
°C	degrees celsius
3D	three dimensions
AVE	elution buffer
AVL	viral lyses buffer
AW1	wash buffer one
AW2	wash buffer two
cDNA	complementary deoxyribonucleic acid
Ct	cut-off value
CVL	central veterinary laboratory
ELISA	enzyme linked immunosorbent assay
FAO	food and agriculture organization
FMD	foot and mouth disease
FMDV	foot and mouth disease virus
FP	forward primer
g	gravity
Kb	kilobase
Km^2	kilometer square
LAMP	loop isothermal amplification

ME-SA	middle east and south American
ml	milliliter
mRNA	messenger ribonucleic acid
Neg	negative
nm	nanometer
OIE	world organisation for animal health
PCR	polymerase chain reaction
Pos	positive
QIAamp	qiagen amplification
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
RP	reverse primer
RPM	rotations per minute
s	second
SACIDS	southern African centre for infectious disease surveillance
SADC	southern Africa development community
SAT	southern African territory
SEA	south east Asia
TADs	trans boundary animal diseases

TVLA	Tanzania veterinary laboratory agency
UTR	untranslated region
VP	viral protein
μg	microgram
μl	microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Foot-and-mouth disease (FMD), also known as hoof-and-mouth disease (*Aphtae epizooticae*), is one of the most important viral diseases, known to affect all cloven-hoofed animal species. FMD is endemic in most sub-Saharan African countries including Tanzania. It has been effectively controlled in South Africa, Botswana, Namibia, Swaziland and Lesotho, which manage to maintain FMD freedom without vaccination in large zones of their territories through control zones (Rweyemamu and Astudillo, 2002).

The etiological agent, FMD virus (FMDV), is a *picornavirus* of the genus *Aphthovirus* and family *Picornaviridae*. This virus has an icosahedral symmetry with a positive-sense RNA of about 8.5 kb which encodes a polyprotein that is processed to the four structural proteins (VP1– VP4) (Belsham, 2005). VP1 is one of the three proteins expressed on the surface, and contains the major antigenic determinants of the virus. The VP1 is made of two immunogenic sites, known to elicit neutralizing antibodies, found at amino acid positions 140–160 (the G–H loop) and at residues 200–213 (the C-terminus region) (Mason *et al.,* 2003).

Virus type and antigenic diversity in East Africa (Kenya, Uganda, and Tanzania) remains high, with types A, O, SAT 1 and 2 being recovered from outbreaks in 2005-2006.
Molecular evidence suggests additional introduction of SATs from wildlife although the circulation of types O and A in domestic animals is considered the mechanism for persistence in East Africa.

The virus exists as seven serotypes (A, O, C, Southern African Territories; SAT1, SAT2, SAT3 and Asia1) distributed non-uniformly around the world, with limited cross-protection between them (Paton *et al.,* 2009).

Typical cases of FMD are characterized by a vesicular condition of the feet, buccal mucosa and in females, the mammary glands. Clinical signs can vary from mild to severe and fatalities may occur, especially in young animals. However, in some species the infection may be subclinical e.g. African buffalo (*Syncerus caffer*).

It is known that a significant proportion of animals become long-term carriers after recovery from acute infection and may serve as reservoirs of infection in endemic areas. FMD is prevented by strict import controls, controlled animal movements, slaughter of at-risk livestock in the event of an outbreak of the disease and vaccination to live using appropriate type of vaccines (Rowlands, 2008).

1.2 FMD context in Tanzania

The first report of FMD in Tanzania referred to an outbreak in Kahama district in 1927 was attributed to type O virus, Anon (1927) as cited by Swai *et al.* (2009). Since then FMD has been reported annually in almost every region of the country and generally is assumed to be endemic in East Africa (Vosloo *et al.,* 2002).

Types O, A, SAT-1 and SAT-2 were noted in late 1960s, Rweyemamu (1970) as cited by Swai *et al.* (2009) and serological evidence for SAT-3 has been reported in 1996 in Arusha Region (Kivaria, 2003). Seasonal and trade-related movements of cattle across borders help maintain FMD in Tanzania. The country has many African buffalo (*Cyncerus caffer*), which are carriers of FMD virus (Thomson *et al.,* 2003). Contact

between cattle and buffalo is common in pastoral and agro-pastoral areas particularly in the dry season when animals meet in masses around watering points. From 2001 to 2006, 878 FMD outbreaks were reported in 605 different villages of 5815 populated places (Picado *et al.,* 2007) The spatial distribution of FMD outbreaks was concentrated along the Tanzania-Kenya, Tanzania-Zambia borders, and the Kagera basin bordering Uganda, Rwanda and Tanzania.

FMD has a great impact on Tanzania's livestock sector, one of the main economic activities in the country with over 16 million herds of cattle in 2002–2003 (FAO, 2003) FMD control, which should help reducing poverty in Tanzania (Perry and Rich, 2007), requires improving the current knowledge on the disease dynamics and factors related to FMD occurrence so that the rational control measures can be implemented more efficiently. FMD causes losses by death of animals especially during an outbreak and loss of milk production. Animals which recover from infection are always weak and have reduced productivity. Vaccination programmes are expensive and vaccinated animals requires booster doses after almost every six months and there is no cross protection between different viral strains.

The persistence and spread of the FMD in Tanzania is because of the movement of livestock within and across international borders (Kivaria, 2003). The main impact of FMD is its economic effects due to quarantine measures in areas where control measures are in place and more significantly its ascribed to the lost trade opportunities with countries free of the disease. The FMD status, given by the presence of disease and the control methods applied, influence the trade between countries according to OIE regulations. So far, four serotypes (A, O, SAT 1 and SAT 2) have been incriminated to cause FMD outbreaks in many parts of Tanzania (Kasanga *et al*., 2012).

Of late in Africa, only three countries are considered completely free of FMD (Lesotho, Madagascar and Swaziland) thus free to trade, and three countries (Botswana, Namibia and South Africa) have FMD-free zones and can therefore export some of their livestock products (OIE, 2010). Serotype diversity studies and molecular characterization of the virus has been an important epidemiological tool in controlling most livestock viral diseases.

1.3 Problem statement

FMD virus is an important cause of disease in livestock and wildlife throughout the world. The disease has led to serious financial losses due to costs incurred during its prevention and control. The virus that causes FMD has about seven different serotypes. Three of these serotypes namely the three Southern African Territories (SAT) are unique to Africa. Serotype C has also been recently reported in Africa (Sangula *et al*., 2010).

The epidemiology of FMD in sub-Saharan Africa is more complicated than most places in the world. This is due to the involvement of wildlife that plays a very important role in the epidemiology of this disease in Southern parts of Africa than in other parts of Africa (Vosloo, 2002).

In Tanzania, attempts to control FMD outbreaks are hampered by lack of an appropriate vaccine and presence of other disease outbreaks such as tick born diseases which are given more attention and priority. Actual situation concerning FMD is still under reported in Tanzania. This can be due to poor monitoring and recording systems which can be largely contributed by geographical distribution of livestock keeping regions, remoteness of some villages, poor communication systems and few numbers of veterinarians as compared to animal load in Tanzania. In this situation, little is known on the spatial and

temporal occurrence of FMD serotypes and topotypes particularly in livestock-wildlife interface areas. Rapid detection and characterization of the field strains is of the paramount importance as it aids in selection of appropriate vaccine candidate strains for FMD control in Tanzania and the neighboring countries. FMD virus is still a re-emerging disease with newly emerging topotypes within the serotypes. It is important to understand the FMDV infection status so as to better manage the risks for transmission and spread of the virus. This knowledge as well as understanding the virus distribution will help in development of relevant strategic control program(s) for the disease such as targeted vaccinations in predetermined geographic locations.

1.4 Problem justification

Foot-and-mouth disease (FMD) is a difficult disease to control and eradicate because of its contagiousness and the variety of mechanisms by which the virus can be transmitted, which includes carriage of the virus by wind and presence of multiple serotypes and topotypes that are antigenically different.

It appears that there are waves of epidemics in Tanzania and to understand or study the epidemiology of FMD it is necessary to rapidly detect the virus and perfom serotyping.
Although FMD is of economic importance and FMDV is among viruses that had been identified to cause animal diseases both in livestock and wildlife, there is no much information about FMDV infection in livestock-wildlife interface areas in Tanzania. Currently, FMD surveillance is based on passive case detection and reporting by veterinary officers in the field. Diagnosis of FMD is based on clinical signs and confirmed by a number of laboratory tests such as Ag-ELISA test and RT-PCR (Kasanga *et al.,* 2012).

Knowing the characteristics of foot and mouth disease and its causative agent will help in developing a good database for epidemiologists which is an important step in controlling the disease. Surveillance for FMDV genome provides answers to questions such as what is the FMDV infection status in selected areas of Tanzania.

1.5 Objectives

1.5.1 General objective

To investigate the prevalence of FMDV genomes in buffaloes and cattle in selected livestock-wildlife interface areas of Mikumi, Ruaha and Mkomazi focusing on the development of appropriate control method of FMD.

1.5.2 Specific objectives

i. Identification of FMDV genome from FMD-suspected and clinically normal cattle and buffalo in selected wildlife-livestock interface areas of Mikumi, Ruaha and Mkomazi.

ii. Elucidating association between cattle and buffalo in disease dynamics and predict FMDV persistence infection in selected wildlife-livestock interface areas of Mikumi, Ruaha and Mkomazi.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Global situation of FMD

FMDV serotypes are not uniformly distributed in the regions of the world where the disease still occurs. The cumulative incidence of FMDV serotypes show that six of the seven serotypes of FMDV (O, A, C, SAT-1, SAT-2, SAT-3) have occurred in Africa, while Asia contends with four serotypes (O, A, C, Asia-1), and South America with only three (O, A, C). Periodically, there have been incursions of types SAT-1 and SAT-2 from Africa into the Middle East (Valarcher *et al.*, 2004).

The advent of molecular biology technology has enabled the genetic characterization of virus strains and thereby the tracing of strains isolated from outbreaks can be carried out with far greater accuracy than was possible previously with serological techniques (Knowles and Samuel, 2003). As a result, it is now possible to group countries into epidemiological clusters according to the topotypes within each serotype that occur there. It should be noted that as a result of globalization, the spread of FMD epidemics can change from local and regional spread to wide international spread, even to distant areas as happened with the type O Pan-Asian lineage (Knowles *et al.,* 2005).

As type O is the most widely prevalent serotype in the world, the topotype distribution of this serotype gives an indication of possible epidemiological clustering. It is apparent that South America has had a genetically stable type O virus for nearly 50 years. Five different type O topotypes could be identified in Africa. The Sudan-Sahel strain in West Africa appears to have been responsible for the type O outbreaks.

There seems to be a strain that is common between Uganda and Kenya while the topotype that was identified in Tanzania in 1998 seems to have spread on one hand to Rwanda, Burundi, Kenya, and Uganda and on the other to Malawi and Zambia (Knowles *et al.,* 2004).

In Asia, several sub lineages are circulating. The dominant topotypes seem to have been the ME-SA topotypes and more particularly the Pan-Asia strain that originated from South Asia (Knowles *et al.,* 2005).

However, other topotypes are still present in East Asia such as the Cathay and the SEA topotypes

2.2 The causative agent

The aetiological agent FMDV is classified within the *Aphthovirus* genus as a member of the *Picornaviridae* family being a non-enveloped, icosahedral virus of about 26 nm in diameter and containing positive sense RNA of around 8.4 kb.

FMDV like many RNA viruses are known to have high levels of genetic and antigenic diversity. Previous studies done in Africa have shown that SAT viruses in within each of the prevailing serotypes on the continent evolved independently of each other in different locations. These are what known as topotypes.

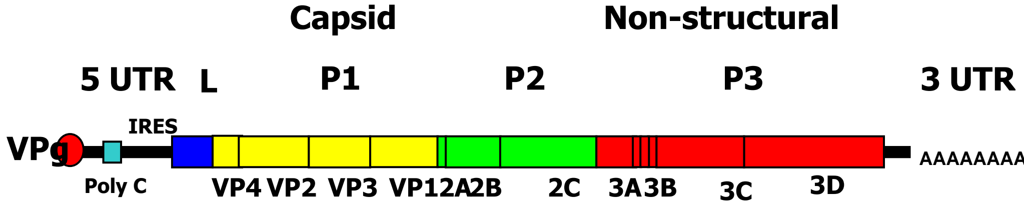

Figure 1: Genome structure and orientation of FMDV showing various gene organization.

2.3 FMD transmission

The most common mechanism of FMD transmission is by mechanical transfer of virus from infected to susceptible animals, the virus entering through cuts or abrasions or through the mucosa, or infection by the deposition of droplets or droplet-nuclei (aerosols) in the respiratory tract of recipient animals. Carriage of the virus by wind has also been reported as the route of disease transmission.

2.4 Pathogenesis of FMD

Susceptible livestock may be infected by FMDV as a result of direct or indirect contact with infected animals or with an infected environment.

When infected and susceptible animals are in close proximity, the aerial transfer of droplets and droplet nuclei is probably the most common mode of transmission. Long-range airborne transmission of virus is uncommon but important route of infection, requiring the chance combination of particular factors, including the animal species, the number and location of the transmitting and recipient animals, and favorable topographical and meteorological conditions (Sorensen *et al.,* 2000). Pathogenesis studies have been carried out in animals infected by simulated natural methods (direct or indirect

contact with infected donors or virus aerosols from such donors) or in animals infected by artificial methods, including subcutaneous, intradermal, intramuscular and intravenous inoculation, intranasal instillation and exposure to artificially created aerosols.

2.5 Tools used for diagnosis of FMD

Laboratory tests such as serology, virus isolation and antibody detection are the basis for diagnosis at the herd level. Although they are less time consuming, the shortcoming of serological tests such as virus neutralization and Enzyme Linked Immunosorbent Assay (ELISA) is that they do not allow for the differentiation between infected and vaccinated animals. A definitive diagnosis is based on detection of virus in fluids or epithelium from vesicular lesions. Virus isolation is the most reliable diagnostic method, but it is labor-intensive, time-consuming, and requires properly equipped facilities.

Sandwich ELISA is a much faster approach to detect viral antigens, but it has low sensitivity, so its primary indication is to confirm and type the FMDV after isolation in cell culture. Faster diagnostic methods for FMD based on amplification of specific sequences of the viral genome by conventional serotype specific RT-PCR using serotype specific primers, which can be applied to different kinds of biological samples such as fluids and tissues, and in some cases, this approach allows identification of infected animals even before development of clinical signs or positive virus isolation as well as identification of positive animals at the end of the course of infection when virus isolation may be negative.

The RT-LAMP assay is a technique carried out in a single tube, incubating the mixture at a constant 65◦C for less than an hour in a standard water bath or heat block. The advantages of the method are due to its simple operation, rapid reaction, and potential for

visual interpretation. This could make the technique far more suitable for field operation. LAMP has been reported to be capable of detecting as little as 6 copies of starting template and RT-LAMP has been shown to be 10 times more sensitive than end-point RT-PCR (Dukes *et al.,* 2006).

2.6 Epidemiology of FMD

The epidemiology of FMD in southern Africa is unique in that it mainly revolves around specific serotypes (SAT 1, 2 and 3) of viruses maintained and spread by wildlife, African buffalo in particular.

Although the precise mechanism of spread of FMD from buffalo to cattle is only broadly understood, it is facilitated by direct contact between these two species. Once cattle are infected, they may maintain SAT infections without the further involvement of buffalo. Despite the disease been endemic with epidemics being experienced almost each year; serotyping studies for FMD infection are inadequate in Tanzania. The occurrence and distribution pattern of the known serotypes remain un-exploited and hence poorly understood. Establishing and quantifying spatial distribution of serotypes will contribute to our understanding of the FMD epidemiology across eco-climatic zone, particularly to researchers, donors and policy makers who make decisions affecting animal movements and financial allocation for disease control (Swai *et al*., 2009).

2.7 Regional prevention and control of FMD

Cross-border epidemiological events related to FMD in southern Africa have clearly occurred on a number of occasions in the recent past. It should therefore be expected that unless improvement of FMD control in the region as a whole takes place, increasingly frequent FMD outbreaks in the region are likely. The leaders of SADC countries have

recently agreed to collaborate on renewed efforts to better manage FMD regionally and as evidence of this, in July 2011 the Government of Botswana donated 2 million doses of vaccine to Zimbabwe to vaccinate 222,000 cattle every four months for the next two Years along the Botswana- Zimbabwe border (University of Pretoria, 2011)

However, the reach of this collaboration apart from mutual material assistance, may be insufficient in that the wider community (e.g. farming interests, rural development agencies and conservation organizations) does not appear to be involved or well informed. That is of concern as the FMD situation is technically complicated.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Tanzania is a tropical country which lies between Latitude 1° and 12° South and Longitude 29° and 41° East, covers an area of 945,000 km^2. Two thirds of the country land resource is rangelands, suitable for livestock keeping activities (Nyamrunda *et al*., 2007). The climate is seasonal, unimodal (December to April) and bimodal (October to December and March to May). Rainfall varies widely across and between regions. However duration and precipitation level varies from season to season and from year to year. Temperature variation (daily average) is from 25°C to 31°C in dry months (December to March) and 15°C to 25°C in wet months (April to July).

Study area was composed of where samples were collected and where analysis was done. Sample collection was carried out in three different locations which were Mikumi, Ruaha and Mkomazi national parks together with areas around them as shown in Figure 2.

Mikumi National Park is a national park in Mikumi near Morogoro Tanzania. The park was established in 1964 and currently it covers an area of about 3230 km^2. It is the fourth largest national park in the country. The park is bordered to the south with Selous game reserve. Two other natural areas bordering the national park are Udzungwa and Uluguru mountains.

Ruaha national park which was established in 1964 is among the largest national parks in Tanzania. It covers an area of about 13,000 km^2. It is located in the middle of Tanzania about 130 km from Iringa. The park is part of a more extensive ecosystem, which includes

Rungwa and Usangu game reserves. The name of the park is derived from the great Ruaha river which flows along its south-eastern margin and is the focus for game-viewing.

Mkomazi national park is located in north eastern Tanzania on the Kenyan border contiguous with Kenya's Tsavo east national park. It was established in 1951 and it is found in both Kilimanjaro and Tanga regions. The Reserve covers about 3,200 km^2. The area commonly called Mkomazi is the union of two game reserves which are Umba game reserve in the east (in Lushoto district, Tanga region) and Mkomazi game reserve in the west (in Same district, Kilimanjaro region).

Figure 2: Map of Tanzania showing areas where sample collection was done. The red mark indicates the location of the three national parks: Ruaha, Mikumi and Mkomazi which are found in Iringa, Morogoro and a region between Tanga and Kilimanjaro regions respectively.

Laboratory analysis of samples was carried out in the FMD laboratory located at central veterinary laboratory (CVL) in Temeke, Dar es Salaam.

3.1.2 Study design

The study was a cross sectional study design reinforced with laboratory based experiments.

The laboratory work was divided into two main groups which were RNA extraction from the virus and running of Real time RT-PCR.

3.2 Samples and sample collection

Probang (oesophageal-pharyngeal fluid) was obtained from both clinically normal and FMD suspected animals. Samples were collected from three different parts in Tanzania which were Mikumi, Mkomazi and Ruaha National parks. In all the areas, bovine and buffalo samples were collected.

Table 1: Number of samples collected in each location.

Location	**Number of samples**		**Total**
	Buffalo	**Bovine**	
Mikumi	20	31	51
Ruaha	2	34	36
Mkomazi	11	42	53
Total	**33**	**107**	**140**

3.2.1 Handling of probang samples

Sample collection was done in a pre labeled 2ml collection tube which had a sample code for the animal and indicate the animal specie and area. Following collection, samples were placed in a cold box for transportation into the laboratory. In the laboratory, samples were stored at minus 20°C freezer prior to analysis.

3.3 Laboratory analysis of samples

Laboratory analysis of samples started with sorting the samples according to study location and animal species. Samples stored in the freezer were thawed in a biosafety cabinet and to avoid contamination as well as economical use of samples, all samples were aliquot into 2ml eppendorf tubes, transferring 500 microliter of probang into each tube. Remaining samples were returned into the freezer. Sorted samples were followed by nucleic acid extraction using QIAamp® Viral RNA Mini Kit (QIAGEN, Germany). qRT-PCR using AgPath-ID one step RT-PCR was the final procedure to obtain Ct values which were used as source of data (Appendix 2.0).

3.3.1 Preparation of nucleic acids

Probang material was added into buffer AVL, a lyses solution containing carrier RNA in a tube and incubated at room temperature (Appendix 2.1). Absolute ethanol was added and the mixture was filtered, discarding the tube containing the filtrate. This step was repeated to concentrate RNA. Wash buffer 1 (AW1) was added, filtrate discarded followed by wash buffer 2 (AW2) (Appendix 2.3). The mini columns were transferred into clean micro centrifuge tubes and an elution buffer (AVE) equilibrated at room temperature was added to elute the nucleic acid (Appendix 2.4). Obtained nucleic acid which is the viral RNA was stored at -20°C waiting for qRT-PCR to be performed.

3.3.2 Buffer solutions

Relevant buffer solutions incorporated in respective nucleic acid preparation kits were used in this study. Examples include extraction buffers and PCR buffers.

3.3.3 qRT-PCR

Amplification of RNA targets was done using a rapid single-tube qRT-PCR strategy which was recommended for viral RNA amplification (Fig. 3).

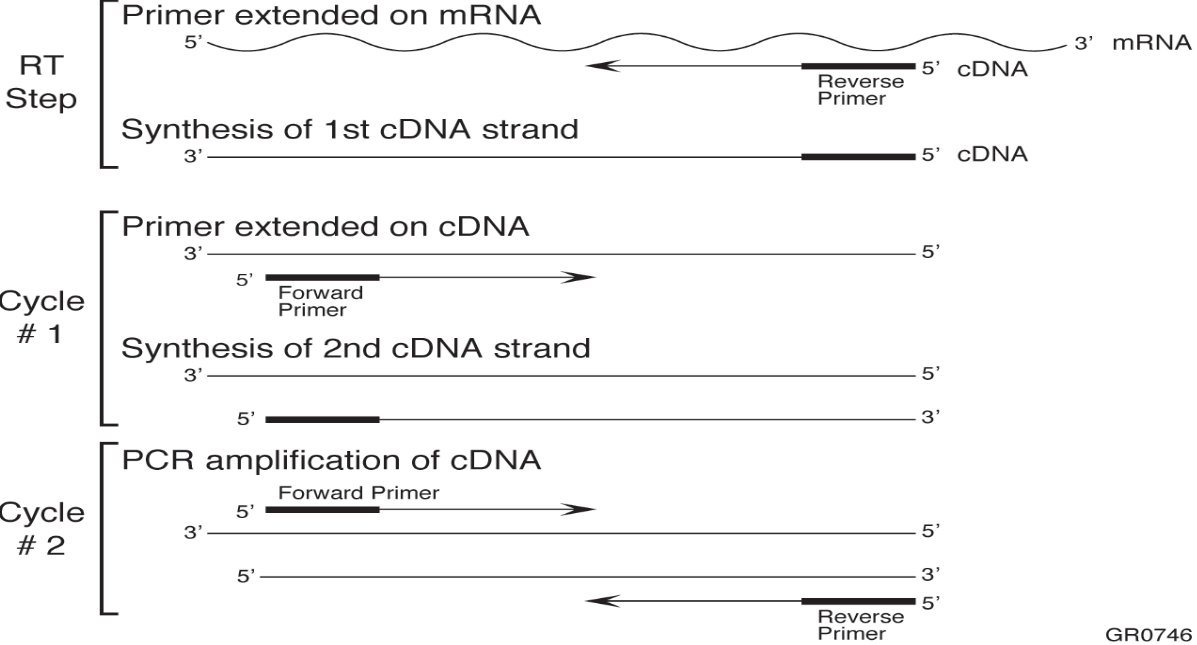

Figure 3. One-step qRT-PCR for FMDV genome detection. In the reaction, the viral genome is converted to cDNA followed by PCR amplification

3.3.3.1 Setup of the qRT-PCR

The set up of qRT-PCR was done into three experiments and data obtained were the Ct values.

Table 2: Master mix for the qRT-PCR showing ingredients and proportions for the reaction mixture

S/N	Reagents	1x reaction	74x reaction
1	2x RT PCR Buffer	12.5	925
2	Forward Primer	1	74
3	Reverse Primer	1	74
4	Probe	1	74
5	25x RT PCR Enzyme	1	74
6	Detection enhancer	1.67	123.58
7	Nuclease free water	0.83	61.47
	TOTAL	**19**	**1406.05**

-7 µl RNA sample was added into a tube containing 18 µl of master mix to make a final volume of 25 µl.

Table 3: Thermo cycling conditions for qRT-PCR

Temperature (°C)	**Time (min)**	**Number of cycles**
45°C	10	1
95°C	10	1
95°C	15	40
60°C	45	40

3.3.3.2 Interpretation of the qRT-PCR data

The sources of data were cut off point values which varied from sample to sample.

From the principle used by World Organisation for Animal Health (OIE) Ct value is 32.

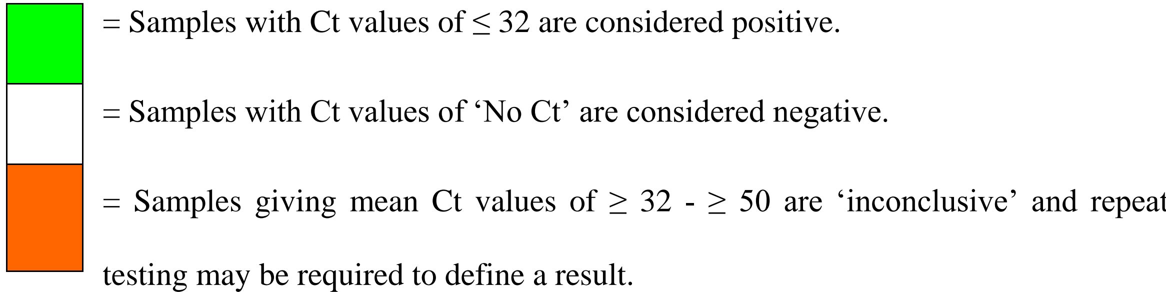

Figure 4: Results interpretation for the qRT-PCR indicating various Ct values. The green colour Ct values was indicative of presence of the viral genome, the white colour has no Ct value reading therefore negative for the virus while the red colour was inconclusive requiring repetition of the experiment (Appendix 4).

3.3.4 Primer combination

Primers used in this study were those that targeted the VP-1 region of the viral genome, also known as pan serotypic primers which were non-specific to serotypes.

These primers and probes with their sequences included:

Forward primer (3DF)	5'-ACTGGGTTTTACAACCTGTGA-3'
Reverse primer (3DR)	5'-GCGAGTCCTGCCACGGA-3'
Taqman probe (3DP)	5'-TCCTTTGCACGCCGTGGGAC-3'

3.4 Methods

3.4.1 Principle

QIAamp® Viral RNA Mini Kit (QIAGEN, Germany) provides the fastest and easiest way to purify viral RNA for reliable use in amplification technologies. Viral RNA can be purified from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids including probang samples.

The kit combines the selective binding properties of a silicagel- based membrane with the speed of microspin or vacuum technology and is ideally suited for simultaneous processing of multiple samples.The sample is lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers. High-quality RNA is eluted in a special RNase-free buffer, ready for direct use or safe storage. The purified RNA is free of protein, nucleases, and other contaminants and

inhibitors. Purification of viral RNA was done using spin protocol. This protocol is for purification of RNA from probang using a microcentrifuge (Appendix 3).

3.5 Statistical data analysis

Results were recorded in Microsoft Office Excel 2007. Obtained data was analysed by descriptive statistics that compared relevant parameters such as geographic location, species of animals and sex.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

The FMD situation of East Africa has been illustrated as being the most complicated in the world due to wildlife- animal interface (Rweyemamu *et al.,* 2008). Endemic phases are mainly due to pastoralist areas which include area around the National parks of Tanzania. However, the risk factors for transmission differ in each region (Swai *et al*., 2009).

The probang samples were collected from cattle and African buffaloes in livestock-wildlife interface areas of Mikumi, Mkomazi and Ruaha National Parks in Tanzania in 2011, which included National Parks and the area around. The detection rates of FMDV genome calculated from Microsoft excel, were 5.88% (n = 3), 19.44% (n = 7) and 41.18% (n = 21) in Mkomazi, Ruaha and Mikumi National Parks, respectively. FMDV detection rates in Ruaha and Mikumi were significantly higher in the African buffaloes ($p < 0.05$) compared to that in cattle. There was no correlation of FMDV detection with either age or sex of the animals in the three National Parks. These findings indicate that cattle and buffaloes in Mikumi, Ruaha and Mkomazi were naturally infected with FMDV. Furthermore, the higher FMDV detection rates in buffaloes suggest that buffaloes could potentially act as reservoirs for FMDV and possibly play a significant role in transmission of the virus to other in-contact susceptible animals.

However, it should be noted that there are other possible reasons for variation in FMDV detection rates for different animal species as well as study locations.

The reasons may include poor sample collection and storage which is contributed by lack of portable facilities for field use. Due to low budget, it was also not possible to obtain

equal number of samples in each location and for each animal species. Buffalo samples are very expensive as compared to bovine samples, a reason to lower number of buffalo samples as compared to bovine samples.

Depending on the stage of FMDV replication in an animal, low RNA concentration can also be a factor. It should also be taken into account that probang samples, although are good for qRT-PCR having low concentration of PCR inhibitors, have lower stability and low RNA detection as compared to other samples such as serum sample. The situation of FMD in Mikumi, Ruaha and Mkomazi was seen to be endemic. Buffaloes are seen to be reservoir of the disease. Animals in this area come into contact by sharing the same water points and grazing from the same grounds. From simple observations and talking to the pastoralists living in the interface, it was noted beyond doubt that in draught seasons when feeds and water are scarce, cattle are pastured in wildlife conserved area. It is well established that SAT serotype viruses are maintained by healthy, free-living African buffalo populations although, once established in cattle, SAT viruses appear to be capable of persistence in cattle populations without the need for further contact with infected buffalo.

Therefore, the approach of most of southern Africa is as far as possible, to prevent contact between buffalo and cattle and to eliminate outbreaks as soon as they occur (Foot and mouth bulletin, 2010).

Table 4: Summary of FMDV genome detection on probang samples from buffaloes and cattle in Ruaha, Mikumi and Mkomazi areas

Location	Species	Genome detection		Total
		Positive	Negative	
Ruaha	Bovine	6	28	**34**
	Buffalo	1	1	**2**
Mikumi	Bovine	9	22	**31**
	Buffalo	14	6	**20**
Mkomazi	Bovine	1	41	**42**
	Buffalo	2	9	**11**
Total		**33**	**107**	**140**

Results were obtained by simple mathematical calculation using Microsoft excel.2007 The detection rates of FMDV genome was computed by dividing number of genome positive animals with total number of animals in each study location (Table 3).

For Mkomazi, total sample size was 53, number of infected animals was 3 and hence the detection rate was 5.8%

In Ruaha, sample size was 36 and infected animals were 7; hence detection rate of 19.44%

The sample size of Mikumi was 51 animals of which 21 were FMDV positive giving out the detection rate of 41.18%.

Calculation based on animal species indicated the detection rate to be high on African buffaloes as compared to cattle.

Total number of african buffalo samples used were 33 of which 17 were FMDV positive giving the detection rate of 51.52%. On the other hand, cattle having the sample size of 106 had only 14 FMDV positive animals giving the detection rate value as low as 13.2%.

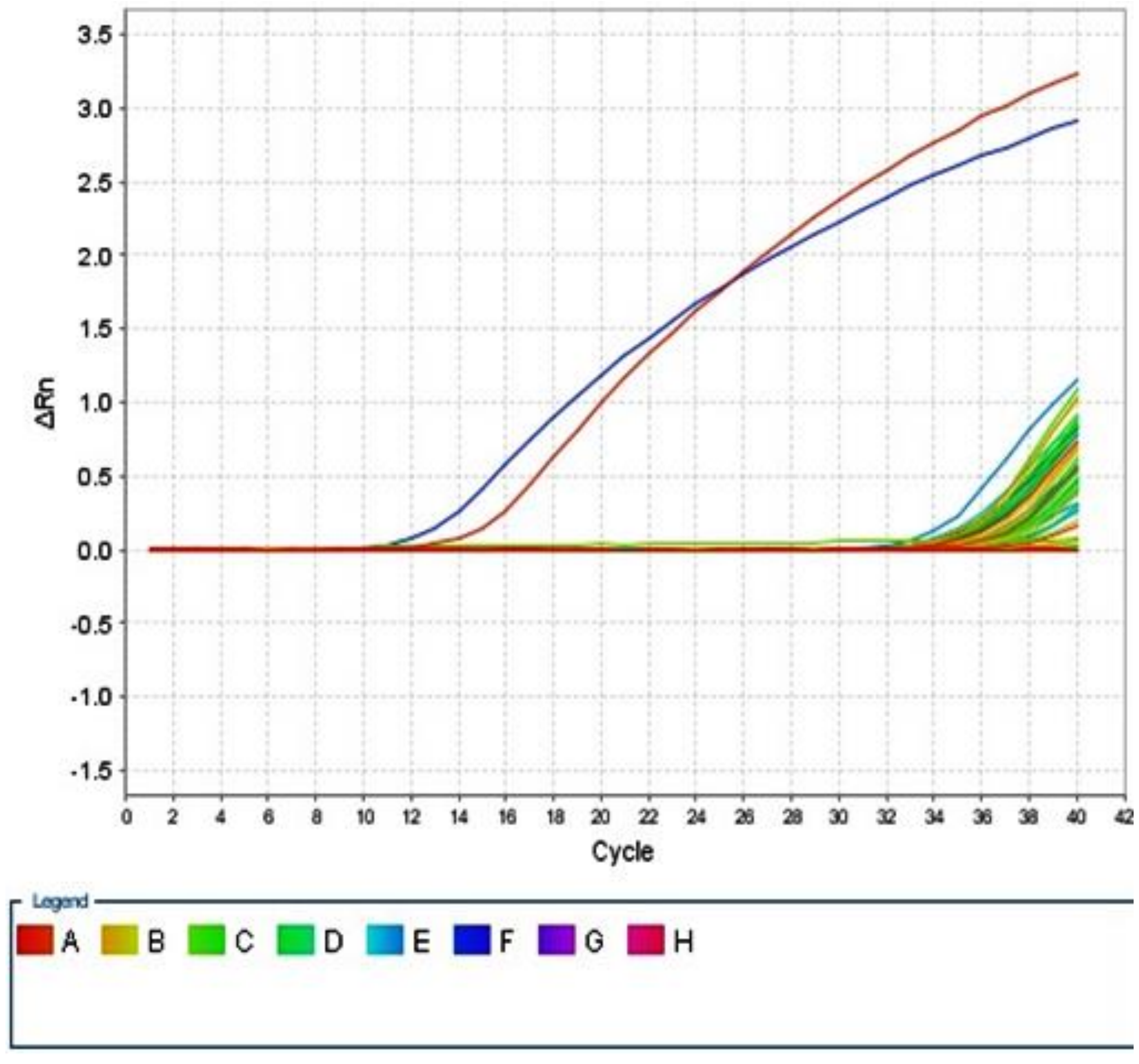

Figure 5: Amplification plot for qRT-PCR displaying FMDV genome detection

Table 5: Table showing sex and species of animals in each location.

Location FMDV genome	Species	Male		Female		Uncategorized		Total
		Positive	Negative	Positive	Negative	Positive	Negative	
Ruaha	Bovine	3	10	2	9	1	9	**34**
	Buffalo	1	0	0	0	0	1	**2**
Mikumi	Bovine	0	2	9	20	0	0	**31**
	Buffalo	10	2	4	4	0	0	**20**
Mkomazi	Bovine	0	0	0	0	5	37	**42**
	Buffalo	0	6	2	3	0	0	**11**
Total		**14**	**20**	**17**	**36**	**6**	**47**	**140**

Footnote: Some of the animals were not grouped as female or male; they therefore belong to the group of uncategorised animals.

When sampling it was important to ensure samples collected included animals from both gender groups but the study did not consider having equal number for both groups. In this case, data obtained (Table 3) is not indicative of the disease situation between male and female animals. Age of animals was also not considered in sample collection although preferred samples were of middle aged animals.

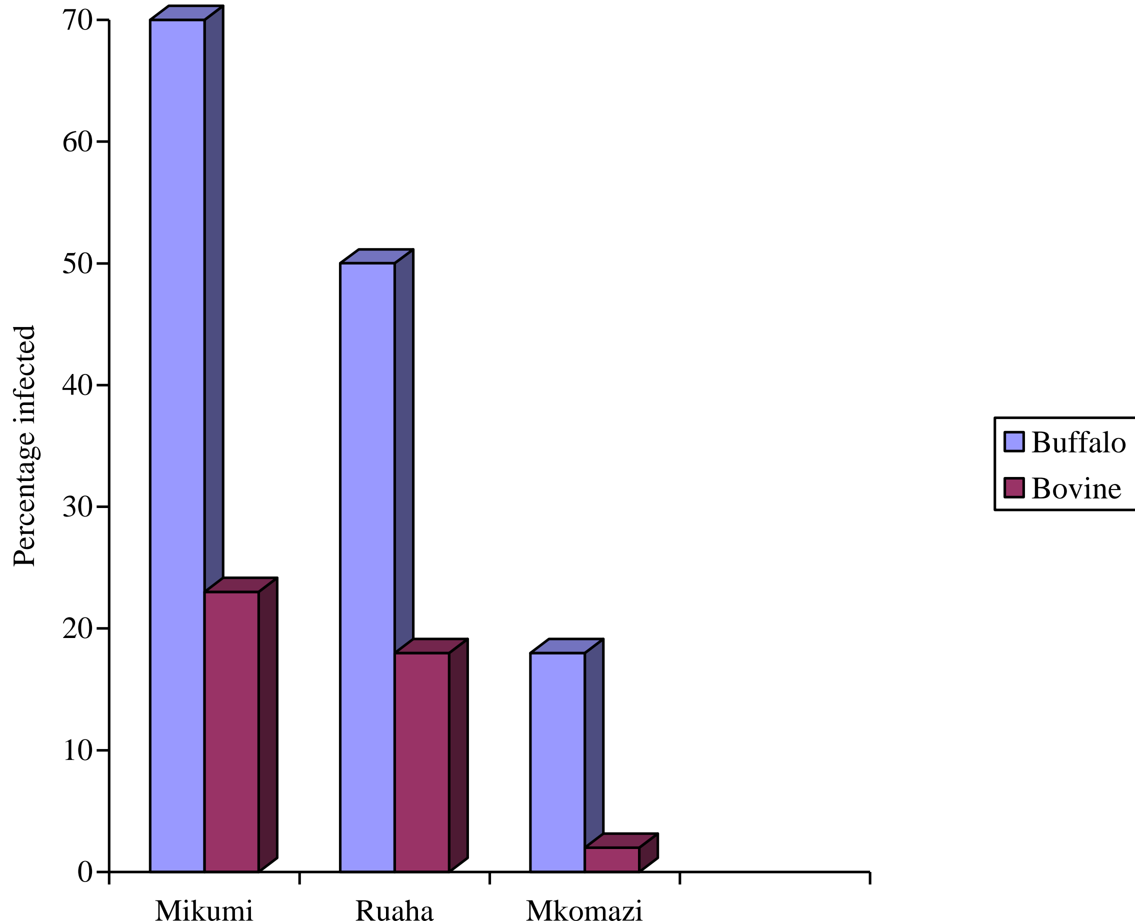

Figure 6: Graph showing percentage of FMD infected animals in each location

In Tanzania, there is variation of infection from place to place like in many other places in the world. Mikumi had the highest number of infected animals as compared to Ruaha and Mkomazi (Figure 3). Further investigation may be needed to come out with a reason to this observation but this may probably be due to high number of animals in the area. In this study it was noted that the two animal species, bovine and buffaloes were seen to vary in infection. Buffaloes were seen to have higher numbers of infection 51.52% (n=17) as compared to bovine species 13.21% (n=14). Except for Ruaha where only two buffalo samples were collected (n=2), the rest of the data clearly indicated that FMD is more in wildlife as compared to livestock. Negative results also shows large number of cattle as FMDV negative compared to what was observed in buffaloes. Of thirty one (31) FMDV positive animals, seventeen (17) were buffaloes and out of sixty eight (68) FMDV negative animals, only nine (9) were buffaloes. This can probably point out that buffalo species can be reservoir of the disease.

Livestock, wildlife, people and materials that are infected or have had contact with infected animals can spread FMD. Direct contact is the most probable method of infection because infected animals produce a great amount of saliva containing the virus.

This study could not clearly indicate whether the movement of the disease is from livestock to wildlife or if the vice versa is true. The question remains, which is to blame between wildlife and livestock?

In order to clarify FMDV transmission and/or carrier status between livestock and wildlife or vice versa, experimental infection of susceptible animals is highly recommended by this study.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Knowing the disease dynamic is an important step in controlling the disease in Tanzania. This study has found that buffaloes have higher levels of FMDV infection as compared to livestock. It was also noted that infection varied from one location to another but there was no significant difference in the infection status for male and female animals.

5.2 Recommendations

The finding that wildlife is more infected as compared to livestock calls for deeper investigation concerning FMD status of wildlife in all Tanzanian national parks. Efforts should be done to control the disease in wildlife-livestock interface on both grazing and water points. I recommend a much more intensive study which should aim at a wider coverage and large number of samples especially in wildlife. The study should also cover other wildlife apart from buffaloes.

Many FMD vaccines are made from killed virus and provide short-term protection. Previously vaccinated animals need to be re-vaccinated after some months. FMD has seven serotypes and about sixty subtypes. Currently, there is no single vaccine that is effective against all the variants.

The subtype can change during an outbreak, necessitating a change in vaccine and leaving animals vaccinated against a different subtype vulnerable to the new viral strain. Vaccinated animals get a much milder version of the disease and may become a source of infection for other animals.

For this reason this study is recommending further investigation on the serotypes circulating in Tanzania as well as coverage of the study in wider range. Sequencing should also be performed so as to understand the virus circulating in our country.

I also recommend the study to go deep into molecular serotyping and sequencing so as the exact type of circulating FMD genomes can be known together with their sequences as this will give an answer about the disease movement.

Further studies, including serotyping, virus isolation, experimental infection and sequencing of the viruses, are required to elucidate the complex epidemiology of FMD in cattle and buffaloes in the livestock-wildlife interface areas in Tanzania.

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APPENDICES

Appendix 1: Table showing animals in Ruaha, their Ct values and FMD status

S/N	National park	Animal species	Ct value	FMD status
1	Ruaha	Buffalo	35	Negative
2	Ruaha	Buffalo	31	Positive
3	Ruaha	Bovine	25	Positive
4	Ruaha	Bovine	34	Negative
5	Ruaha	Bovine	33	Negative
6	Ruaha	Bovine	35	Negative
7	Ruaha	Bovine	27	Positive
8	Ruaha	Bovine	36	Negative
9	Ruaha	Bovine	34	Negative
10	Ruaha	Bovine	35	Negative
11	Ruaha	Bovine	33	Negative
12	Ruaha	Bovine	30	Positive
13	Ruaha	Bovine	28	Positive
14	Ruaha	Bovine	34	Negative
15	Ruaha	Bovine	36	Negative
16	Ruaha	Bovine	38	Negative
17	Ruaha	Bovine	34	Negative
18	Ruaha	Bovine	36	Negative
19	Ruaha	Bovine	35	Negative
20	Ruaha	Bovine	33	Negative
21	Ruaha	Bovine	36	Negative
22	Ruaha	Bovine	34	Negative

S/N	National park	Animal species	Ct value	FMD status
23	Ruaha	Bovine	35	Negative
24	Ruaha	Bovine	31	Positive
25	Ruaha	Bovine	33	Negative
26	Ruaha	Bovine	35	Negative
27	Ruaha	Bovine	34	Negative
28	Ruaha	Bovine	37	Negative
29	Ruaha	Bovine	34	Negative
30	Ruaha	Bovine	37	Negative
31	Ruaha	Bovine	29	Positive
32	Ruaha	Bovine	33	Negative
33	Ruaha	Bovine	36	Negative
34	Ruaha	Bovine	34	Negative
35	Ruaha	Bovine	37	Negative
36	Mikumi	Buffalo	33	Negative
37	Mikumi	Buffalo	34	Negative
38	Mikumi	Buffalo	33	Negative
39	Mikumi	Buffalo	24	Positive
40	Mikumi	Buffalo	34	Negative
41	Mikumi	Buffalo	31	Positive
42	Mikumi	Buffalo	26	Positive
43	Mikumi	Buffalo	28	Positive
44	Mikumi	Buffalo	30	Positive
45	Mikumi	Buffalo	26	Positive
46	Mikumi	Buffalo	36	Negative

S/N	National park	Animal species	Ct value	FMD status
47	Mikumi	Buffalo	29	Positive
48	Mikumi	Buffalo	28	Positive
49	Mikumi	Buffalo	33	Negative
50	Mikumi	Buffalo	30	Positive
51	Mikumi	Buffalo	31	Positive
52	Mikumi	Buffalo	34	Negative
53	Mikumi	Buffalo	25	Positive
54	Mikumi	Buffalo	26	Positive
55	Mikumi	Buffalo	28	Positive
56	Mikumi	Buffalo	29	Positive
57	Mikumi	Bovine	36	Negative
58	Mikumi	Bovine	33	Negative
59	Mikumi	Bovine	27	Positive
60	Mikumi	Bovine	30	Positive
61	Mikumi	Bovine	29	Positive
62	Mikumi	Bovine	34	Negative
63	Mikumi	Bovine	36	Negative
64	Mikumi	Bovine	33	Negative
65	Mikumi	Bovine	33	Negative
66	Mikumi	Bovine	34	Negative
67	Mikumi	Bovine	37	Negative
68	Mikumi	Bovine	35	Negative
69	Mikumi	Bovine	33	Negative
70	Mikumi	Bovine	36	Negative

S/N	National park	Animal species	Ct value	FMD status
71	Mikumi	Bovine	34	Negative
72	Mikumi	Bovine	24	Positive
73	Mikumi	Bovine	33	Negative
74	Mikumi	Bovine	35	Negative
75	Mikumi	Bovine	29	Positive
76	Mikumi	Bovine	33	Negative
77	Mikumi	Bovine	36	Negative
78	Mikumi	Bovine	35	Negative
79	Mikumi	Bovine	33	Negative
80	Mikumi	Bovine	33	Negative
81	Mikumi	Bovine	34	Negative
82	Mikumi	Bovine	37	Negative
83	Mikumi	Bovine	27	Positive
84	Mikumi	Bovine	34	Negative
85	Mikumi	Bovine	36	Negative
86	Mikumi	Bovine	34	Negative
87	Mikumi	Bovine	29	Positive
88	Mkomazi	Buffalo	36	Negative
89	Mkomazi	Buffalo	26	Positive
90	Mkomazi	Buffalo	35	Negative
91	Mkomazi	Buffalo	33	Negative
92	Mkomazi	Buffalo	34	Negative
93	Mkomazi	Buffalo	37	Negative
94	Mkomazi	Buffalo	34	Negative

S/N	National park	Animal species	Ct value	FMD status
95	Mkomazi	Buffalo	30	Positive
96	Mkomazi	Buffalo	33	Negative
97	Mkomazi	Buffalo	34	Negative
98	Mkomazi	Buffalo	36	Negative
99	Mkomazi	Bovine	36	Negative
100	Mkomazi	Bovine	35	Negative
101	Mkomazi	Bovine	34	Negative
102	Mkomazi	Bovine	33	Negative
103	Mkomazi	Bovine	34	Negative
104	Mkomazi	Bovine	34	Negative
105	Mkomazi	Bovine	36	Negative
106	Mkomazi	Bovine	36	Negative
107	Mkomazi	Bovine	33	Negative
108	Mkomazi	Bovine	33	Negative
109	Mkomazi	Bovine	34	Negative
110	Mkomazi	Bovine	35	Negative
111	Mkomazi	Bovine	37	Negative
112	Mkomazi	Bovine	34	Negative
113	Mkomazi	Bovine	35	Negative
114	Mkomazi	Bovine	34	Negative
115	Mkomazi	Bovine	35	Negative
116	Mkomazi	Bovine	34	Negative
117	Mkomazi	Bovine	35	Negative
118	Mkomazi	Bovine	36	Negative

S/N	National park	Animal species	Ct value	FMD status
119	Mkomazi	Bovine	33	Negative
120	Mkomazi	Bovine	30	Positive
121	Mkomazi	Bovine	34	Negative
122	Mkomazi	Bovine	34	Negative
123	Mkomazi	Bovine	36	Negative
124	Mkomazi	Bovine	34	Negative
125	Mkomazi	Bovine	35	Negative
126	Mkomazi	Bovine	37	Negative
127	Mkomazi	Bovine	35	Negative
128	Mkomazi	Bovine	34	Negative
129	Mkomazi	Bovine	34	Negative
130	Mkomazi	Bovine	33	Negative
131	Mkomazi	Bovine	34	Negative
132	Mkomazi	Bovine	35	Negative
133	Mkomazi	Bovine	37	Negative
134	Mkomazi	Bovine	35	Negative
135	Mkomazi	Bovine	33	Negative
136	Mkomazi	Bovine	33	Negative
137	Mkomazi	Bovine	35	Negative
138	Mkomazi	Bovine	35	Negative
139	Mkomazi	Bovine	36	Negative
140	Mkomazi	Bovine	34	Negative

Appendix 2: Buffers for extraction, purification of RNA and PCR

2.0. RNA extraction and PCR kits

- QIAamp Viral RNA Mini Kit.

This kit was for 50 minipreparations and contained 50 QIAamp Mini columns, carrier RNA, Buffers and 2ml collection tubes.

- AgPath-ID™ One-Step RT-PCR kit.

The kit was for 100 preparations of 25 microliter and contained 2x RT PCR buffer, forward primer, reverse primer, probe, qRT PCR enzyme, detection enhancer and nuclease free water.

2.1. Viral lyses buffer

The sample is lysed under denaturing conditions provided by viral lyses buffer (AVL) to inactivate RNases and to ensure isolation of intact viral RNA.

Buffer AVL was checked for precipitate and was incubated at room temperature until the precipitate was dissolved. Since there was large number of samples, volumes were calculated using the following sample calculation equation:

n x 0.56 ml = **y** ml

y ml x 10 µl/ml = z µl

Where:

n = number of samples to be processed simultaneously

y = calculated volume of Buffer AVL

z = volume of carrier RNA–Buffer AVE to add to Buffer AVL

Mixing was done by inverting the tube 10 times. Vortexing was not applied so as to avoid foaming.

Carrier RNA, added to Buffer AVL, improves the binding of viral RNA to the QIAamp® membrane especially in the case of low-titer samples, and limits possible degradation of the viral RNA due to any residual RNase activity.

2.2. Carrier RNA

Carrier RNA enhances binding of viral nucleic acids to the QIAamp Mini membrane, especially if there are few target molecules in the sample. Addition of large amounts of carrier RNA reduces the chance of viral RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in Buffer AVL. If carrier RNA is not added to Buffer AVL this may lead to reduced viral RNA recovery.

2.3. RNA wash buffer

Two wash buffers were used.

Buffer AW1 or wash buffer 1 is supplied as a concentrate contained 19ml of the buffer. Before using for the first time, 25ml of absolute ethanol was added to make a final volume of 44ml AW1 as indicated on the bottle.

Buffer AW2 or wash buffer 2 is also supplied as a concentrate containing 13ml of the buffer and 30ml of absolute ethanol was added to make a final volume of 43ml AW2 as indicated on the label of the bottle.

Appendix 2: Continues

2.4. RNA elution buffer

Elution buffer AVE is RNase-free water that contains 0.04% sodium azide to prevent microbial growth and subsequent contamination with RNases. Sodium azide affects Spectrophotometer absorbance readings between 220 and 280 nm but has no effect on RT-PCR.

2.5. Decontamination solution

70 percent ethanol was used as a sterilisation reagent to sterilise the safety cabinet and pipettes. Vircon buffer which has virucidal action was used as decontamination solution. Desderman gel was used as a hand disinfectant.

2.6. RNA extraction.

FMDV is an RNA virus and thus the genome used to study the virus was RNA genome. The pre-aliquoted samples were optimized at fridge temperature to enable thawing. Room temperature was avoided since FMD viruses are unstable at room temperature.
RNA extraction was done using the QIAMP MinElute Spin Kit.

Protocol used was purification of viral RNA (Spin protocol). This protocol is for purification of RNA from 140 micro liter of probang using a microcentrifuge.

Appendix 3: Protocal for RNA extraction.

1. 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
2. 140 µl of probang material was added to Buffer AVL–carrier RNA in the microcentrifuge tube and vortexed for 15 seconds.
3. The mixture was incubated at room temperature (25°C) for 10 minutes.
4. Centrifugation of the tubes was done to remove drops from the inside of the lid.
5. 560 µl of 100% ethanol was added to the sample, and mixed by pulse-vortexing for 15 seconds. After mixing centrifugation was done to remove drops from inside the lid.
6. 630 µl of the solution from step 5 was applied to the QIAamp Mini column (in a 2 ml collection tube) carefully without wetting the rim. Caps were closed, and centrifuged at 6000 x g (8000 rpm) for 1 minute. QIAamp Mini columns were placed into clean 2 ml collection tubes, and tubes containing the filtrate were discarded.
7. QIAamp Mini columns were carefully opened and step 6 was repeated.
8. QIAamp Mini columns were carefully opened and 500 µl of Buffer AW1 was added. Caps were closed and the solution was centrifuged at 6000 x g (8000 rpm) for 1 minute. QIAamp Minicolumns were transfered in clean 2 ml collection tubes and tubes containing the filtrate were discarded.
9. QIAamp Mini columns were opened and 500 µl of Buffer AW2 was added. Caps were closed and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min.

Appendix 3: Continues

10. To eliminate any possible chances of buffer AW2 contamination, QIAamp Mini columns were placed in new 2 ml collection tubes and the old collection tubes with the filtrate were discarded. Centrifugation was done at full speed for 1 min.
11. QIAamp Mini columns were placed in clean 1.5 ml microcentrifuge tubes. Old collection tubes containing the filtrate were discarded. QIAamp Mini columns were carefully opened and 60 µl of Buffer AVE equilibrated to room temperature was added. Caps were closed and the mixture incubated at room temperature for 1 minute. Centrifugation at 6000 x g (8000 rpm) for 1 minute was performed.

Viral RNA was stored at –20°C waiting for Real Time PCR to be performed.

Appendix 4: Experiment 1: qRT-PCR loading sequence and results sheet.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A				Neg	Pos	1	2	3	4	5	6	7
B	8	9	10	11	12	13	14	15	16	17	18	19
C	20	21	22	23	24	25	26	27	28	29	30	31
D	32	33	34	35	36	37	38	39	40	41	42	43
E	44	45	46	47	48	49	50	51	52	53	54	55
F	56	57	58	59	60	61	62	63	64	65	66	67
G	68	69	70									
H												

Results interpretation for qRT-PCR.

= Samples with Ct values of ≤ 32 are considered positive.

= Samples with Ct values of 'No Ct' are considered negative.

= Samples giving mean Ct values of ≥ 32 - ≥ 50 are 'inconclusive' and repeat testing may be required to define a result.

Appendix 4: Continues

Experiment 2. Master mix.

S/N	**Reagents**	**1x**	**74x**
1	2x RT PCR Buffer	12.5	925
2	Forward Primer	1	74
3	Reverse Primer	1	74
4	Probe	1	74
5	25x RT PCR Enzyme	1	74
6	Detection enhancer	1.67	123.58
7	Nuclease free water	0.83	61.47

-7 µl RNA sample was added into a tube containing 18 µl of master mix to make a final volume of 25 µl.

Experiment 2(b) PCR conditions.

Temperature	Time (min)	Cycles
45°C	10	1
95°C	10	1
95°C	15	40
60°C	45	40

Appendix 4: Continues

Experiment 2: qRT-PCR loading sequence and results sheet.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A				Neg	Pos	71	72	73	74	75	76	77
B	78	79	80	81	82	83	84	85	86	87	88	89
C	90	91	92	93	94	95	96	97	98	99	100	101
D	102	103	104	105	106	107	108	109	110	111	112	113
E	114	115	116	117	118	119	120	121	122	123	124	125
F	126	127	128	129	130	131	132	133	134	135	136	137
G	138	139	140									
H												

Results interpretation for qRT-PCR.

= Samples with Ct values of ≤ 32 are considered positive.

= Samples with Ct values of 'No Ct' are considered negative.

= Samples giving mean Ct values of ≥ 32 - ≥ 50 are 'inconclusive' and repeat testing may be required to define a result.

Appendix 4: Continues
Repeat experiment.
Experiment 3 Master mix.

S/N	Reagents	1x	50x
1	2x RT PCR Buffer	12.5	625
2	Forward Primer	1	50
3	Reverse Primer	1	50
4	Probe	1	50
5	25x RT PCR Enzyme	1	50
6	Detection enhancer	1.67	83.5
7	Nuclease free water	0.83	41.5

Experiment 3: qRT-PCR loading sequence and results sheet.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A				Neg	Pos	2	8	16	21	46	63	64
B	65	73	74	76	78	81	88	91	93	94	97	98
C	99	101	103	104	106	107	108	109	112	114	115	116
D	117	119	122	124	125	129	131	135	136	138	141	142
E	143	144										
F												
G												
H												

Results interpretation for qRT-PCR.

= Samples with Ct values of ≤ 32 are considered positive.

= Samples with Ct values of 'No Ct' are considered negative.