INVESTIGATING THE PERFOMANCE OF LATERAL FLOW DEVICES IN DIAGNOSIS AND GENETIC CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUS IN TANZANIA

PAULO FUPI RAPHAEL

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED MICROBIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.

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ABSTRACT

A study on rapid detection and diagnosis of foot and mouth disease (FMD) field outbreaks was carried out in field settings using a Svanodip® foot and mouth disease virus (FMDV)- antigen (Ag) test lateral flow device (LFD) in Serengeti and Ngorongoro districts of Tanzania. Epithelial tissues from cattle (n=80) were collected from different villages and tested at penside using Svanodip® FMDV-antigen test to evaluate the sensitivity and specificity of LFDs relative to gold standard reverse transcription polymerase chain reaction (RT-PCR) molecular technique for FMDV. The duplicates of same epithelial tissues were stored in liquid nitrogen and transported to the laboratory for confirmation using gold standard RT-PCR technique targeting conserved region (3D) region of the FMDVgenome. Seventy eight samples showed positive reaction to LFDs antigen test and two samples were negative. All eighty samples collected during this study were positive to RT-PCR gold standard. LFDs and RT-PCR showed sensitivity and specificity of 97.5% and 100% respectively. There was no significant difference on the results of LFDs and RT-PCR in relation to the age of the lesions and type of epithelial tissues used ($P>0.05$). Tested LFDs were kept at-20°C and at room temperature for six and eight months respectively and possibility of FMDV RNA recovery was done. LFDs kept at room temperature for eight months were shown to be good vehicle for recovery, storage and transportation of FMDV RNA, than those kept at -20°C for six months. Antigen ELISA serotyping revealed that serotype A and O were associated with the current FMD outbreaks in the studied villages. Although, the lateral flow devices are panserotypic and provide point of care results for the rapid detection and the diagnosis of FMDV field outbreaks, their use must go in parallel with (OIE) gold standard technique for confirmation of FMDV outbreaks. Further studies are required to validate and deploy the LFDs for a wider application for FMDV outbreak investigations in Tanzania.

DECLARATION

I, **PAULO FUPI RAPHAEL**, 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.

_____ _____

Paulo Fupi Raphael Date

(MSc. Student)

The above declaration is confirmed by;

_____ _____

Prof. Kazwala, R. R. Date

(Supervisor)

_____ _____

Dr. Kasanga, C. J Date

(Supervisor)

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This dissertation is dedicated to my dear wife Regina, my sons Frank and Faustine, my daughters Lucianna and Julianna.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ag	antigen
BBSRC	Biotechnology and Biological Sciences Research Council
BHK-21	baby hamister kidney cell lines
BTY	primary bovine calf thyroid cells
CBPP	contagious bovine pleuropneumonia
CDC	Centre for Disease Control
CEO	Chief Executive Officer
CIDB	Centre for Infectious Disease and Biotechnology
cm^2	centimetre squared
CPE	cytopathic effects
Ct	cycle threshold
DEFRA	Department for Environment, Food and Rural Affairs
DIVA	differentiating infected from vaccinated
DVO	District Veterinary Officer
ELISA	enzyme- linked immunosorbent assay
FAO	Food and Agriculture Organization of United Nations
FAO-EUFMD	Food and Agriculture European Union Foot and Mouth Disease Commision
FMD	foot and mouth disease
FMDV	foot and mouth disease virus
IAH-P	Institute for Animal Health-Pirbright
IATA	International Air Transport Association
IBRS	renal swine cell lines
IF 10	monoclonal antibody

IgA	immunoglobulin A
IRES	intra ribosomal entry sites
kb	kilobase
LFDs	lateral flow devices
LFO	Livestock Field Officer
LPBE	liquid phase blocking elisa
mab	monoclonal antibodies
mg^{2+}	magnesium ion
MLFD	Ministry of Livestock and Fisheries Development
mm	milimetre
MoWLD	Ministry of Water and Livestock Development
MSc	Masters of Science
N	north
n	sample size
nm	nonametre
NSP	non structural protein
OIE	Office des International Epizootics
OP	oropharygeal fluid
ORF	open reading frame
PCR	polymerase chain reaction
P^H	negative logarithm of hydrogen ion concentration
R	correlation coeficient
RNA	ribonucleic acid
Rnase OUT	rembinant ribonucleic acid inhibitor
Rnases	ribonucleases

rpm	rotation per minute
rRT-PCR	real time reverse transcription- poymerase chain reaction
RT-LAMP	reverse transcription- loop mediated isothermal amplification
RT-PCR	reverse transcription- polymerase chain reaction
SAT 1	South African territory strain one
SAT 2	South African territory strain two
SAT 3	South African territory strain three
Snanova	Swedish Company
SOP	standard operating procedure
SP	structural protein
SPCE	solid phase competing elisa
ssRNA+	single stranded ribonucleic acid positive sense
SUA	Sokoine University of Agriculture
TAD	transboundary animal disease
TVLA	Tanzania Veterinary Laboratory Agency
UK	United Kingdom
UTR	untranslated region
VEO	Village Executive Officer
VP1	viral protein segment one
VP2	viral protein segment two
VP3	viral protein segment three
VP4	viral protein segment four
VPg	viral protein g
WRLFMD	World Reference Laboratory for Foot and Mouth Disease
ZVC	Zonal Veterinary Centre

µl	microlitre
µM	micrometre
®	registered trade mark
°C	degree celsius
146s	virus sedimentation rate
3ABC	recombinant non structural protein of FMDV
3D	conserved region of foot and mouth virus genome

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Foot and Mouth disease (FMD) is severe, highly contagious viral disease of cloven hoofed domestic and wild animals (Reid *et al*., 2001; Sutmoller *et al*., 2003; Ayalet *et al*., 2009; Polychronova *et al.*, 2010). The disease is caused by FMD virus (FMDV) a picornavirus, a member of genus *Aphthovirus* of the family *Picornaviridae*.

Although, FMD is reported worldwide (Polychronova *et al.,* 2010), sub-Saharan Africa harbours a widest range of FMDV strains circulating both in domestic animals and wildlife and widespread outbreaks of clinical disease are reported every year (Sahle *et al*., 2004; Rweyemamu *et al*., 2008). Throughout sub-Saharan Africa and in some parts of South Asia sub-continent (Middle East, Indian subcontinent and Southeast Asia) and South America, FMD is an endemic transboundary viral disease (Kitching, 1998; Vosloo *et al*., 2002; King *et al.*, 2012). FMDV exists as seven discrete serotypes in the world namely O, A, C, ASIA 1, South African territory 1, 2 and 3 (abbreviated as SAT 1, SAT 2 and SAT 3 respectively) (Sutmoller *et al*., 2003). In sub-Saharan Africa five serotypes are endemic, these include all the three SAT serotypes, serotype A and serotype O (King *et al.*, 2012). The existence of sixth serotype, serotype C in Africa has been reported also (Vosloo *et al.,* 2002; Ayalet *et al.,* 2009). Only Asia 1 has never been reported in Africa (Ayalet *et al*., 2009; Maree *et al.*, 2011). East African countries have endemic status and in some areas sporadic outbreaks are common every year (Namatovu *et al.,* 2013).

Since its official documentation in 1927 in Tanzania, the FMDV were isolated for the first time in 1954 (Atang, 1968) and since then isolation and characterization were carried out

by different workers. In 1972, Characterization and serotyping of circulating serotypes was done in Tanzania (Rweyemamu and Loretu, 1972).

Although, isolation and characterization of foot and mouth disease virus (FMDV) were carried at different times by different researchers, lack of comprehensive epidemiological background on the disease, uncontrolled livestock movement, budgetary constraints, lack of data on actual disease impact, gross lack of political commitment and presence of wildlife reservoirs of FMDV hindered effective disease control programmes. In Tanzania, four serotypes namely O, A, SAT 1 and SAT 2 are commonly documented and known to circulate in domestic animals (Mlangwa, 1983; Rweyemamu, 2008; Kasanga *et al.*, 2012., Kasanga *et al*., 2014).

The Ministry of Livestock and Fisheries Development (MLFD) in Tanzania in 2006 identified FMD as an important viral transboundary animal disease (TAD) in cattle in Tanzania (Swai *et al*., 2009) and was listed as the second important TAD of cattle after contagious bovine pleuropneumonia (CBPP) (Ministry of Water and Livestock development) (MoWLD., 2003). The disease has attained an endemic status especially in the northern part of Tanzania and Lake Victoria basin (Kivaria, 2011) and sporadic outbreaks of the disease occur across the country yearly (Swai *et al.,* 2009; Picado *et al*., 2011; Kasanga *et al.,* 2014).

In Tanzania, FMD is most often diagnosed based on clinical signs by livestock officers and District veterinary officers (DVOs). Confirmation of the disease has been carried out by viral isolation and serotyping at the World Reference Laboratory for Foot and Mouth Disease (WRLFMD) Pirbright, United Kingdom (UK). As in most of East African Countries, National laboratories (Tanzania Veterinary Laboratory Agency (TVLA)

through its centre Centre for Infectious Disease and Biotechnology (CIDB) performs diagnosis of FMDV. CIDB in Tanzania commonly used antigen enzyme linked-immunosorbent assay (ELISA) and antibody detection for FMDV diagnosis (Namatovu *et al.*, 2013), capacity for reverse transcription-polymerase chain reaction (RT-PCR) FMDV genome detection now established.

As part of FMD surveillance, clinical samples are collected and submitted to Tanzania Veterinary Laboratory Agency (TVLA) from districts through strategically placed Zonal Veterinary Centres (ZVC) and TVLA Zonal Centres. In turn TVLA regularly ships selected samples to WRLFMD, Pirbright for isolation and characterization.

Typical cases of FMD are characterized by a vesicular condition of the feet, buccal mucosa and, in females, the mammary glands (Thomson, 1994). Clinical signs may vary from mild to severe, and fatalities may occur, especially in young animals (OIE, 2009). Animals infected with the virus develop clinical signs after 2-14 days (Alexandersen *et al.,* 2003).

FMD shares indistinguishable clinical signs with other vesicular diseases namely Swine vesicular disease, Vesicular stomatitis and Vesicular exantherma of swine (Alexendersen *et al.,* 2003; Hole *et al.,* 2010). FMD is endemic in Tanzania but due to the expansion of trade in live animals, animal movements and increased trade in animal products and greater mobility of people poses a risk of exotic disease introduction with similar clinical signs as FMD (Sutmoller *et al.,* 2002). The rapid detection of the agent of the disease in cattle and swine is required to differentiate between FMD and other vesicular diseases for proper and rapid control implementation.

Outbreaks of FMD in Tanzania result in severe economic losses and have a considerable impact on both national and international trade within the livestock sector (MoWLD,

2003). Globally, FMD has immense economic losses threatening agricultural industry and is considered as major animal health problem (Vosloo *et al.*, 2002; Knowles and Samuel, 2003; Longjam *et al.,* 2011a).

Control of FMD is possible through mass vaccination, control of livestock movements, fencing, quarantine and stamping out of infected animals and in contact animals. In Tanzania, efforts to control the disease have not been successful due to number of reasons. Kivaria (2003) stated reasons for FMD control difficulties in Tanzania to be due to a lack of control of livestock movements within and between international borders, lack of comprehensive epidemiological studies on the disease, antigenic variations of different circulating serotypes and subtypes and presence of wildlife reservoirs of the virus, lack of coherent vaccination strategy. Movement of infected animals has been regarded as important milestone in the spread of FMD in endemic settings (Rweyemamu *et al.,* 2008; Maree *et al.*, 2011, Kasanga *et al*., 2014). Lack of information on circulating strains prevents selection of locally-appropriate strains for vaccination, lack of effective surveillance of disease, which prevents implementation of a prompt response to outbreaks (e.g. movement restrictions).

Animals infected with FMDV harbour the virus for considerable variable periods without apparent clinical signs (Sutmoller *et al.,* 2002), but there is still much uncertainity about the role of carrier animals in the transmission of FMD (Chang et *al.,* 2013). Previous studies revealed that approximately 50% of infected cattle become carriers irrespective of their vaccination status (Moonen *et al.,* 2000). The vaccinated animals as well may be infected and serve as silent shedders of the virus without apparent clinical signs (Parida *et al.,* 2006*)*. These two factors provide considerable challenge to control strategies of the disease.

Prompt diagnosis and confirmation are important control components to prevent devastating effects of FMD to livestock reliant farmers and national economy. Provisional diagnosis of FMD disease by clinical signs needs to be supported by rapid diagnostic test at penside using a point of care devices such as lateral flow devices that can provide a quick and accurate rapid diagnosis of FMD. The rapid diagnosis enhances accurate and rational disease control strategy and mitigating solutions to the farmers and national economy. The laboratory confirmation of the disease using gold standard technique for FMDV is important to avoid consequences of misdiagnosis. As it was spelt out that FMD shares indistinguishable clinical lesions with other vesicular diseases and therefore confirmation is important component of disease control. Routine viral isolation and virus strain characterization do not produce rapid diagnostic results, although, they have been used as an official confirmation procedure adopted in referral laboratories. ELISAs techniques are important serological methods for detecting antigen and antibody reactions targeting both structural proteins and non structural components of FMDV genome (Bronsvoort *et al.,* 2006; Inoue *et al.,* 2006).

Currently, new high throughput molecular techniques have been developed and adopted for rapid diagnosis and confirmation. RT-PCR and reverse transcription loop mediated isothermal amplification (RT-LAMP) are now in place for rapid confirmation of FMDV in the laboratory (Notomi *et al*., 2000).

Immunity against FMD is complex and partly related to complexity and diversity of FMDV and existing serotypes and subtypes; calves may be protected by maternal antibodies shortly and immunity to vaccines is short lived (Doel *et al.,* 1996) and immunity to one FMD serotype do not provide cross-protection to other serotype and

variable cross protection across subtypes (Kenneth *et al.*, 1992; Mattion *et al.*, 2004). In developed countries the control of FMD is usually achieved by mass destruction of infected animals and in contact animals whereas as in developing countries large scale vaccination programmes accompanied by restriction of animal movements are used as a pathway for FMD control.

Conventional laboratory antigen and virological methods invariably incur some delay in confirmation of cases, which can have major consequences for effectiveness of measures to control and contain outbreaks (Notomi *et al.*, 2010). Delays in developing countries are further compounded by difficulties in transport of samples to reference laboratories. There is therefore a clear need for accurate field diagnostic tests that can provide timely information for responses to outbreaks.

A further advantage of decentralized testing is greater engagement of district veterinary officers and livestock field officers in FMD surveillance, as delays in feedback of diagnostic test results from central laboratories are likely to act as a major disincentive to reporting and investigation of outbreaks by field teams.

Recently, rapid field diagnostic kits have been used successfully and have been recommended for rapid field diagnosis of FMDV as options of decentralized laboratory testing (FAO-EuFMD, 2009). The kits have a possibility of improving diagnosis of FMD and its control strategy in many countries (FAO-EuFMD, 2009). SVANODIP® FMDV Ag –test made up of IF10 monoclonal antibodies is one of those kits that can be used at the penside to rapidly detect FMD. This study aims to introduce, evaluate and validate the use of SVANODIP® kit in diagnosis of FMDV field outbreaks in Tanzania, to determine performance of the device under field conditions and at different stages of clinical disease

of lesions and will provide recommendations for the feasibility of its use in FMDV field diagnosis and outbreak investigations in Tanzania.

1.2 Problem Statement and Justification

FMD is endemic in Tanzania and has been clearly defined by the MLFD as an important viral transboundary animal disease in cattle in Tanzania (Swai *et al.,* 2009). Rapid detection and accurate diagnosis of FMD is central to the implementation of effective measures to control the spread of FMD. As FMD virus is a highly variable RNA virus, field diagnostic tests need not only to have high analytical sensitivity and specificity, but also to detect diverse FMDV field strains. Although capacity exists at the TVLA Centre , (CIDB), Dar es Salaam, for laboratory diagnosis of FMDV through real-time RT-PCR assays and antigen ELISA tests, the requirement to send samples from remote field sites to the CIDB continues to pose logistical challenges, with the resulting delays causing sample degradation, and demotivating field staff to report and investigate outbreaks.

The development of a new lateral-flow diagnostic test kit (SVANODIP® FMDV-Ag test), offers a simple direct method for the detection of FMDV in field settings, which provide valuable diagnostic support for field veterinary officers and DVOs to implement quarantine measures, generate awareness and collect timely epidemiological data, and guide selection of material for laboratory confirmation (Ferris *et al.*, 2009). A further advantage of the lateral flow device (LFD) is the potential for recovery of genetic material from the "test cassette'' following storage at -20^{o}C and ambient temperature. This could potentially provide a useful archive of genetic material from field outbreaks for virus characterization using simple, cheap and robust storage systems.

1.3 Objectives

1.3.1 General objectives

To assess the performance of LFDs in rapid field diagnosis of FMDV outbreaks in Tanzania.

1.3.2 Specific objectives

1) To assess the diagnostic test sensitivity and specificity of the SVANODIP® lateral flow test for detection of FMDV in field outbreaks.
2) To evaluate the effect of the age of the lesion at different clinical stages of the disease and the type of epithelium on the reactivity of the lateral flow devices
3) To investigate the feasibility of extracting FMD viral RNA from lateral flow cassettes after storage at different temperature conditions.
4) To identify circulating serotypes of FMD in studied villages.

CHAPTER TWO

2.0 LITERATURE REVIEW

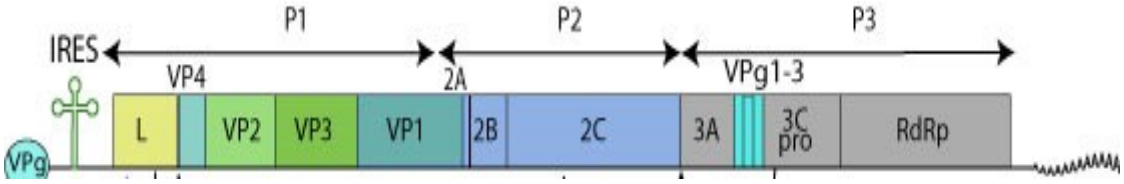
2.1 Foot and Mouth Disease

Highly contagious viral disease caused by Picornavirus. FMD is considered as most contagious disease of mammals and has a great potential of causing severe economic loss in susceptible cloven hoofed animals (Reid *et al.*, 2001, Ryan *et al*., 2008, OIE, 2009; Longjam *et al.*, 2011a). The disease is usually characterized by fever, salivation and vesicular erosions in the buccal cavity, rhinarium, interdigital space and in teats in females (Thomson, 1994). FMD affects extensive areas worldwide and is one of notifiable disease to the World Organization for Animal Health because of devastating effects to livestock trade. The disease is considered as serious epidemic from sixteenth century threatening animal industry and still regarded as major global animal health problem (Longjam *et al.,* 2011a).

2.2 Foot and Mouth Disease Virus

FMDV is a non enveloped positive single stranded RNA virus designated to family *Picornaviridae* of genus *Aphthovirus.* The FMDV is small in size, icosahedral in shape morphologically. The virus exists in the form of seven serologically and genetically distinguishable serotypes namely O, A, C, Asia1, SAT 1, SAT 2 and SAT3 and within each types exist topotypes and different lineages that evolved within each serotypes (Pereira *et al.*, 1977). The FMDV has a size of 22-28nm (Belsham *et al.,* 2011) and has a sedimentation coefficient of 146S. The FMDV capsid is made of 3 copies of 60 capsomers externally placed in which VP1, VP2 and VP3 are main components, and VP4 is internally sandwiched. VP1 is main and highly active diverse component responsible for many antigenic diversity containing G-H and B-H loops (Domingo *et al.,* 1996).

During replicative phase the virus has high replication ability, however its RNA polymerase enzyme lacks proof reading ability and mutations are common (Domingo *et al*., 2004). FMDV is labile to weak acids such as 0.2% citric acid and weak bases such as sodium hypochlorite and survives best around pH 7.2-7.6 (FAO., 2009; OIE., 2010; Krug *et al*., 2011) .



Alternative initiation ribosomal initiation

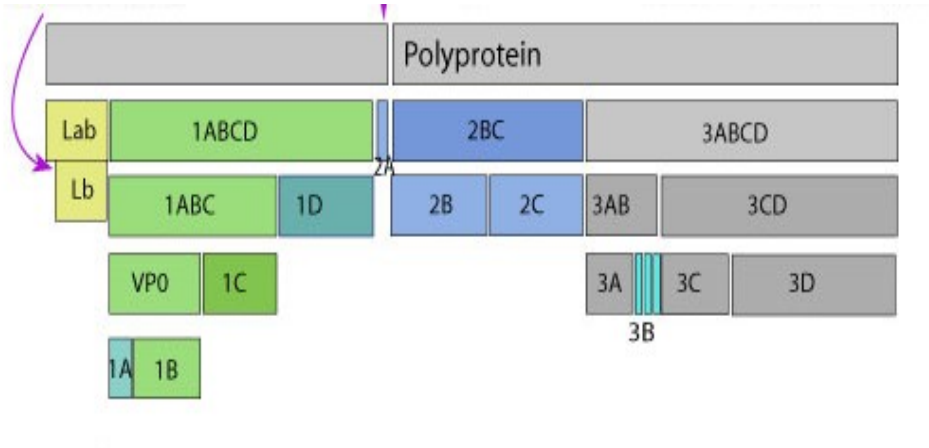


Figure 1: **Genome of FMDV**

Source: viral zone (Swiss Institute of Bioinformatics, 2010).

Key: Linear **ssRNA (+) genome** of 7.5-8.5 kb, polyadenylated, composed of a single **ORF** encoding a polyprotein. Viral genomic **RNA** has a viral protein (VPg) at its 5'. The long **UTR** at the 5' end contains an **internal ribosome entry site** type II. The P1 region encodes the structural polypeptides. The P2 and P3 regions encode the nonstructural proteins associated with replication. Encodes a N-terminal leader protease (L protease) in addition to the 3C protease. The shorter 3' **UTR** is important in (-) strand synthesis.

2.3 Cleavage of FMDV Polyproteins

It is demonstrated that the FMDV3C protease, in addition to the FMDV Lb protease induce cleavage of the translation initiation factors into different structural and non structural proteins (Belsham *et al*., 2000).

2.4 Clinical Signs and FMD Lesions

In susceptible animals FMD is characterized by the development of fever accompanied by the formation of blisters in epithelial tissues in buccal mucosa, in feet and on the mammary glands of female animals. Blisters later ruptures and form erosions. The presence of vesicles and ruptured tissues is characterized by limping animals and profusely salivating animals which in turn affects feed intake (Klein *et al.,* 2009). The resultant effect is emaciation and reduced productivity. In severe cases animals are reluctant to move and sloughing of hoofs is evident and in young animals multifocal myocarditis is the common cause of calf mortalities (Klein *et al.,* 2009).

2.5 Immunity to FMD

Constant antigenic variation relying on frequent mutation contributed complexicity of immunity against the FMDV. Both humoral and cell mediated immunity against the disease are not explicitly described. Natural recovery from disease provides short lived immunity against the same serotypes no crossprotection is conferred against other serotypes (Doel *et al.*, 1996). Maternal antibodies provide immunity shortly to calves and vaccine induced immunity is also short lived with periods below 6 months. Mucosal immunity which protects mucosal surface attributed by persistent stimulation due to carrier states imitates continuous secretion of IgA. The contribution of mucosal immunity for the protection against FMD is not well founded (Summerfield *et al.,* 2009).

2.6 Carrier States in FMD

Studies shows animals that recover from clinical and subclinical FMD in most cases become subsequent shedders of FMDV (Alexandersen *et al.*, 2003). Their study further observed some animals become carriers of the virus and persistently shed foot and mouth disease virus 28 days post infection. In the carriers animals, FMDV remain dormant in oro-pharygeal (OP) fluid and detection ability by collection of probang samples has been low and unreliable (Alexandersen *et al.,* 2003; Parida *et al.,* 2006). Prevalence of carriers was high after experimental infection (Parida *et al.,* 2006). It is also estimated that approximately 50% of the cattle infected with FMDV become carriers, irrespective of their vaccination status (Moonen *et al.*, 2000; Cox *et al.,* 2005).

Different species have variable carrier states after infection. In cattle it's known that they may remain carriers up to 3.5 years (Hedger., 1976; Hargreaves., 1994). In goats carrier state exists up to 9 months (Anderson, *et al.,* 1976; Singh, 1979), 2-3months in sheep (Burrows., 1968; Mc Vicar and Sutmoller., 1968) and in variable durations in wild species (Anderson *et al*., 1975; Ferris *et al.,* 1989). African buffalo (*Syncerus caffer*) are known to carry FMDV for 5 years (Condy *et al.,* 1985; Thomson., 1995). In isolated buffalo herds it's reported to be 24 years (Condy *et al.,* 1985). Studies involving pigs showed that pigs infected with FMD shed the virus within limited periods and do not serve as carriers of FMDV but studies showed that they may also harbour the FMDV for up to 300 days (Mezencio *et al.*, 1998). Previous studies suggested that in order to better control FMDV in the future, the role of carrier animals in FMDV transmission and the mechanism of FMDV persistence need further study (Moonen *et al.*, 2000).

2.7 Diagnosis of FMD and FMDV

Foot and Mouth disease is diagnosed by viral isolation techniques, detection of FMDV antigens and antibodies. Detection of viral genome is done by collecting epithelial tissue samples for laboratory confirmation from infected animals (Longjam *et al.,* 2011b). Routinely, FMD is diagnosed by the combination of several methods which involves the use of different types of enzyme-linked immunosor bent assay (ELISA), virus isolation techniques, and by reverse transcription PCR (RT-PCR) usually in accredited National, Regional and referral laboratories (Longjam *et al.,* 2011b). However, most of these diagnostic methods require the availability of a dedicated laboratory facility, highly trained laboratory personnel, stable reagents, multistep sample handling or preparation, and management of the logistical considerations associated with sample collection, and transportation (Reid *et al.,* 2002; Longjam *et al.,* 2011b).

2.8 Viral Isolation Techniques

Bovine calf thyroid cell culture (BTY) are primary cell culture for cultivation of foot and mouth disease virus (Snowdon., 1966). BTY cells are highly sensitive and permissive to FMDV detection but are problematic to get them and use in FMD endemic countries (Brehm *et al*., 2009). However, it is preferred cell culture for isolation and characterization of foot and mouth disease virus. Other cells used for isolation and cultivation include baby hamister kidney cell lines (BHK-21) and pig kidney cell lines (IBRS-2). IBRS-2 is highly sensitive to porcinophilic strains of FMDV (De Castro, 1964**)**. The cells are inoculated with infected epithelial suspension of FMDV and incubated at 37°C in a shaker for 4 days and with subsequent observation of cytopathic effects (CPE) for FMDV confirmation. Recently, Goat fetal tongue cell lines are known to be most sensitive, rapid and convenient for the isolation and detection of FMDV and alternative tool to BTY (Brehm *et al.,* 2009).

2.9 Serological Methods

2.9.1 Non structural protein enzyme -linked immunosorbent assays - (NSP-ELISA)

Antibody and antigen complexes can be detected by ELISA raised against foot and mouth disease virus. Several methods are available currently and have been commercialized targeting non structural components of FMDV genome (Bronsvoort *et al.,* 2006; Inoue *et al.*, 2006). FMDV genome encodes 12 viral proteins that are controlled by virus, these proteins are antigenic and have been used singly or in combination to develop various serological methods for identifying exposure to FMDV and vaccine challenge (Inoue *et al*., 2006). Animals that are naturally infected by FMDV can be differentiated from vaccinated (DIVA) animals based on detection of antibodies against the recombinant non structural protein 3ABC. In immunized animals usually vaccines are produced free of NSPs, these antibodies are elicited as a consequences of infection (Bruderer *et al.,* 2004). In countries that use vaccination to control FMD outbreaks it is important to differentiate antibodies due to infection from the field and immune response to vaccination. NSP 3ABC antibodies are indicator of infection. Currently used FMD vaccines are structural proteins of the virus only elicit antibodies against structural proteins.

Recently, a number of inhouse and commercial tests are available to identify carrier animals (Brocchi *et al.*, 2006). 3ABC is considered to be highly immunogenic polypeptide and is most appropriate antigen preparation for diagnostic purposes (Robiolo *et al.*, 2006).

2.9.2 Structural proteins (SP-ELISA)

Structural proteins form the basis of capsid structure, SPs have been shown to determine the antigenicity of FMDV and virion attachment to cell receptors. ELISA techniques such as liquid phase blocking ELISA (LPBE) and solid phase competing ELISA (SPCE) are

important diagnostic assays so far available. Sandwich-based assays have been playing an increasingly important role in the identification of FMDV serotype (Ma *et al.,* 2011). Antigen ELISA has been successfully employed to differentiate FMDV serotypes and is the method of choice although its sensitivity has been outweighed by molecular throughput techniques now.

2.10 Molecular Techniques

2.10.1 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase Chain reaction is the modern tool widely used in field of molecular diagnostics and research (Hoffman *et al.,* 2009). Reverse transcriptase polymerase chain reaction is currently employed for detection of diverse pathogens including FMD (Mckillen *et al*., 2011). Amplification of specific nucleic sequences using reverse transcription polymerase chain has been employed for laboratory detection of FMDV (Reid *et al.*, 2003, Reid *et al*., 2009; Wright *et al*., 2011; King *et al.,* 2012).

Sequencing of FMDV genome resulted into discovery of conserved and diverse parts of the genome (Wright *et al*., 2011). Conserved segments as well as diverse segments of the genome are employed for diagnostic purposes. In detection of FMDV initially, was based on amplification of conserved regions of the genome 3D (Meyer *et al*., 1991; Rodrıguez *et al.,* 1994 and 5' untranslated region 5' UTR, Reid *et al.,* 2000) utilised agarose gel electrophoresis for the detection of amplified products. However, these labour intensive procedures have a high risk of generating false positives due to carry-over of chain reaction (PCR) amplicons and are therefore not generally considered ideal for routine testing of large numbers of samples (Hoffman*.,* 2009).

Real-time RT-PCR (rRT-PCR) assays have now largely replaced agarose gel based assay formats. These fluorescence-based approaches are highly sensitive enabling simultaneous amplification and quantification of FMDV specific nucleic acid sequences. In addition to enhanced sensitivity, the benefits of these closed-tube rRT-PCR assays over conventional endpoint detection methods include a reduced risk of cross-contamination, their large dynamic range, an ability to be scaled up for high-throughput applications and the potential for accurate target quantification. Several assays have been developed to detect FMDV that use 5'-nuclease assay (TaqMan®) system to detect PCR amplicons (Callahan *et al.,* 2002; Reid *et al*., 2002; Oem *et al.,* 2005). Real time RT-PCR is gold standard technique currently available for molecular diagnosis of FMD (Callahan *et al.*, 2002; Reid *et al.*, 2009, OIE, 2012).

Currently*,* most of throughput PCR modification are used in centralised laboratories, deployment of rRT-PCR technologies and portable PCR are being developed and validated to suit field settings for the purpose for confirming the disease in shortest possible time (Madi *et al.*, 2012). These test formats may be particularly suitable for use in FMD endemic areas such as countries within sub-Saharan Africa where samples have to transported over long distance where usually confirmation will done before rational disease control strategies are put in place. Demand for point of care devices that provide diagnostic services within few hours has increased recently. Work in this area has explored the use of new hardware platforms to allow PCR testing to be deployed into the field for use by non-specialists (Callahan *et al.,* 2002; Hearps, *et al.,* 2002; King *et al.,* 2008). The devices such as lateral flow devices and portable PCR have been developed to suit that purpose (Madi *et al.,* 2012). The use of automated robotic machines which are simple-to-use and robust template extraction process such that all the steps of the assay

can be performed without user intervention are in demand which in turn reduce human error.

The use of homogeneous systems has previously been recognised an important aspect for the implementation of molecular methods for field detection of FMDV (Hearps *et al.,* 2002). Currently, there is only limited access to these technologies in most of developing countries where as in developed countries they have been part and parcel of every diagnostic utility. In addition to the performance of these equipment and assays, the availability and cost of consumables, as well as mechanisms to locally service the machines (in the event of equipment failure) will be important factors for the routine use of these tests in countries within sub-Saharan Africa.

2.10.2 Reverse transcription-loop mediated isothermal amplification (RT- LAMP)

Reverse-transcription loop-mediated amplification is modification of reverse transcription polymerase chain reaction that utilizes a single amplification temperature. Japanese group chemical company invented this technology for the first time and it appears to highly sensitive and specific when compared to RT-PCR (Notomi *et al.,* 2000; Shao *et al*., 2010). This is an isothermal autocyling strand-displacement DNA synthesis technique which utilises four specific primers to recognise six regions of the target genome (Notomi *et al.,* 2010; Chen *et al*; 2011). RT-lamp has been used successfully in rapid detection of foot and mouth disease virus amplification (Shao *et al*., 2010; Chen *et al*., 2011). Previous studies by Notomi *et al*. (2000) showed RT-Lamp appears to overweigh other throughput PCR techniques of amplification such as nucleic acid sequence based amplification, Conventional PCR and sequence replication amplification. The Formation of loop structures enables explosive polymerase-based enzymatic amplification, which generates

double-stranded, multi-sized amplicons. Pan-serotypic RT-LAMP assays have been designed for FMDV (Dukes, *et al.,* 2006; Li *et al.,* 2009).

Validation data indicates that RT-LAMP has equivalent analytical sensitivity to rRT-PCR and may be less sensitive to inhibition by problematic sample matrices such as Oropharygeal fluid (OP) fluids and faecal samples (Parida *et al*., 2005) Reverse-transcription loop-mediated amplification products are generated in abundance and can be detected using equipment to monitor turbidity, agarose gels or real-time PCR machines (Parida *et al.*, 2006).

Furthermore, RT–LAMP technology enables scientists to visualise dual-labelled LAMP amplicons using novel lateral flow devices (James *et al.,* 2010). The importance of magnesium ions in the RT-LAMP has been minimized instead a dye indicator such as hydroxynaphthol blue has been extensively used in RT -LAMP (Bearinger *et al.,* 2011). The results obtained by Notomi *et al*. (2010) and Yamazaki *et al*. (2013) showed RT-Lamp to be simple, rapid, cost effective, efficient and quicker method that can fully utilized for rapid detection of FMDV genome. The two workers described further RT-lamp as simple, reliable method to prepare template RNA, enabling RT-LAMP to be proven useful technology in field settings.

2.11 Antigen Tests

2.11.1 Lateral flow devices

The development of rapid chromatographic strip test or lateral flow devices were described recently for the pen-side diagnosis of FMD based on monoclonal antibody (mab) which reacted against FMDV of all seven serotypes (Ferris *et al.*, 2009). Previous studies conducted by Grazioli *et al*. (2006) on monoclonal antibodies showed a significant

neutralization of antigenic determinants of SAT1 and SAT 2 of FMDV by monoclonal antibodies. Their study identified three monoclonal antibodies that sufficiently neutralized the antigenic determinants for SAT 2 serotypes. The monoclonal antibodies included 2H6, 3C5 and 4A6. In SAT1 three antigenic sites were also identified (Grazioli *et al.*, 2006). Similarly, Monoclonal antibodies against other serotypes were developed and validated for incorporation into lateral flow devices (Ferris *et al*., 2011)

Monoclonal antibodies are valuable diagnostic tool for the development of antigen and antibody typing (Grazioli *et al.*, 2006). Studies on the performance of monoclonal IF10 embedded on lateral flow showed low sensitivity to SAT 2 serotypes (Ferries *et al.*, 2009). Similar results were documented by other researchers on the low sensitivity of lateral flow devices on SAT serotypes (Nordengrahn *et al*., 2008; Ferris *et al*., 2009). Another development on the incorporation of 2H6 monoclonal antibodies was also validated by Ferris *et al*. (2008) showed to have high sensitivity against all seven serotypes of FMDV. These developments led to introduction of rapid and easy-to-perform test, which would allow for on-site diagnosis in the case of a suspected FMD outbreak in different countries.

The need for the penside diagnosis was given a special focus during European Union for foot and mouth technical meeting (FAO-EUFMD., 2009). Since, then number of lateral flow devices were developed and are currently marketed by different commercial companies. SVANODIP FMDV-Ag test is one of those Antigen tests. SVANODIP FMDV – Ag test have the ability to provide an accurate results within 10 minutes. This ability forms bases for rapid diagnosis and provision of early warnings for risk analysis for FMD (Polychronova *et al.*, 2010).

The introduction of lateral flow devices in detection of FMDV antigens in Tanzania, would prevent problems associated with the transportation of samples to the laboratory and would be especially useful for a faster diagnosis in areas where the disease is endemic (Oem *et al*., 2009). Availability of "pen-side" diagnostic tests would have the advantage of rapid, user friendly, correct diagnosis of FMD in field condition. SVANODIP kit for pen-side diagnosis based on a monoclonal antibodies is sensitive against FMDV of all seven serotypes (Reid *et al.,* 2002). The SVANODIP® FMDV-Ag test is useful for early detection of infection as first line diagnostics for veterinarians in slaughter houses, in farms and in simply equipped regional laboratories, to control the spreading of infections (Belen., 2004).

2.11.2 Sensitivity and Specificity of lateral flow devices

Many lateral flow devices are available in the market, LFD IF10 was used in the laboratory in UK for the diagnosis of FMDV and it showed a high sensitivity comparable to that of antigen ELISA with sensitivity of 84% compared to 85% of conventional antigen ELISA. The specificity of the device during the same study was 99% compared to 99.9% of antigen ELISA (Ferris *et al.,* 2008). In another study the diagnostic sensitivity of the lateral flow device (LFD) for FMDV types O, A, C, and Asia 1 was similar, at approximately 87.3%, to that of 87.7% obtained with antigen enzyme-linked immunosorbent assay (Ag ELISA) but the specificity of the LFD was 98.8%, compared to 100% for the Ag ELISA (Oem *et al.,* 2009).

Validation studies was also carried on a new lateral flow device (LFD) for the detection of foot-and-mouth disease virus (FMDV) of the SAT 2 serotype which was developed using a monoclonal antibody (mab 2H6) for providing rapid and objective support to veterinarians in their clinical judgment of the disease. The validation studies showed the

LFD with mab 2H6 using clinical samples to detect accurately diverse FMDV type SAT 2 strain infections. The LFDs showed a high sensitivity (88%) and specificity (99%) (Ferris *et al.,* 2008).

In a different study conducted in China using the lateral flow devices to validate the performance of the devices using FMDV serotype O, A and Asia 1 showed the sensitivity of 88.3% compared to 89.7% by the indirect sandwich ELISA. For FMDV Asia 1sensitivity was slightly higher (92.1%) compared to 90.5% for the ELISA. The specificity of the LFDs was 97.1% compared to 97.4% for ELISA (Jiang *et al.,* 2011). The results obtained by Jiang are in close agreement with those observed by Chinese fellow workers who obtained similar sensitivity and specificity of lateral flow devises (Chang *et al.,* 2011). Study by Yang *et al*. (2013) recommended the lateral flow devices to have ability to produce rapid results and high specificity rendering it to be valuable tool for early detection of FMDV O, A and Asia 1 in the field .

A pen-side diagnosis provides benefits in FMD emergencies, relevance to FMD control programmes which operate in endemic regions of the world such as Sub Saharan Africa for increasing disease awareness where efforts to control disease may be difficult (Reid *et al.,* 2002). In each circumstance the availability of a pen-side device for diagnosis would reduce the necessity for sending routine diagnostic samples to FMD laboratory and thereby reduce the delay in diagnosis, which can in some areas be considerable.

2.12 Future FMD Diagnostic Prospects

The future of FMD diagnostics relies on the use of the new technologies that are capable of producing rapid results that can be done in the field settings without the need of

centralized laboratories (Sammin *et al*., 2010; King *et al.,* 2012). The new technologies that are being evaluated in different countries currently include the use of immunoassays that includes the use of lateral flow devices and immunochromatograhic assays that can be produce point of decision results without the need for transportation of sample to the laboratory (Sammin *et al*., 2010). The importance of rapid diagnostic assays has been exemplified in the UK 2001 FMD outbreaks where animals were slaughtered within 24 hours without waiting for laboratory results (Anderson, 2002).

Portable tests, field tests, on site tests and penside tests have a potential to produce more rapid diagnostic solutions to the farmers and thereby producing reputable decisions to policy makers to contain the highly contagious disease such as FMD. These tests are being evaluated currently for the sensitivity and specificity and their cost effectiveness for deployment to wider applications (King *et al.,* 2012).

The use of automated portable polymerase chain reaction, improvement on laboratory based RT-PCR and RT-LAMP has been carried out at different times and their performance has been evaluated (Callahan *et al.,* 2002; Dukes *et al.,* 2006; Hole *et al.*, 2010; Notomi *et al.,* 2010; Madi *et al.*, 2012). The detection ability and applicability RT-LAMP for FMDV has been improved and validated by previous workers (Chen *et al*., 2011) and recommended to be simple, rapid and highly sensitive to minute FMDV RNA (Notomi *et al.,* 2010). The discovery of highly sensitive goat tongue epithelium for FMDV isolation is imperative at this juncture (Brehm *et al.*, 2012). The focus of this current study is to validate the use of the lateral flow devices for detection and diagnosis of foot and mouth disease virus in endemic settings to see its applicability in Tanzania where it's known that at least four serotypes are circulating.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Duration

This study focused on FMDV outbreaks reported to DVOs by Livestock Field Officers (LFO), Village Executive Officers (VEOs) and livestock keepers from districts adjacent to wildlife-protected ecosystems in Northern Tanzania, involving Serengeti and Ngorongoro Districts. The study was conducted for duration of 9 months from September 2012 to June 2013 to closely monitor FMD outbreaks for purposes of collecting and testing the epithelial tissues and vesicular fluids at pen side using SVANODIP® FMDV-Ag test containing IF10 monoclonal antibodies.

Serengeti and Ngorongoro districts are within a great Serengeti-Ngorongoro ecosystem. Serengeti National Park is restricted area for wildlife conservation. Figure 2 shows the location of studied villages with reference to Serengeti national park and Ngorongoro Conservation Area Authority (NCAA). The studied villages included Nyamburi, Rwamchanga, Nyichoka , Parknyigot, Mbilikili and Nata (Table 1). These villages are located in western corridor of Serengeti National Park where there is huge contact between livestock and wild animals. Outside the borders of Serengeti national parks there are game controlled areas and wildlife management areas owned by villages close to national park. Livestock keepers are around this wildlife management areas due to availability of abundant pasture. In Ngorongoro district, only one village, Oleparkash was involved. It's located outside the NCAA in the northern part of NCAA.

Oleparkash village constantly receives spillover of wildlife from NCAA. In all studied villages from both districts practiced pastoral and agropastoral livestock production

systems. The livestock keepers in these districts kept Tanzania short horn zebu and few crosses of Boran. This study was conducted in this district due continuous and frequent incursions of foot and mouth disease (District Livestock Reports, 2010). The names of the studied villages and number of epithelial tissues collected from the both districts are stipulated in the Table 1.

Table 1: Summary of villages from which samples were collected and tested by SVANODIP® FMDV-Ag test

District	Villages	No. of samples collected and tested
Serengeti	Nyichoka	4
	Parknyigot	27
	Mbilikili	26
	Bonchungu	3
	Nata	1
	Rwamchanga	9
Ngorongoro	Oleparkash	10
Total		**80**

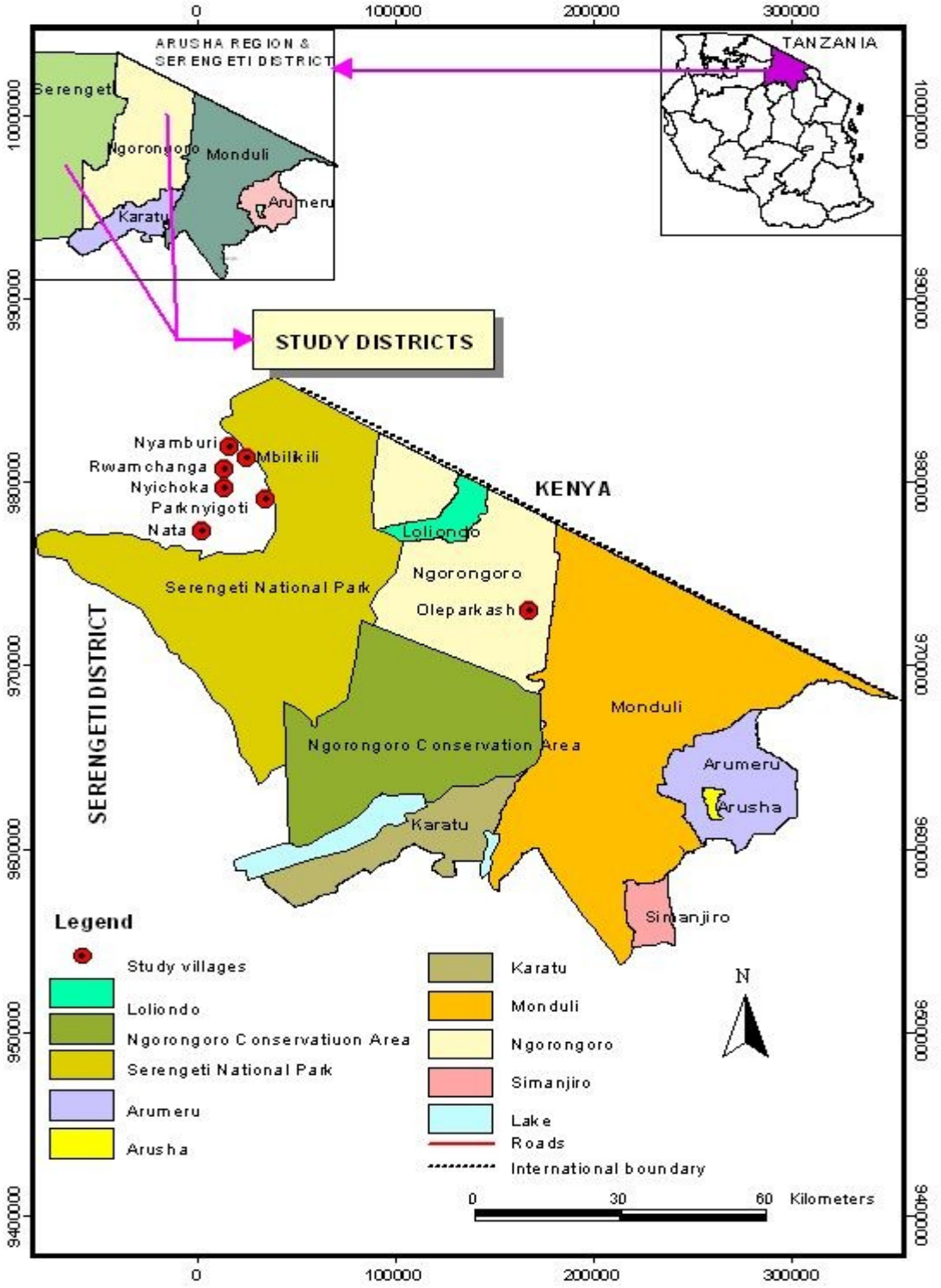


Figure 2: The map of study villages

3.2 Study Design

Cross-sectional research design was used to collect the vesicular fluid and epithelial tissues from FMD suspected cattle**.** FMD suspected outbreaks were reported to district officers and FMD project field team (FMD project - A collaborative FMD project between Sokoine University of Agriculture (Tanzania), University of Glasgow (UK), Pirbright Institute (UK) and Tanzania Veterinary Laboratory Agency (Tanzania) funded by BBSRC) were provided with the information pertaining new outbreaks of the disease. In Tanzanian perspectives where field livestock officers are limited at ward and village levels, FMD cases are usually reported directly to district livestock department by village executive officers. Due to lack of livestock field officers and poor livestock disease reporting system resulted many outbreaks to go unreported.

3.3 Sampling Strategy and Sample Size

Purposive sampling strategy was employed only dependent on occurrence of FMD outbreaks. All cattle from selected villages and from different herds with FMD like suspected clinical signs were restrained and closely examined. Epithelial tissues from all animals (cattle) with mouth and foot lesions were collected and tested at penside using FMDV antigen test. A total of eighty (80) tissues samples were collected and tested during the study. The samples included four (4) vesicular fluids from mouth and foot blisters and remaining seventy six (76) were from foot and mouth epithelial tissues.

3.4 Field Sample Collection

3.4.1 Physical examination

The FMD suspected animals with salivation and lameness are the main signs observed during clinical examination. Cattle with those signs were restrained and closely examined by detailed physical examination to determine presence of any foot and mouth lesions. The presence of FMD like lesions in the examined cattle provided incentive for the

collection of important biodata of the animal including the body temperature, respiration rate, age and the sex.

3.4.2 Lesion sampling

For mouth or foot lesions at least 2 cm^2 of epithelium from unruptured or freshly ruptured vesicles were collected using scissors or scalpel blade and forceps. Lesion samples were placed into cryovials and in liquid nitrogen immediately after collection. For vesicular fluids, fluid was collected with a syringe and needle and placed in a cryovial, and stores in liquid nitrogen immediately after collection.

Aliquots of vesicular fluid or lesion samples were processed according to manufacturer's recommendations for testing on lateral flow test kits. For each lesion tested, photographs were taken of the lesion to assist with assessment of the stage of the lesion, and a photograph taken of the cassette showing the test result. Animal ear-tag number, type of epithelial tissue tested and date were recorded on the lateral-flow cassettes, tested cassettes were stored in a sealed, dry zip-lock bag. A total of fifty three LFDs were kept at room temperature and twenty seven LFDs were kept at -20^0C regardless of their reactions. The LFDs were kept at these temperatures to see the possibility of recovering intact FMDV RNA from nitrocellulose membrane of LFDs. LFDs results obtained during field testing were reported immediately to the herd owner and to the District Veterinary Officer.

3.4.3 Sample storage

Collected epithelial tissue were labelled with eartag number of the animal from which the sample were collected and put in cryovial and stored in cool box until the collection of epithelial samples in a particular herd were completed.

3.4.4 Sample transportation to laboratory

All collected epithelial tissues were kept and transported to laboratory by the use of liquid nitrogen container. Dry shipper were used to transport all the samples to CIDB, Dar es Salaam. All collected samples sent to WRLFMD were transported according to international standards for transportation of dangerous infectious agents and using international air transport association (IATA) regulations.

3.4.5 Sample preparation for lateral flow testing

3.4.5.1 Sample processing

Sample processing and extraction for the lateral flow testing was done as per by Manufacturer's recommendation (Svanova, Boehringer Ingelheim, Uppsala, Sweden).

3.4.5.2 Protocol for processing epithelial tissue and testing

The epithelial tissue of about 0.2 gram were put into bijou bottle made up of sterile sand. The epithelial tissue were cut into small pieces using sterile scissors and about 24 drops of buffer are added. Gently the epithelial tissues were ground and homogenate were formed. The suspension were left for about three minutes. Each cassette were labelled with eartag number, type of epithelium tested and date of testing. The supertanant were collected using pipette and loaded into loading pad of lateral flow cassette, 8 drops are enough to produce the results. The result of lateral flow cassettes are read after 10 minutes. Pink band at test site were considered as positive result. In negative result no band was evident. Positive control is conjugated in the cassette for reference. Both positive and negative results were photographed for future evidence. The tested lateral flow devices were kept at room temperature and some at -20°C without drying for possibility of FMDV RNA recovery in the laboratory.

3.4.5.3 Testing of vesicular fluids

Vesicular fluids were added with 500µl of buffer, mixed stirred thoroughly well and 8 drops of solution were loaded into loading pad and results are obtained within 10 minutes.

3.4.6 LFDs results and their scores

The results of lateral flow were recorded as strong positive, weak positive and negative results. For the purposes of obtaining the scores of the lateral flow devices the results were further categorized into different levels of identification as stipulated here below. A negative result was scored as 0; a very weak reaction is recorded as 1; weak 2; weak to moderate,3; moderate 4; moderate to strong, 5; and strong reaction was recorded as 6. The scores results obtained were plotted against Cycle threshold (Ct) values of LFDs elutions.

Reaction with score 6 is the strong positive reaction in which test band is clearly visible (Figure 3), followed by moderate strong and various weak reactions (Figure 4) and zero scores is considered as negative reaction in which no band is evident in test zone (Figure 5). Cycle threshold (Ct) values of RT-PCR amplification below 32 were considered as positive for RT-PCR of FMDV RNA recovered from the lateral flow devices. Ct values between 32-39 were considered as weak positive for FMDV RNA amplification and 40 was cut-off value. Ct values above 40 were regarded as negative sample for FMDV.

3.4.7 Estimation of the age of the lesion

Ageing of each lesion of epithelial tissue were estimated by using procedure described by FAO in its training booklet published in 2005 (FAO, 2005). During this study the lesions were closely examined and decided whether is a fresh (new), or old lesion. Animals with

blisters (vesicles) and fresh lesion are considered to have a lesion of 2-3days. Usually fresh lesions are ones in which epithelial tissue that have just ruptured and the wound was fresh. An old lesion usually characterized by deposition of fibrinous tissue and healing was evident, were estimated to be more than seven days. Estimation of age of the lesion was important epidemiologically in tracing back when the disease occurred for the first time in a herd. The livestock keepers were interviewed on when they saw the first sick animal in his herd.

3.4.8 Extraction of FMDV RNA from lateral flow cassettes

Elution of FMDV RNA from lateral flow devices was conducted both at the Pirbright institute, UK and at the CIDB, Tanzania. No standard operating procedure is currently available for elution of FMDV RNA from nitrocellulose membrane. This study tries to develop a standard operating procedure for elution of FMDV RNA from lateral flow devices. The following protocol was used in this experiment for the recovery of FMDV RNA from the lateral flow devices**.**

The lateral flow cassettes were split between the two embedding plastic covers to reach the nitrocellulose membrane using sterile gloves, scissors and forceps. Using sterile scissors the nitrocellulose membrane was cut about 1mmx4mm at the band of the positive reaction and also a second cut just above the positive band as of the same size. The cut nitrocellulose membrane were stored in the labelled eppendorf tubes ready for elution.

The nitrocellulose membrane were added with 50µl of nuclease free water containing RNase OUT (recombinant ribonucleic acid inhibitor, invintrogen) made from 1:50 dilution of RNase OUT nuclease free water (20µl RNase out into1000µl sterile nuclease free water) and stirred for a 20 seconds. The supernatant of RNA of 7µl was transferred

into labeled eppendorf tubes. The harvested RNA was used directly as template for RT-PCR after preparing the PCR master mix. The remaining cut nitrocellulose membrane were kept at room temperature for future use.

3.4.9 Extraction of total tissue RNA from epithelial tissues

The initial steps used during the epithelial suspension preparation was to weigh the epithelial tissues using weigh balance. In case of large epithelial tissue, the samples were preserved for future use at -80°C. The pieces of epithelial tissues are ground using mortar and pestle in sterile sand. The suspension were left for a while for particles to settle. Supernatant were collected into cryovials and stored at 2-8°C until required for FMDV RNA extraction.

Qiagen protocol using its QIAamp viral RNA mini spin procedure were adopted in extracting FMDV RNA during this experiment. The biosafety cabinet class three was put on and allowed to work for 30 minutes before extraction. The microcentrifuge tubes were added with 560µlof prepared buffer AVL containing carrier RNA. Followed by addition of 140µl of epithelial suspension, mixed thoroughly from homogenized solution to be formed followed by incubation of 10 minute at room temperature. The solution was centrifuged for 1 minute at 10 000rpm. The above solution was added with 560µl of ethanol (96-100%) and mixed by vortexing for 15 seconds, the solution was centrifuged briefly.

630µl of ethanol 96-100% was added to the solution and was centrifuged at 8000rpm for 1 minute using spinning column fixed on the collection tubes. The collection tubes were discarded and spinning columns were fixed to new collection tubes. The next step was the addition of 500µl of washing buffer AW1 followed by centrifugation at 8000rpm for one

minute. The collection tubes were discarded and spinning column was retained, Another 500µl of washing buffer AW2 was added and centrifuged for 3minutes at 14 000rpm.

Spinning column was retained and collection tubes were discarded. Spinning column was fixed with new collection tubes and added with 60µl of buffer AVE. The solution was equilibrated at room temperature and centrifuged at 8000rpm for 1minute. The spinning column was fixed on eppendorf tubes and centrifuged for 1minute at 8000rpm. The spinning column was discarded and eppendorf tubes with FMDV RNA was collected and stored at -70°C until required for polymerase chain reaction.

3.4.10 RT-PCR

Amplification of FMDV was conducted using 7500 fast Real time RT- PCR Applied biosystems targeting conserved region (3D) of FMDV genome. The master mix were prepared and run as one step RT –PCR.

Table 2: Reaction mixture used for one step RT- PCR reaction for one reaction

Reagents	Volume (µl)
Buffer	12.5µl
Nuclease free water	1µl
Forward primer 0.5µM	2µl
Reverse primer 0.5µM	2µl
Taqman probe 0.2µM	1.5µl
SuperScript III platinum TaqMan enzyme	0.5µl
Rox1:10 prediluted	0.5µl
RNA template	7µl
Total	**27µl**

The forward and reverse primers were designed from highly conserved regions of FMD viral genome as described by Ferris *et al*. (2009).

Table 3: Set of primers and probe used during amplification of 3D region of FMDV genome

Primers	Sequences
Forward primer 3DF	5'-ACTGGGTTTACAAACCTGTGA-3'
Reverse primer 3DR	5'-GCGAGTCCTGCCACGGA-3'
Probe 3Dprobe	6- FAM5'-TCCTTTGCACGCCGTGGGAC-3' TAMRA

3.4.11 RT-PCR set up

The RT-PCR thermocycler was set to run at 45°C for 10 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 45 seconds and amplification was run for 40 cycles for approximately two hours. The samples amplified were FMDV RNA from the lateral flow devices and FMDV RNA from epithelial tissues as well as positive and negative controls. Samples for specificity tests were also included. All the real time RT-PCR reactions were carried on an automated PCR machine and data were generated using accompanying software. Two plates were used during amplification procedure 7µl of each sample RNA and controls were used during this amplification.

3.4.12 Sensitivity and specificity of the LFDs

Aliquots of lesion material were sent to the CIDB (TVLA), Tanzania for confirmatory testing using RT-PCR. Samples were tested in parallel at the Institute for Animal Health, Pirbright (IAH-P) for further validation. Test sensitivity and specificity for the LFD was determined by comparison with rRT-PCR an OIE gold standard technique for FMD confirmation. Specificity studies were carried out using ten epithelial tissues collected from apparently healthy animals with no FMD signs. These ten epithelial samples were subjected to LFDs and RT-PCR tests. Strips from the lateral flow tests were cut from the cassettes and processed for RT-PCR analysis. Crosscut values were compared for (a) lesions at different clinical stages of disease; (b) different types of lesion material

(vesicular fluid, epithelial tissue) and (c) cassettes stored under differing conditions. Antigen ELISA was done for few samples to identify the serotypes circulating in the study area to inform the policy makers.

3.5 Antigen ELISA for Serotyping protocol

Serotyping of few selected samples were done in WRLFMD, Pirbright using the Ag ELISA. Procedure adopted in the serotyping was the same procedure continuously used as standard operating procedure (SOP) by WRLFMD. The protocol was described previously (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988; Anon, 2008). A of total sixteen epithelial tissue samples were serotyped at WRLFMD, Pirbright and the remaining samples will be serotyped by on going FMD project in Tanzania. Ag ELISA is an indirect sandwich test in which different rows in multiwell plates are coated with 50µl rabbit antisera to each of the seven serotypes of FMDV. These are the capture sera. Test sample suspensions 50µl were added to each of the rows, and 12.5µl test controls are also included. 50µl guinea pig antisera to each of the serotypes of FMDVwere added, followed by 6.3µgl rabbit anti-guinea pig serum conjugated to an enzyme. Plates are washed thoroughly in each stage to remove unbound reagents. A colour reaction on the addition 50 µl of enzyme substrate and chromogen indicates positive reactions and reactions are stoped by addition of 50µl stop solution and results can be visualized by naked eyes which were also read at 492nm. Test samples were also confirmed by viral isolation technique in which cytopathic effects were apparent in cell culture. All samples were also confirmed by the current OIE gold standard technique for molecular diagnosis of FMD RT-PCR. Serotyping results are shown in Table 9.

3.6 Data Analysis

The scores of lateral flow device (SVANODIP) both positive and negative results were recorded and results compared by results of conventional RT-PCR. Epinfo version 7.0.8.0 (CDC) software was employed for sensitivity and specificity results. Chi-squared test was used to compare effects of age and type of epithelium on the reactivity of LFDs. The effect of temperature on the recovery of FMDV RNA were compared using Chi-square. Scatter plots were made to explore possible correlation of cycle threshold values.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical Presentation of Examined Cattle

Animals from which epithelial tissues were collected had variable FMD clinical signs. Clinical signs observed included salivation and lameness. Epithelial tissues were collected from all age groups and from both sexes. Examined animals had mouth and foot lesions on epithelial tissues of foot, dental pad, tongue and mouth mucosal surfaces. Figure 3, 4 and 5 show some of FMD lesions observed during sampling.

Figure 3: Eroded dental pad epithelium caused by FMD in cattle

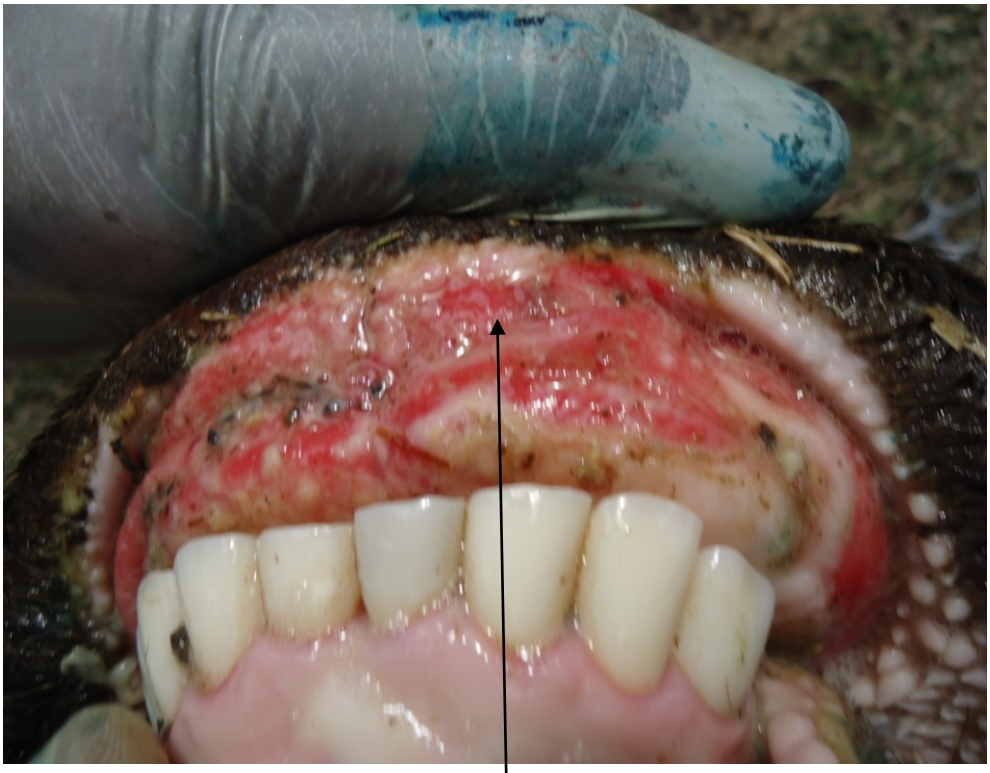


Figure 4: Extensive erosion of dental pad epithelium caused by FMD in cattle

Figure 5: Foot interdigital space of cattle with FMD lesion

4.2 Lateral flow device results

Tested lateral flow devices generated16.2% (13 out of 80) strong positive results, 81.2% (65 out of 80 cassettes) weak positive results and negative results of 2.5% (2 out of 80). Therefore the overall sensitivity of LFDs in detecting FMDV was 97.5% (78 out of 80). All the collected samples (n=80) used in validating the sensitivity of LFDs were positive to RT-PCR. Figure 6, 7 and 8 below shows some LFDs results.

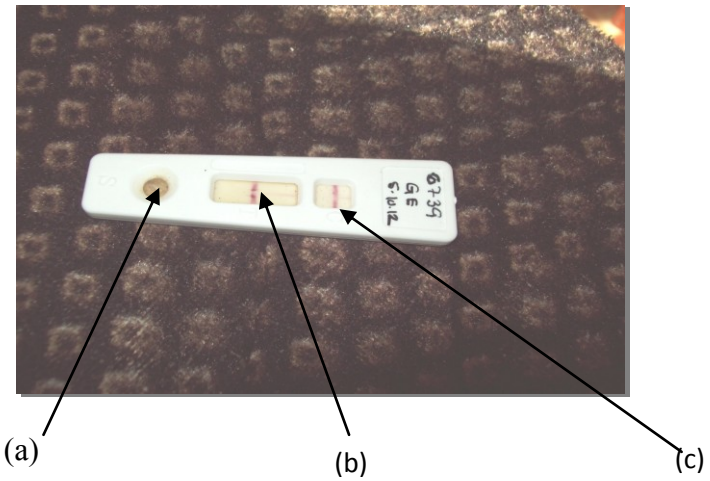


Figure 6: Strong positive result of LFDs

Note: (a) = Sample window
(b) = Test sample window showing strong positive reaction
(c) = Positive control window

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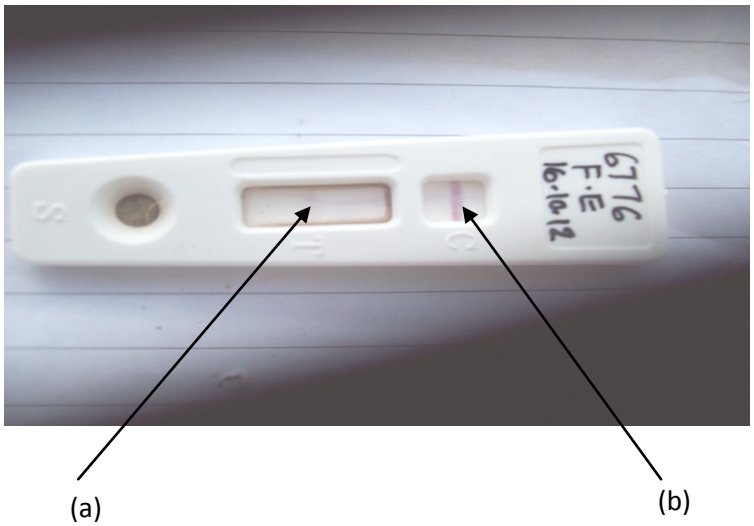

Figure 7: Weak positive LFDs result

Note: (a) = Weak pink band apparent, weak positive results
(b) = Positive control band

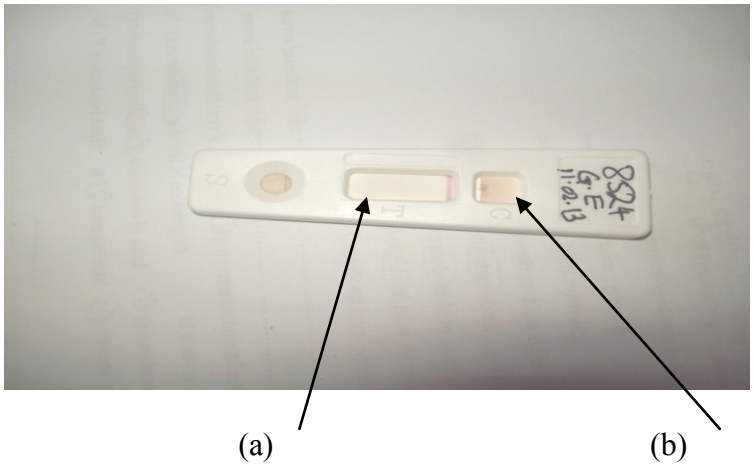

Figure 8: Negative LFDs result

Note: (a) = No pink band was apparent in test window
(b) = No band apparent in positive control window

The LFDs results were classified into different scales of scores 0, 1, 2,3,4,5 and 6. The strong positive was recorded as 6 and negative results were recorded as 0. Figure 9 below shows results of different LFDs results recorded as scores plotted against the Ct values of eluted LFDs and Ct values of same epithelial tissues preserved in liquid nitrogen container. Figure 6 narratively show the Ct values of the eluted cassettes in relation to the scores of LFDs in the field settings in comparison with laboratory RT-PCR amplification results. Lowest Ct were observed in the LFDs with score 6 which provided strong positive LFDs results due higher viral loads in the tested samples. In eluted LFDs Ct value 40 were taken as no Ct values which means a negative result.

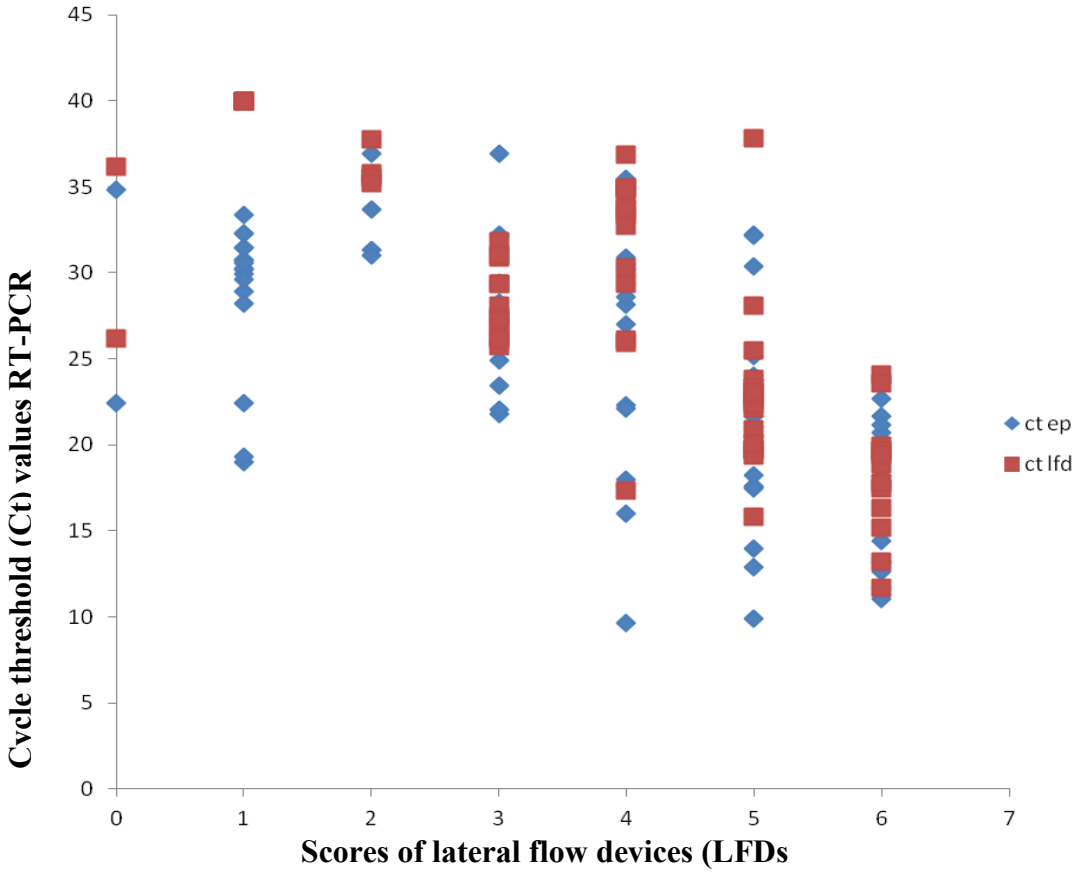

Figure 9: LFDs results plotted as scores with reference to eluted LFDs Ct and Ct of the same epithelial tissues

The scores were plotted using different Ct values obtained from lateral flow devices and those from amplification of the same epithelial tissues. Score 6 represents a lateral flow cassettes with strong positive results whereas weak positive results were presented with score 5-1 depending on the intensity of positive results of test band. Two cassettes provided negative result and were presented as 0 score. RT-PCR of the epithelial tissues showed strong Ct values and two negative results of LFDs were also positive to RT-PCR.

Table 4: A Comparative sensitivity of Lateral flow results with those of rRT-PCR epithelial results

LFDs results				rRT-PCR epithelial results			
No. Tested	Positive	Negative	Sensitivity %	No. Tested	Positive	Negative	Sensitivity %
80	78	2	97.5	80	80	0	100
Total	78	2	97.5	80	80	0	100

Table 5: A Comparative specificity of LFDS with those of rRT-PCR epithelial tissues

LFDs results				rRT-PCR epithelial results			
No. Tested	Positive	Negative	Specificity %	No. Tested	Positive	Negative	Specificity %
10	0	10	100	10	0	10	100
10	0	10	100	10	0	10	100

Specificity was tested using samples collected from health animals and proved to be useful because no sample was positive to both tests.

4.2 Results of LFDs Based on Type of Epithelial Tissue Tested

The influence of type of epithelial tested on the results of lateral flow devices were assessed by testing different tissues at different occasions.

Table 6: The summary of the results of LFDs by different epithelial tissues

Type of Epithelial tissue tested	Number tested n(n)	LFDs results n (%)		
		Strong positive	Weak positive	Negative
Vesicular fluid	4(80)	4(100)	0(0)	0(0)
Gum epithelium	21(80)	6(28.5)	13(61.9)	2(9.5)
Foot epithelium	43(80)	4(9.3)	39(90.6)	0(0)
Tongue epithelium	9(80)	0(0)	9(100)	0(0)
Mouth epithelium	3(80)	0(0)	3(100)	0(0)

There is no significant statistical difference caused by type of epithelial tissues on the reactivity of lateral flow devices when positive and negative reactions were compared by Chi-squared test (P>0.05).

4.3 Age of Lesions

To determine the effect of the age of the lesion on the reactivity of lateral flow devices, samples of different ages of lesions were used. The ageing of FMD lesion was based on the FAO training manual for ageing FMD lesions (FAO, 2005). The criteria used in deciding the age of the lesion is freshness of lesion and presence of blisters, healing and apparently dry lesions are considered as old and are estimates. The Table 7 below shows different estimated ages of lesions and their results of LFDs test.

Table 7: The effect of age on the reactivity of lateral flow devices

Type of tissue tested	Age of lesion	Total no of sample tested	LFDs results n(%)		
	Days		Strong positive	Weak positive	Negative
Vesicular fluids	2-3days	4	4(100)	0 (0)	0(0)
Foot epithelium	2-3 days	2	0 (0)	2 (100)	0 (0)
Gum epithelium	2-3days	3	2 (66.6)	0 (0)	1(33.3)
Tongue epithelium	2-3 days	1	0 (0)	1 (100)	0 (0)
lesions with	**4-9days**				
Foot epithelium	4-9 days	13	1(7.6)	12(92.3)	0(0)
Gum epithelium	4-9days	18	4(22.2)	8(72.2)	1(5.5)
Tongue epithelium	4-9 days	6	0(0)	6(100)	0(0)
Mouth epithelium	4-9 days	1	0(0)	1(100)	0(0)
Lesions over	**> 10 days**				
Foot epithelium	10days	30	2(6.6)	24(93.3)	0(0)
Gum epithelium	10days	2	0(0)	2(100)	0(0)

Chi-squared test was used to compare the positive and negative results at different age groups of the lesions. The lesions with 2-3 days were compared with lesions over 5 days. The Chi-squared tests revealed insignificant statistical difference ($P>0.05$).

4.4 Recovery of FMDV RNA from LFDs Kept at Prolonged Periods of Months

All the tested cassettes were subjected into different storage periods of time and at different temperature storage conditions, 53 cassettes were kept at room temperature and the remaining 27 out 80 were kept at -20°C to explore possibility of recovery of FMDV RNA for up to 6-8 months. Cassettes at room temperature were kept for 8 months and the results showed that, 49 out of 53 (92.45%) gave Ct values implying that FMDV RNA 3D region was intact and those kept at -20ºC, 14 out of 27 (51.85%) FMDV RNA was recovered. The effect of time of storage on the availability of FMDV on the nitrocellulose membrane was investigated using Chi-squared test and there was no evidence of significant statistical differences between the two storage time schedule on FMDV RNA recovery (P>0.05). Figure 10: Shows storage time with respect to availability of FMDV RNA recovered.

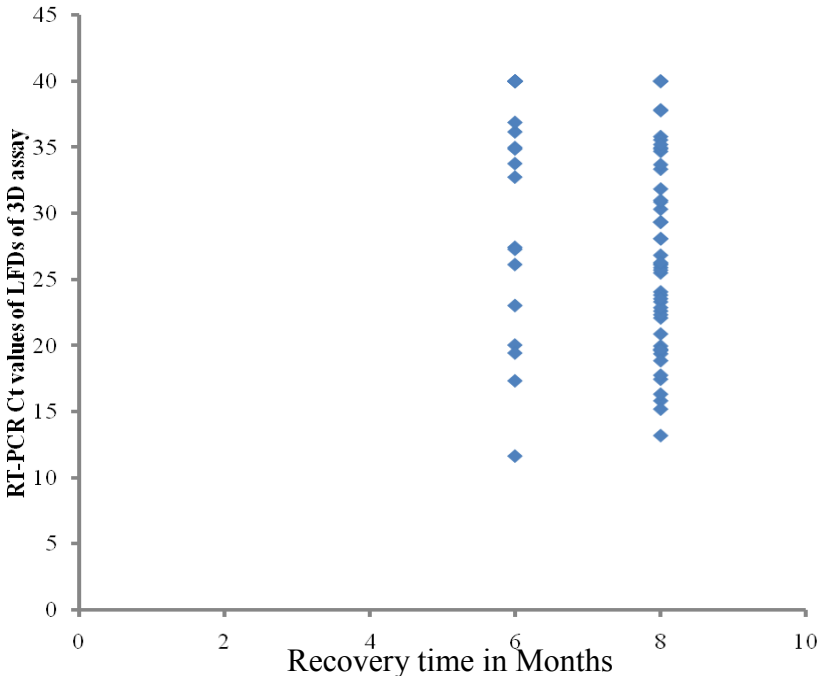

Figure 10: Graphical presentation of the effect of time on recovery of FMDV RNA at different times (months)

4.5 Results of different storage conditions of temperature on the recovery of FMDV RNA

To investigate the effect of different temperature storage condition of LFDs cassettes on the recovery of FMDVRNA. The LFDs cassettes were stored in two different temperature conditions, at room temperature 53 cassettes (25°C) and 27 cassettes were kept at (-20°C). The elutions were done to cassettes of both storage condition and Ct values obtained were plotted against storage temperature. Figure 11 provides detailed results of effect of temperature on FMDV RNA recovery. Ct value of 40 were regarded as cut-off point.

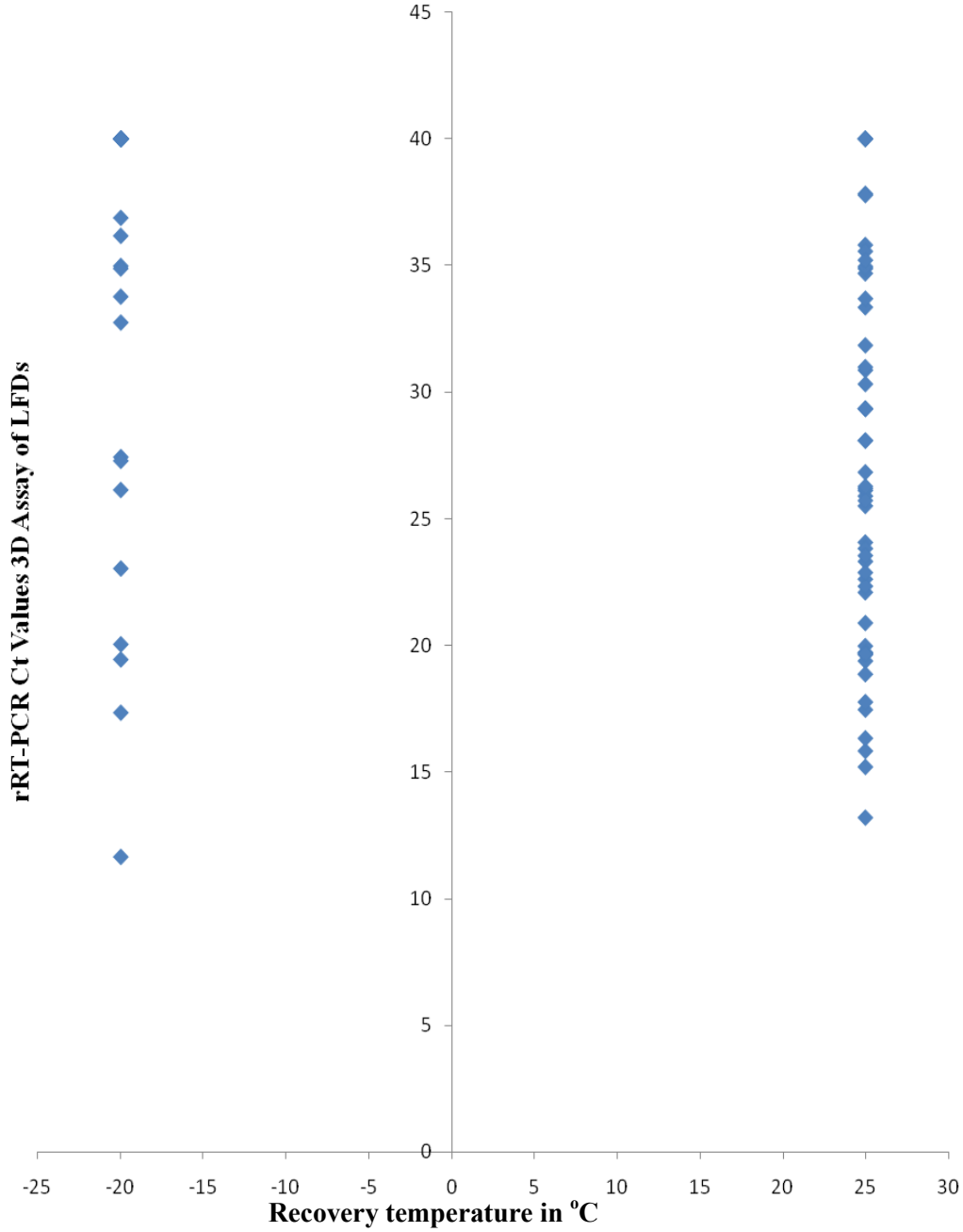

Figure 11: **Graphical presentation of the effect of different temperature on recovery of FMDV RNA**

Table 8: Comparative results showing ct values from amplification of epithelial tissues and FMDV RNA from LFDs eluted

Range of Ct values epithelial tissues	Frequency	Average Ct value	Range of Ct values LFDs	Frequency	Average Ct value
9-15	10	12.27	9-15	4	13.95
16-20	12	18.08	16-20	14	18.93
21-28	26	23.34	21-28	20	25
29-32	24	30.97	29-32	10	30.60
33-40	8	35.03	33-40	32	37.82

The Ct values from epithelial tissues appear to be stronger (lower Ct values meaning have higher viral load) than those obtained from LFDs elutions. The scatter plot was made to compare the Ct values of LFDs elution and those obtained by amplification of the same duplicate epithelial tissues to obtain a line of best fit. Only few ct values were plotted in the scatter plot and it looked there were close correlation between the two procedure. Figure 12 show line of best fit for the Ct values of elution cassettes and those from duplicate epithelial tissues.

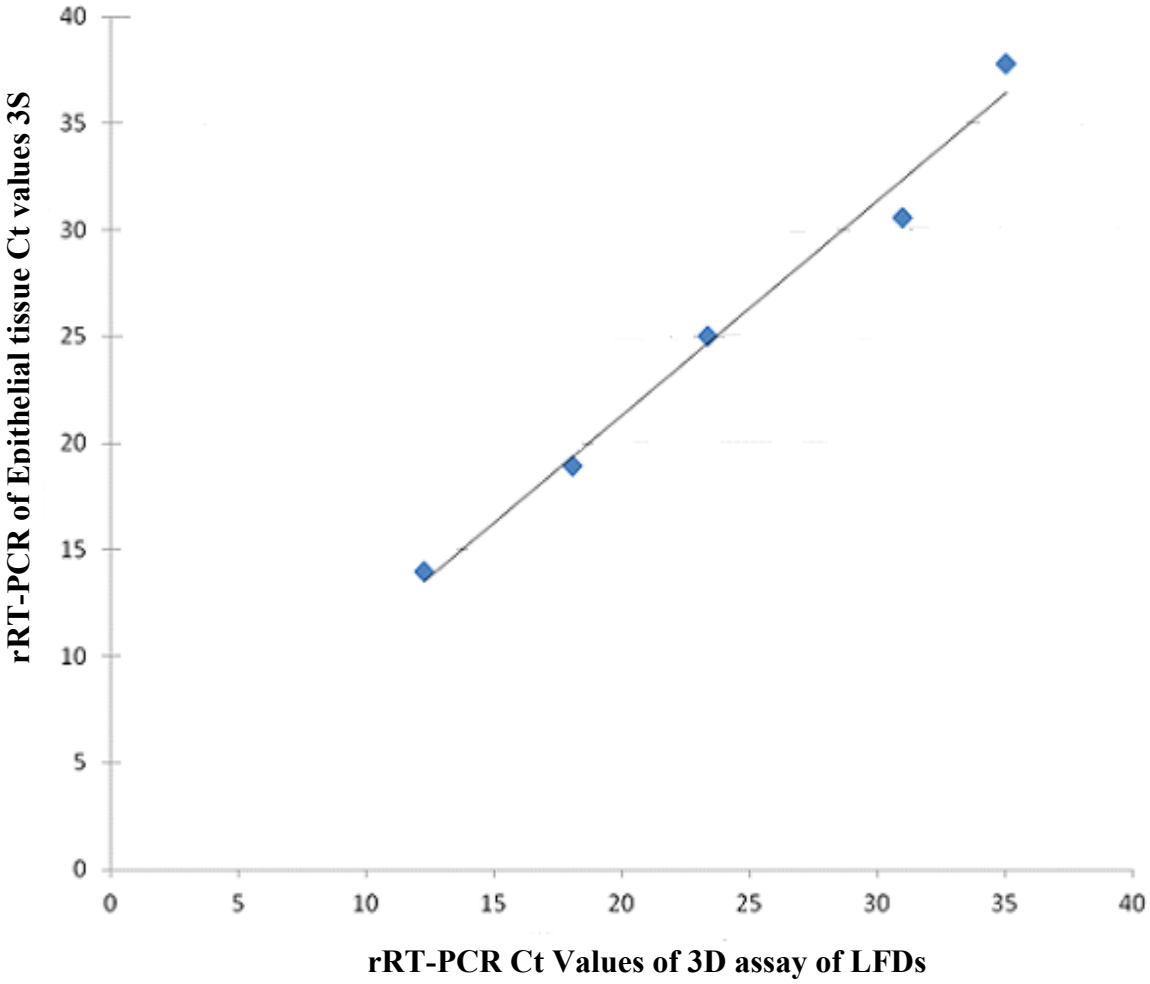

Figure 12: A comparisons of few selected Ct values obtained from the lateral flow devices and those obtained from epithelial suspension of the same epithelial tissues using scatter plot

Table 9: **Serotyping results: The results revealed the presence of serotype A and O in the studied village and these serotypes were associated with the current outbreaks of FMD**

District	Village	Eartag No.	Serotype
Serengeti	Nyichoka	7053	A
	Nyichoka	8426	A
	Parknyigot	8492	A
	Parknyigot	8491	A
	Parknyigot	6737	A
	Parknyigot	6740	A
	Parknyigot	8485	A
	Parknyigot	8489	A
	Parknyigot	6772	A
	Parknyigot	8493	A
	Parknyigot	8494	A
	Parknyigot	8495	A
	Parknyigot	8496	A
	Parknyigot	8498	A
	Parknyigot	6703	O
	Mbilikili	7887	A

The sixteen samples serotyped showed the existence of two serotypes circulating in the studied villages. The currently circulating serotypes were A and O. Serotype A and O were circulating in Parknyigot. Nyichoka and Mbilikili villages had serotype A. Samples from the remaining villages and serotypes circulating will be serotyped by FMD Project currently present in Tanzania. Figure 13 shows the distribution of serotypes among the studied villages.

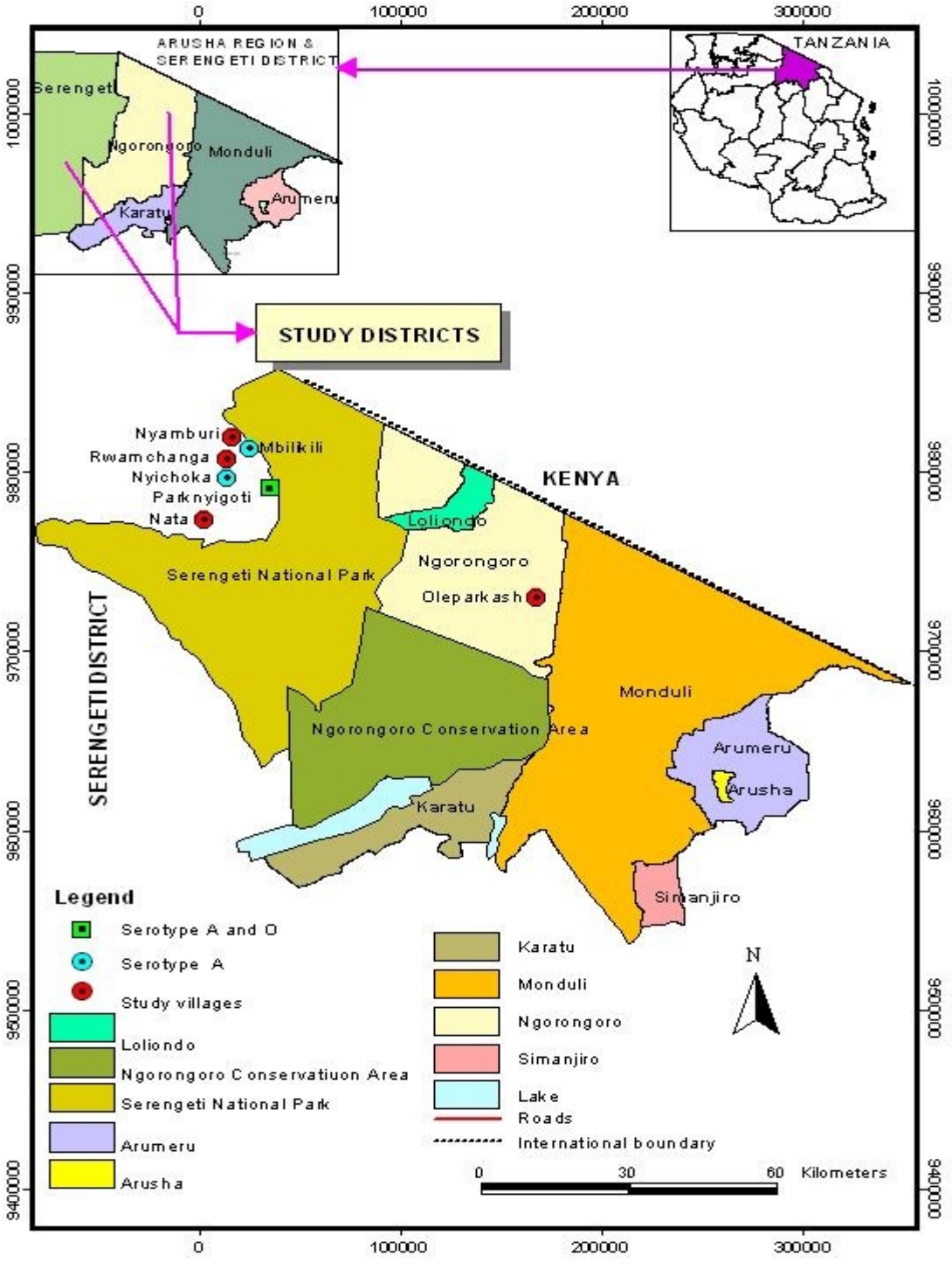

Figure 13: Spatial distribution of FMD serotypes in study villages

CHAPTER FIVE

5.0 DISCUSSION

FMD have resulted in tremendous economic losses. Thus, the development of a rapid and easy to perform test for FMD detection is important for immediate reporting, imposing quarantine and controlling a FMD outbreaks and finally containing its spread. Development of new robust diagnostic technologies that suit field settings and at the same time with high sensitivity and specificity with ability to provide point of care results has been slow for past years both in developed and developing countries. Rapid detection and diagnosis of highly contagious diseases like FMD is important in minimizing losses and provide solution for rapid containment of the disease. The through put technologies such as reverse transcription polymerase chain reaction have revolutionized the diagnostic field in recent years but are expensive and there is little possibility of deployment into wider application into field settings for diagnosis rather than laboratory use (King *et al.,* 2012). The necessity of transporting the samples to national laboratories remains inevitable for confirmation which usually takes protracted time delaying rational disease control strategies.

The use of lateral flow devices in detection and diagnosis of foot and mouth disease virus in field settings will improve rapid diagnosis of FMD in Tanzania. The performance of lateral flow devices in detection and diagnosis was evaluated by previous researchers (Ferris *et al.*, 2001; Ferris *et al.*, 2009) using clinical samples submitted to WRLFMD. The lateral flow device during the current study were used in penside detection and diagnosis of foot and mouth disease virus field outbreaks in Tanzania.

In the current study seventy six epithelial tissues and four vesicular fluids, total eighty samples (Table 1) collected from FMD suspected presenting cattle from various villages in Serengeti and Ngorongoro districts were tested using lateral flow devices. The results of this study showed that lateral flow devices effectively diagnosed 78 samples out of 80 samples to have FMDV antigens. Only two samples tested negative to lateral flow devices. These results enabled the lateral flow devices to have sensitivity of 97.5%. To confirm the results obtained from the lateral flow devices, RT-PCR was employed using duplicate epithelial tissues, RT-PCR showed 100% sensitivity (Table 4). The two negative LFDs cassettes results were also diagnosed by RT-PCR to be positive for FMDV genome. The two negative samples, one of two cassettes appeared normal but when tested, pink positive band could not appear in test window and there was no positive control band, therefore it was difficult to conclude the result of the test. The remaining negative sample could not produce a pink band on the test zone however positive control was evident. The two negative results were also positive to RT-PCR amplification for FMDV indicating that the FMDV genome was detected in those negative cassettes.

The sensitivity of LFDs findings presented during the current study are in contrary with results obtained by (Ferris *et al.*, 2009; Jiang *et al*., 2011; Chang *et al.,* 2011 and Yang *et al*., 2013) whose results had shown the sensitivity of the lateral flow devices to be between 87.3% to 92.1% using different FMDV serotypes. The specificity of the lateral flow devices in this study was 100% (Table 5) which is in close agreement with previous studies (Ferris *et al.*, 2009; Oem *et al.*, 2009; and Jiang *et al.*, 2011) that showed a specificity of 99%, 98.8% and 97.1% respectively. The current study used small sample size for specificity studies due to lack of samples of other vesicular like diseases in Tanzania that can be included in this study.

During the present study, various epithelial tissues were subjected into the lateral flow devices in order to assessing the contribution of different sources of epithelial tissues (from mouth, foot and vesicular fluids) in the reactivity of the lateral flow devices (Table 6). The type of epithelial tissues had a little contribution on the performance "variation" of the lateral flow devices results. The scatter plot (Figure 12) provide an negative slope of trend line indicating there was no correlation between the type of epithelial tissue tested to the performance of lateral flow devices. The comparisons of cycle threshold values (Ct values) of the amplification of lesions with different epithelial tissues could not reveal a significant statistical differences ($P>0.05$).

In this study the influence of age of lesion to the results of lateral flow tests was demonstrated by the results of more recent lesions. The age of lesion had a profound effect on the performance of the lateral flow devices and was stipulated by the performance of all vesicular fluids (n=4) which were estimated to be of 2-3 days, all had strong positive reactions to lateral flow devices (Table 7 and Appendix 1). The possible explanation to this is that most recent lesions including vesicular fluids contained high concentration of FMDV. The results of vesicular fluids and most recent lesions of 2-3 days would need much bigger sample size to generalize these results. This study proposes studies using vesicular fluids and other recent lesions with much bigger samples sizes for validation. Similarly, old lesions provided weak reactions (Table 7 and Appendix1) due to low concentration of the FMDV in the collected tissues as most of FMDV were shed into the environment. This finding is in agreement with the previous studies made by Ferris *et al*. (2009). All the foot lesions with over 10 days (Table 7 and Appendix 1) of age had weak reactions to the lateral flow devices probably because most of FMDV were shed into the soil during movement. However, when tested using chi-squared test there was no significant statistical difference caused by the effect of age on the lesions

(P>0.05). This may be because of small samples size of strong positive results from the recent lesions compared to old lesions. The previous studies could not reveal the effect of age of the lesion on the sensitivity of lateral flow devices (Ferris *et al.,* 2009). However, the scatter plot (Figure 12) showed strong positive correlations signifying the effect of age on the reactivity of the lateral flow devices which was obtained by using average Ct values of the age groups.

The cycle threshold values (Ct values) of RT-PCR of LFDs cassettes amplification of recent lesions, most of them showed good Ct values below 20 and those lesions older than 10 days most of them showed weak Ct values (Figure 9 and Appendix 1) and are above 30 Ct values which could be contributed by the fact that recent lesions have higher concentrations of virus than those old lesions.

The current study was able to recover FMDV RNA from the cassettes kept at room temperature and those kept at -20°C (Figure 11, Appendix 3 and 4). The recovery of FMDV RNA from elution of nitrocellulose membranes embedded in LFDs was possible. Therefore it's feasible scientifically to recover the FMDV viral RNA from the lateral flow devices. The eluted FMDV RNA from the lateral flow devices were amplified together with epithelial tissues in one plate using the same master mix. The Ct values from the eluted RNA were quite comparable to Ct values obtained from those from the same epithelial tissues (Figure 11 and Appendix 1). These results, obtained from the current study are in agreement with other study conducted by Bankowski *et al.* (2012), as their results revealed that it was possible to recover FMDV RNA from lateral flow devices for up to 3 months. The current study was able to recover the FMDV RNA up to 8 months for the cassettes kept in room temperature and to those cassettes kept at -20°C consistently gave Ct values up to 6 months (Figure 10).

The lateral flow cassettes from the both storage conditions gave Ct values below 20 for some LFDs cassettes which is good indication (Appendix 3 and 4). This study confidently reveals that the lateral flow cassettes can be effectively kept in room temperature at much longer periods over eight months. This finding is in agreement with another study which indicated that long term storage of dry LFD does not affect the RNA preserved on the nitrocellulose membrane (Bankowski *et al.*, 2012). Therefore from this study we can conclude that lateral flow devices can be used for storage, transportation and recovery of FMDV RNA from nitrocellulose membrane in longer periods.

The RT-PCR thermocycler was unable to amplify 7.5% (n=4) from the cassettes that were kept at room temperature and 51.8% (n=14) (Appendix 3 and 4) of those kept at -20°C could not be amplified and this may be contributed by loss of FMDV RNA during transportation, storage and effect of humidity. The cassettes were not dried just after testing. The humidity is known to degrade the preserved RNA from the nitrocellulose membrane (Bankowski *et al*., 2012). The effect of humidity may have caused most of cassettes from the freezer (-20°C) to provide negative amplification. The effect of humidity on the recovery of FMDV RNA from lateral flow devices needs further study.

RT-PCR Amplification of FMDV RNA using the both approaches provided this work with useful information in the detection of foot and mouth disease virus. The RT-PCR thermocycler was able amplify all the epithelial tissues subjected into the thermocycler (n=80) which is 100% sensitivity. FMDV RNA eluted from the lateral flow devices in the other hand gave a positive amplification of 78.7% (n=80) of all samples eluted from the lateral flow devices and samples with no Ct values accounted for 21.2% signifying that their Ct values are above 40 which indicates that they are either negative or provided much weak Ct values. The result obtained through elution of LFDs provides data that

testing using epithelial tissues could remain OIE gold standard RT-PCR for the confirmation of foot and mouth disease virus.

The amplification results from epithelial tissues as well as eluted RNA from the lateral flow devices gave a strong Ct values which are closely comparable (Figure 9 and Appendix 1). The eluted RNA from LFDs were able to provide strongest Ct value of 11.66 and samples from epithelial suspension gave a strongest value of 9.66. However, in general, the Ct values obtained from epithelial suspension are better (strong) than those obtained from the elution of LFDs. The above statement does not rule out the use of the lateral flow devices for diagnostic purposes. The LFDs when used according to the manufacturer's recommendations have a potential to provide a reliable diagnostic sensitivity that is comparable to those obtained from the epithelial suspension using RT-PCR.

The scatter plot (Figure 12) showed a strong correlation between the Ct values with positive slope with correlation coefficient (r^2=0.90) which showed a highest relationship between the two Ct values almost approaching to R^2=1 for a linear relationship and this provides evidence showed relatedness between the two approaches of detecting FMDV genome.

Serotype A and O appear to cocirculate in the studied villages (Table 9). The villages from which lesions samples were collected and tested are in close vicinity (Figure 13) which implies the animals were grazed in the same communal land and they also share the same watering points.

This finding is in agreement with previous studies which showed that FMD in endemic countries, livestock movement is the main source of transmission between animals (Vosloo *et al.,* 2002; Kivaria *et al.*, 2003; Rweyemamu *et al.,* 2008., Swai *et al.,* 2009.,Kasanga *et al.*, 2014). The study on serotype that is circulating in buffaloes currently in the Serengeti national park and Ngorongoro could not be identified to correlate with the serotype circulating in the domestic animals. However, buffaloes are known to carry SAT serotype in previous serosurveillance studies conducted by Fyumagwa and his colleagues in 2012. The current studies on the serotyping could not reveal a presence of SAT serotypes in domestic animals, implying that it could be possible that despite domestic animals sharing close contacts with the buffalo and other wild animals there was no transmission between the wild animals and domestic animals.

This finding is consistent with previous studies that documented a transmission from buffaloes to domestic animal to be erratic (Vosloo *et al*., 1996). Another reason could be that the serotype A could be circulating in the domestic animals alone. Previous study showed a limited occurrence of Serotype A in Tanzania (Kasanga *et al.*, 2012). Study conducted by Kasanga *et al*. (2012) reported the occurrence of serotype A to be exclusively in Eastern coastal zone of Tanzania. This study further reports the presence of serotype A in villages around Serengeti ecosystem. The presence of serotype A in the northern part of Tanzania and northern lake zone is in agreement with previous studies (Rweyemamu and Loretu, 1972). Studies by Swai *et al.* in 2009 could not reveal the existence of serotype A in Tanzania for up to 38 years from 1971-2009. Coexistence of serotype A in the northern part of Tanzania was said to be contributed by uncontrolled livestock movement from neighbouring countries (Kasanga *et al*., 2014). The presence of serotype O around Lake Zone and in the northern highland of Tanzania was reported previously (Kasanga *et al.,* 2012). The existence of serotype O in study area is consistent

with other studies (Kasanga *et al*., 2012) that reported the presence of this serotype in northern highlands and around lake zones of Tanzania.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The results of the present study suggest that lateral flow devices can be effectively used in low resource funding countries such as Tanzania as rapid diagnostic test in detection and diagnosis of foot and mouth disease virus field outbreaks. However, the study proposes the use of lateral flow device to go parallel with RT-PCR OIE gold standard technique for FMDV outbreak confirmation.

In this study it's also evident that the recent lesions provided stronger positive reactions as compared to the old lesions which gave weak reactions.

The LFDs can be adopted and used for transportation and storage of FMDV positive material from field settings to the National laboratories for disease confirmation using OIE gold standard technique (RT-PCR).

The current study revealed that serotypes A and O are cocirculating in the studied villages. The potential limitation of this study is that commercially available lateral flow devices today are panserotypic to all the seven serotypes of FMDV but unable to reveal the type of serotype. This study recommends the development serotyping lateral flow devices with monoclonal antibodies that can provide instant serotyping results in field settings especially at district level so that serotype responsible for the FMDV outbreak can be confirmed without need of transporting the sample to laboratories for serotyping. Such developments will quicken rational disease prevention and control. This is because

the current lateral flow devices can give a positive reaction to FMDV antigen without telling type of serotype you are dealing with.

6.2 Recommendations

i. This study recommends adoption of lateral flow devices for use by ZVCs and TVLA Zonal Centres in the field for rapid diagnosis of the FMDV outbreaks situations.

ii. The use of LFDs should be considered for use as a diagnostic tool for FMDV field outbreaks as long as are cost effective and their meet OIE standards for inclusion as rapid diagnostic tests for FMDV outbreaks in Tanzania.

iii. Further study is recommended on the possibility of using RNA template obtained from the lateral flow devices as source of VPI sequences and phylogenetic studies. Much more studies are also recommended to correlate elution temperatures of LFD membranes at variable time and conditions to further validate the current findings.

iv. Further studies are required to validate and deploy the LFDs for a wider application for FMDV outbreak investigations in cattle and other species in Tanzania.

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APPENDICES

Appendix 1: Results of LFDs in relation to the age of the lesion and Ct Values of RT-PCR from epithelial tissues and those from LFDs elutions.

Village	Date of collection	Eartag No	Type of Tissue tested	Age of lesion	LFD results	RT-PCR epithelial tissues	RT-PCR of LFD RNA
Nyichoka	9/14/2012	7053	Gum Epithelium	10-12days	Weak positive	12.9	15.84
Nyichoka	9/15/2012	8426	Vesicular fluid	2days	Strong positive	11.2	13.21
Nyichoka	9/15/2012	8427	Gum Epithelium	3-4days	Weak positive	22.28	29.34
Parknyigot	10/3/2012	8492	Gum Epithelium	2-3dys	Strong positive	12.61	15.21
Parknyigot	10/3/2013	8491	Foot Epithelium	12-16days	Strong positive	20.69	17.47
Parknyigot	10/5/2012	6736	Tongue Epithelium	7-10days	Weak positive	32.22	37.83
Parknyigot	10/5/2012	6737	Foot Epithelium	8-10days	Weak positive	9.66	25.9
Parknyigot	10/5/2012	6738	Gum Epithelium	5-7days	Weak positive	16.03	30.32
Parknyigot	10/5/2012	6739	Gum Epithelium	3-4days	Strong positive	17.60	19.98
Parknyigot	10/5/2012	6740	Gum Epithelium	5-7days	Weak positive	13.98	28.09
Parknyigot	10/5/2012	6741	Gum Epithelium	10-12days	Weak positive	30.77	No ct
Parknyigot	10/5/2012	8485	Gum Epithelium	8-10days	Strong positive	17.81	24.07
Parknyigot	10/5/2012	8489	Gum Epithelium	2-3days	Strong positive	13.18	19.64
Parknyigot	10/16/2012	6772	Foot Epithelium	10+days	Weak positive	18.00	33.35
Parknyigot	10/16/2012	6773	Foot Epithelium	18days	Weak positive	30.18	34.69
Parknyigot	10/16/2012	6774	Foot Epithelium	14days	Weak positive	36.91	26.84
Parknyigot	10/16/2012	6775	Foot Epithelium	12-14days	Weak positive	30.18	No ct
Parknyigot	10/16/2012	6776	Foot Epithelium	8-10days	Weak positive	25.19	19.71
Parknyigot	10/3/2012	8493	Vesicular fluid	2-3days	Weak positive	9.93	19.73
Parknyigot	10/3/2012	8494	Gum Epithelium	7-10days	Strong positive	14.40	16.34
Parknyigot	10/4/2012	8495	Tongue Epithelium	8-10days	Weak positive	17.90	19.39
Parknyigot	10/4/2012	8496	Vesicular fluid	2-3days	Strong positive	18.19	17.77
Parknyigot	10/4/2012	8497	Foot Epithelium	10-12days	Weak positive	17.60	22.62
Parknyigot	10/4/2012	8498	Tongue Epithelium	5-7days	Weak positive	23.45	23.83
Parknyigot	10/4/2012	8499	Gum Epithelium	8-10days	Weak positive	17.51	25.51

Village	Date of collection	Eartag No	Type of Tissue tested	Age of lesion	LFD results	RT-PCR epithelial tissues	RT-PCR of LFD RNA
Parknyigot	10/4/2012	6703	Gum Epithelium	3-5days	Weak positive	22.09	26.12
Parknyigot	10/4/2012	6705	Tongue Epithelium	3-4days	Weak positive	33.71	35.81
Parknyigot	10/4/2012	6706	Foot Epithelium	7-10days	Weak positive	23.79	23.32
Ngorongoro	2/7/2012	7536	Foot Epithelium	10+days	Weak positive	34.05	34.88
Mbilikili	2/11/2013	7537	Foot Epithelium	10days	Weak positive	27.24	30.87
Mbilikili	2/11/2013	7538	Foot Epithelium	14days	Weak positive	21.77	29.35
Mbilikili	2/11/2013	7539	Foot Epithelium	5-7days	Weak positive	32.23	29.35
Mbilikili	2/11/2013	7540	Foot Epithelium	14days	Weak positive	32.09	28.09
Mbilikili	2/11/2013	7541	Foot Epithelium	14days	Weak positive	22.41	No ct
Mbilikili	2/11/2013	7542	Foot Epithelium	14days	Weak positive	36.96	35.56
Mbilikili	2/11/2013	7543	Foot Epithelium	14days	Weak positive	31.32	37.78
Mbilikili	2/11/2013	7544	Foot Epithelium	10+days	Weak positive	18.22	22.1
Mbilikili	2/11/2013	7545	Foot Epithelium	10+days	Weak positive	32.17	22.34
Mbilikili	2/11/2013	7546	Foot Epithelium	10+days	Weak positive	29.41	31.85
Mbilikili	2/11/2013	7547	Foot Epithelium	14days	Weak positive	28.17	34.96
Mbilikili	2/11/2013	7548	Tongue Epithelium	5-7days	Weak positive	35.48	33.69
Mbilikili	2/11/2013	7549	Gum Epithelium	5days	Strong positive	22.67	19.67
Mbilikili	2/11/2013	7550	Tongue Epithelium	2-3days	Weak positive	24.04	20.89
Mbilikili	2/11/2013	7885	Gum Epithelium	8-10days	Weak positive	31.01	35.21
Mbilikili	2/11/2013	7886	Foot Epithelium	14days	Strong positive	21.70	18.87
Mbilikili	2/11/2013	7887	Foot Epithelium	10+days	Weak positive	22.08	30.99
Mbillikili	2/11/2013	8525	Foot Epithelium	2-3days	Weak positive	30.37	22.88
Mbilikili	2/11/2013	8524	Gum epithelium	2-3days	Negative	22.42	26.2
Mbilikili	2/11/2013	8523	Foot Epithelium	14days	Weak positive	24.90	25.72
Mbilikili	2/11/2013	8522	Gum epithelium	8-9days	Weak positive	27.47	26.28
Mbilikili	2/11/2013	8521	Foot Epithelium	8-10dys	Strong positive	21.19	23.55
Mbilikili	2/11/2013	8520	Foot Epithelium	7-8days	Weak positive	28.92	No ct
Bonchungu	21/03/2013	8583	Foot Epithelium	10days	Weak positive	32.3	No ct
Bonchungu	21/03/2013	8584	Foot Epithelium	7days	Weak positive	30.72	No ct
Bonchungu	21/03/2013	8585	Foot Epithelium	6days	Weak positive	28.6	32.75
Nata	21/03/2013	8535	Foot Epithelium	2days	Weak positive	29.96	No ct

Village	Date of collection	Eartag No	Type of Tissue tested	Age of lesion	LFD results	RT-PCR epithelial tissues	RT-PCR of LFD RNA
Mbilikili	21/03/2013	8537	Gum Epithelium	5days	Negative	34.82	36.17
Mbilikili	21/03/2013	8545	Foot Epithelium	8days	Weak positive	30.24	No ct
Mbilikili	21/03/2013	8546	Foot Epithelium	5days	Weak positive	18.99	No ct
Mbilikili	21/03/2013	8547	Foot Epithelium	7days	Weak positive	21.04	20.05
Mbilikili	21/03/2013	8549	Foot Epithelium	7days	Weak positive	21.67	23.04
Mbilikili	21/03/2013	8550	Gum Epithelium	7days	Weak positive	19.34	No ct
Mbilikili	21/03/2013	8551	Gum Epithelium	8days	Weak positive	17.62	17.35
Ngorongoro	7/5/2013	8621	Foot Epithelium	10+days	Weak positive	30.54	34.98
Ngorongoro	7/5/2013	8622	Foot Epithelium	10+days	Weak positive	28.3	27.44
Ngorongoro	7/5/2013	8623	Foot Epithelium	10+days	Weak positive	31.45	No ct
Ngorongoro	7/5/2013	8624	Foot Epithelium	10+days	Weak positive	33.34	No ct
Ngorongoro	7/5/2013	8625	Foot Epithelium	10+days	Weak positive	30.55	No ct
Ngorongoro	7/5/2013	8573	Foot Epithelium	7days	Weak positive	29.36	33.77
Ngorongoro	7/5/2013	8626	Foot Epithelium	10+days	Weak positive	30.88	36.88
Rwamchanga	30/05/2013	8653	Foot Epithelium	10+days	Weak positive	32.3	No ct
Rwamchanga	30/05/2013	8654	Foot Epithelium	10+days	Weak positive	29.6	No ct
Rwamchanga	20/06/2013	8655	Foot Epithelium	10+days	Weak positive	31.49	No ct
Rwamchanga	20/06/2013	8531	Foot Epithelium	3-4days	Slightly strong positive	13.12	11.66
Rwamchanga	20/06/2013	8531	Vesicular fluid	2-3days	Slightly strong positive	11.71	19.45
Rwamchanga	20/06/2013	8554	Tongue Epithelium	4days	Weak positive	23.44	27.29
Rwamchanga	20/06/2013	8571	Mouth Epithelium	5 days	Weak positive	25.99	26.14
Rwamchanga	20/06/2013	8572	Gum Epithelium	4days	Weak positive	28.2	No ct
Rwamchanga	20/06/2013	8574	Gum Epithelium	6days	Weak positive	27.02	34.88

Appendix 2: Positive and negative controls used in the amplification of FMDV genome and elution of LFDs membranes

Names of Controls	Tissues used	Ct values
N 1 negative control	Nuclease free water	No ct
N 2 negative control	Negative epithelial tissue	No ct
N 3 negative control	Negative epithelial tissue	No ct
N 4 negative control	Negative epithelial tissue	No ct
P 1 positive control	Epithelial tissue from Nzega district	20.32
P 2 positive control	Kenya vaccine strain	18.03
P 3 positive control	Sample 142 TVLA	23.24
P 4 positive control	O manisa	27.28
N 5	Negative epithelial tissue	No ct
N 6	Negative epithelial tissue	No ct
N 7	Negative epithelial tissue	No ct
N 9	Negative epithelial tissue	No ct
N 10	Negative epithelial tissue	No ct

Appendix 3: RT-PCR Ct values of FMDV RNA recovered from LFD kept at room temperature conditions

Results of LFDs kept at room temperature (25°C-30°C)

Eartag No.	Cts values of epithelial tissues	Cts values of RNA Eluted from LFDs
7053	12.9	15.84
8426	11.02	13.21
8427	22.28	29.34
8492	12.61	15.21
8491	20.69	17.47
6736	32.22	37.83
6737	9.66	25.9
6738	16.03	30.32
6739	17.60	19.98
6740	13.98	28.09
6741	30.77	No ct
8485	17.81	24.07
8489	13.18	19.64
6772	18.00	33.35
6773	30.18	34.69
6774	36.91	26.84
6775	30.18	No ct
6776	25.19	19.71
8493	9.93	19.73
8494	14.40	16.34
8495	17.49	19.39
8496	18.19	17.77
8497	17.60	22.62
8498	23.45	23.83
8499	17.51	25.51
6703	22.09	26.12
6705	33.71	35.81
6706	23.79	23.32
7536	34.05	34.88
7537	27.24	30.87
7538	21.77	29.35
7539	32.23	29.35
7540	32.09	28.09
7541	22.42	No ct
7542	36.96	35.56
7543	31.32	37.78
7544	18.22	22.1
7545	32.17	22.34
7546	29.41	31.85
7547	28.17	34.96
7548	35.48	33.69
7549	22.67	19.67
7550	24.04	20.89
7885	31.01	35.21
7886	21.70	18.87
7887	22.08	30.99
8525	30.37	22.88
8524	22.42	26.2
8523	24.90	25.72
8522	27.47	26.28
8521	21.19	23.55
8520	28.92	No ct

Appendix 4: Results of FMDV RNA recovered from LFDS kept at -20°C

Eartag No.	Cts of epithelial tissues	Cts of LFDs RNA at -20°C
8583	32.3	No ct
8584	30.72	No ct
8585	28.6	32.75
8535	29.96	No ct
8537	34.82	36.17
8545	30.24	No ct
8546	18.99	No ct
8547	21.04	20.05
8549	21.67	23.04
8550	19.34	No ct
8551	17.62	17.35
8621	30.54	34.98
8622	28.3	27.44
8623	31.45	No ct
8624	33.34	No ct
8625	30.55	No ct
8573	29.36	33.77
8626	30.88	36.88
8653	32.3	No ct
8654	29.6	No ct
8655	31.49	No ct
8531	13.12	11.66
8531	11.71	19.45
8554	23.44	27.29
8571	25.99	26.14
8572	28.2	No ct
8574	32.3	34.88