ADAPTATION OF REVERSE TRANSCRIPTION LOOP- MEDIATED ISOTHERMAL AMPLIFICATION FOR FIELD DIAGNOSIS OF FOOT-AND- MOUTH DISEASE IN TANZANIA

SENGIYUMVA EMMANUEL KANDUSI

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

2016

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral vesicular disease of cloven hoofed animals and poses major constraints to international trade in livestock production. Methods available for detection of FMD virus (FMDV) require specialized laboratory facilities and equipment. In this study, targeted laboratory-based experiments studies were conducted using reverse transcription loop-mediated isothermal amplification (RT-LAMP) for detection and serotyping of FMDV under field conditions. Pan-serotypic RT-LAMP utilizing labeled and unlabeled primers was used for detection of the virus. Serotype-specific primers for FMDV serotypes A and O were used to type the positive samples using RT-LAMP. Amplification was observed in real-time for unlabeled primers and by molecular lateral flow devices for labeled primers. Also, gel electrophoresis was used for examination of deoxyribonucleic acid (DNA) bands. A total of 35 samples (n = 35) were examined using RT-LAMP. Of these, 40% (n=14) were positive from different regions in Tanzania. The positive samples were from Iringa with 29% (n=4), Morogoro with 14.2% (n=2), as well Kilimanjaro, Mara, Tanga, Tabora, Mtwara, Kagera Dar es Salaam and Mwanza with 7.1% (n=1) each. All the pan- serotypic RT-LAMP positive samples revealed time for positivity ranging from 12-30 minutes. These findings indicate that the standardized RT-LAMP assay reported in this study can be used for field detection of FMDV in suspected FMD outbreaks in Tanzania. These findings suggest a potential use of serotype-specific RT-LAMP for typing FMDV field strains.

DECLARATION

I, Sengiyumva Emmanuel Kandusi, 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 it has neither been submitted nor concurrently being submitted to any other Institution.

Signature.....Date.....

Sengiyumva Emmanuel Kandusi

(MSc. One Health Molecular Biology)

Signature.....Date.....

Dr. Christopher J.

Kasanga (Supervisor)

Signature.....Date.....

Prof. Philemon N.

Wambura (Supervisor)

COPYRIGHT

ACKNOWLEDGEMENT

I would like to thank my supervisors Dr. Christopher J. Kasanga and Prof. Philemon N. Wambura for accepting to supervise this work for valuable time they spent with me from proposal development up to this report write-up. Their constant guidance invaluable scientific comments and criticisms are highly appreciated.

I wish to thank the Wellcome Trust through the Southern African Centre for Infectious Disease Surveillance (SACIDS) grant WT087546MA for sponsoring my study.

I would like to thank Mr. Raphael Sallu for technical assistance during my research work. Thanks to all staff of the Centre for Infectious Disease and Biotechnology (CIDB) for their assistance during laboratory work.

Special thanks go to Dr. Veronica Fowler and Ms. Emma Howson from Pirbright Institute for their constant technical support and guidance throughout my research work.

I would like to thank my husband Mr. Okumu Were for care, support and encouragement during my studies.

I would like to thank my fellow One Health Molecular Biology student Gurdeep Jaswant for the effort and encouragement she gave me.

Last but not the least, I thank all lecturers and staff of the Faculty of Veterinary Medicine at Sokoine University of Agriculture for their guidance and support during my studies.

DEDICATION

I dedicate this work to the Late Rev. Canon Dr. Emmanuel J. Kandusi and the Late Bishop Fortunatus Lukanima.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS ACRONYMS AND SYMBOLS

$(NH_4)_2SO_4$	Ammoniumsulphate
Ag-ELISA	Antigen Enzyme- Linked Immunosobent Assay
B3	Backward outer primer
BIP	Backward inner primer
Bsm	*Bacillussmith*
Bst	*Bacillussteriothermalphillus*
^oC	Degree Celsius
CFT	Complement fixation test
CIDB	Centre of Infectious Disease and Biotechnology
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleosidetriphosphate
DRC	Democratic Republic of Congo
DVO	District Veterinary Officer
ELISA	Enzyme linked immunosorbent assay
F3	Forward outer primer
FAO	Food and Agriculture Organization
FIB	Forward inner primer
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
IBV	Infectious bronchitis virus
Kbp	Kilo basepair
KCl	Potassiumchloride

LAMP	Loop- mediated isothermal amplification
LB	Loop backward primer
LFA	Lateral flow assay
LFD	Lateral flow device
LF	Loop forward primer
MEQ-LAMP	Microfluidic electrochemical loop-mediated isothermal amplification
$MgSO_4$	Magnesiumsulphate
NASBA	Nucleic acid sequence based amplification
ND	Newcastle disease
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
SACIDS	Southern African Centre for Infectious Disease
Surveillance SARS	Severe Acute Respiratory Syndrome
SAT	Southern African Territories
SDA	Strand displacement
TVLA	Tanzania Veterinary Laboratory Agency
uL	Microliter
uM	Micromole

UTR	Untranslated region
VIC	Veterinary Investigation Centre
VNT	Viral neutralization test
VP	Viral protein
w/v	Weight by volume
%	Percentage

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Foot-and-mouth disease (FMD) is caused by single stranded ribonucleic acid (RNA) virus called FMD virus (FMDV), which is a member of the family *Picornaviridae* genus*Apththovirus.* Foot-and-mouth disease is a highly contagious viral vesicular disease of cloven-hoofed animals that causes serious production losses and poses major constraints to international trade in livestock products. Severe mortality may occur in young stock particularly lambs and piglets.

Foot-and-mouth disease is caused by single stranded positive sense RNA virus, with genome about of 8.4 kbp in size (including 5'and 3'untranslated regions) and encodes 12 proteins namely leader proteinase L^{pro}, four structural proteins 1A (VP4), 1B(VP2), 1C (VP3) and 1D (VP1), and seven non-structural proteins 2A, 2B, 2C, 3A, 3B (VPg), 3C, and 3D (Guohui*et al*., 2008).

The viral genome is translated as single polyprotein which is post transcriptionary cleaved by viral proteases (Ryan *et al*) as cited by Longjam *et al*. (2011) into four structural protein (VP 1 ,VP2 ,VP3 and VP4) and several other non-structural (L 2A, 2B, 2C 3A, 3B, 3C and 3D) (Murphy *et al*., 1986). Sixty copies of each structural protein VP 1-4 assemble to form a capsid. Among which VP4 is internal where as others exposed on virion surface, the three surface exposed capsid proteins carry the neutralizing antigenic sites. Among the four structural polyproteins, VP1 is the most

immunogenic protein of FMDV having G-H loop protruding from the surface andmaximally exposed on the capsid surface forming large part (54%) of the virus surface (Longjam*et al*., 2011). VP1 is exposed to the surface of the virion and contains serotype specific amino acid sequence variation (Guohui*et al*., 2008). The VP1 protein plays important role in virus attachment, protective immunity and serotype specificity, and the nucleotide sequences of this region have been extensively used for the molecular epidemiological studies of FMDV (Yang *et al*., 2011). In FMDV VP1 is more variable which results into many serotype and topotypes/genotypes.

Foot-and-mouth disease virus is the sole member of genus *Aphthovirus* belonging to *Picornaviridae* family. Initially, two FMDV types were named O for Oise in France and A type for Allermargne in German (Longjam *et al*., 2011). Later, FMDV serotype C and Southern African Territories (SAT) 1, 2 and 3, which comprises 65 topotypes/genotypes were demonstrated.

The distribution of FMDV serotypes and FMD affected countries is uneven across A and O are reported in Southern America, Asia and Africa, Asia 1 is found in Asia and Middle East and SAT serotypes are generally restricted to Africa. So far in Tanzania there are four reported circulating serotypes, which are SAT 1, 2, O and A (Kasanga *et al.,*2014).

Foot-and-mouth disease virus can infect most or all members of *Artiodactyla* (cloven-hoofed mammals) as well as few species in other orders. Each animal species varies

in its susceptibility to infection and clinical disease as well as ability to transmit virus to other animals. Livestock susceptible to FMD include cattle, pigs, sheep, goats, waterbuffalo and reindeer. Ilama, alpacas and camels can be infected experimentally but do not appear to be very susceptible.

Foot and mouth disease can also infect at least 70 species of wild animals including African buffalo (*Synceruscaffer*) bison, mouse, chamois, giraffe, wildebeest, blackbuck, warthogs, kudu, impala and several species of deer antelopes and gazelles. Susceptible non-cloven hoofed species include armadillos, kangaroos, nutrias, capybaras guinea pigs, rats and mice.

The disease is endemic in most sub-Saharan African countries, but it has low mortality rate about 5% even though it is considered as the most important disease of farm animals since it cause huge losses in terms of livestock productivity and trade (Longjam *et al.,* 2011).

In sub Saharan Africa, two cycles of disease occur where one virus circulates between wildlife and domestic animals and the other, where the virus spreads among domestic animal without involvement of wildlife (Vosloo and Thomson, 2004). In Southern Africa and to a larger extent Eastern Africa the cycle between wild and domesticated animal occurs while in West Africa due to lower number of wildlife disease is maintained predominantly in domestic animals, however, once the disease cross from the wildlife into domestic animals a cycle could be maintained in domestic animals without involvement of wildlife (Vosloo *et al.,* n.d).

It is difficult to control FMD in Southern Africa partly due to large number of wild animals which act as carriers of the virus over long periods of time. Failure to putboundaries between wild animals and domestic animals at interface areas also aggravates the situation.Lack of movement control within countries and international borders for both wildlife and domestic animals aggravates the problem and gives credence to the fact that FMD will remain a problem on the subcontinent for the foreseeable future (Vosloo *et al.,* n.d).

Foot-and-mouth disease is endemic in Tanzania with outbreaks occurring almost each year in different parts of the country. The disease has a great negative impact on Tanzania's livestock sector, one of main economic activities in the country with over 16 million heads of cattle in 2002-2003; this was reported by (FAO, 2003). The disease is the most important livestock disease in Tanzania though it is reported to cause low mortality rate but it poses serious economic losses due to trade restrictions.

The persistence of disease is because of movement of livestock within and across international borders (Kivaria, 2003). Presence of four FMDV serotypes (SAT 1, SAT 2, A and O) and large number of wildlife reserves with susceptible species especially African buffalo complicates the control of the disease (Picado*et al.,* 2010). Antigenic variation among and within serotype is another problem in the control of FMD.

Foot-and-mouth disease is highly contagious in nature, with wide geographical distribution and broad host range. Foot-and-mouth disease outbreaks occur annually in Tanzania; hence rapid diagnosis is a very critical point in order to initiate proper control of the disease. Under this condition there is a need of rational and field

deployable methods such as RT-LAMP which is specific, sensitive, cheap and offersrapid and reliable results compared to other molecular techniques. Rapid detection and identification of circulating new variants of FMDV is essential for determining the source of outbreak(s), understanding the evolutionary characteristics of the virus and advancing the FMDV epidemiology. Therefore rapid and reliable diagnostic technique(s) of FMD can assist in detection of the virus and hence quick preventive measures of the disease can be taken in order to reduce and control the spread of the virus.

Although rapid detection and identification of FMDV is crucial, the available molecular and immunodiagnostic methods such as conventional reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and compliment fixation tests (CFT) are relatively time consuming and they need expertise and highly specialized condition(s) for their application. Due to shortage of laboratory facilities and modern equipment in developing countries including Tanzania, it is important to develop simple, specific, rapid and field-deployable methods/assays for detection of highly contagious pathogens such as FMDV.

Loop mediated isothermal amplification (LAMP) is an established nucleic acid amplification method offering rapid accurate and cost effective diagnosis of infectious diseases which can be deployed even in resource compromised laboratories in developing countries. The current focus on LAMP method is to develop diagnostic method which is simple in application but offers highly sensitive, reliable and specific results for rapid diagnosis of FMD. Loop-mediated isothermal amplification employs a DNA polymerase and a set of four specially designed primers that recognize a totalof six distinct sequences on target DNA. An inner primer containing sequences

of sense and anti-sense strands of target DNA to initiate LAMP. The following strand displacement DNA synthesis primed by the outer primer release a single stranded DNA. These serve as template DNA synthesis primed by second inner and outer primer that hybridize to the other end of the target which produce loop DNA structure.

The LAMP procedure can be carried out in single tube, incubating the mixture at constant 60-65^{o}C for less than an hour in standard water bath or heat block (Notomi *et al*., 2000). For highly contagious animal disease like FMD which pose large economic impact it is important to develop rapid, accurate and sensitive diagnostic methods for identification of pathogen which is pre-requisite for prevention, control and/oreradication of disease. Advantages of LAMP rely on the fact that the method is simple in operation with minimum specialized conditions for its application and potential visual interpretation without instrumentation.

Loop-mediated isothermal amplification is a novel molecular technique which is highly sensitive and specific it can be applied in rapid detection of highly infectious disease agent such as FMDV especially in endemic settings like Tanzania. There are many methods which can be used in diagnosis of FMD in Tanzania but most of these techniques require specialized equipment providing a challenge to most developing countries. Due to this fact, LAMP assay has potential of complementing the use of these modern and expensive techniques Loop-mediated isothermal amplification requires minimum processing and minimum instrumentation and results can be detected by naked eyes. This is an added advantage especially in resource limited countries but with limited facilities for diagnosis of FMD where the disease is endemic.

1.2 Problem statement

Foot-and-mouth disease is highly contagious disease of animals that spreads quickly yet detection methods available such as conventional and real-time RT-PCR, viral neutralization tests (VNT), complement fixation test are time-consuming and they need specialized conditions, which are not easily available in developing countries. This is compounded by shortage of specialized laboratory facilities and expertise. Hence the need for development of rational methods which are cost effective and simple in application but yet providing reliable diagnosis of the disease, identification and characterization of pathogens at early stages of infection. So far there is no reliable RT-LAMP technique that has been developed for detection and identification of FMDV SAT strains in Southern Africa.

1.3 Research questions

1. What methods of RT-LAMP could be used for field diagnosis of FMD in Tanzania?
2. What are the optimum condition(s) for field deployment of serotype specific RT- LAMP for diagnosis of FMD in Tanzania?

1.4 Objective of the study

1.4.1 General objective

To determine the appropriate method and conditions of RT-LAMP for diagnosis of FMD under field conditions in Tanzania.

1.4.2 Specific objectives

To determine the appropriate and optimum conditions for RT-LAMP assay for FMDV detection.

To examine the FMDV occurrence in different regions of Tanzania using standardized RT-LAMP assay.

CHAPTER TWO

2.0 LITERATUREREVIEW

2.1 Brief on foot-and-mouth disease

Foot-and-mouth disease is a highly contagious disease caused by a virus of genus *Aphthovirus*, family *Picornaviridae* as indicated in Fig.1 which affects all cloven hoofed animals including domesticated ruminants and pigs and more than 70 wildlife species (Mwiine *et al.,* 2010). The disease is characterized by high fever, loss of appetite, salivation and vesicular eruption on the feet, mouth and teats (Mwiine *et al.,* 2010). The disease has high morbidity rate although mortality is rare in adult animals. In young animals the FMDV can cause severe lesions in the myocardium leading to high mortality rates (Domingo *et al*., 1990). Infection of susceptible animals with FMDV can lead to appearance of vesicles on feet, around the oral cavity and on mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest line that grows down side of the hoof (OIE, 2012). Even though mortality rate below 5% it is considered the most important disease to farm animals since it causes huge losses in terms of livestock productivity and trade (Longjam*et al.,* 2011).The disease is of high economic importance especially to countries that have intensive animal industries. Efforts to control the disease consist of vaccination and restriction of animal movements in affected areas.

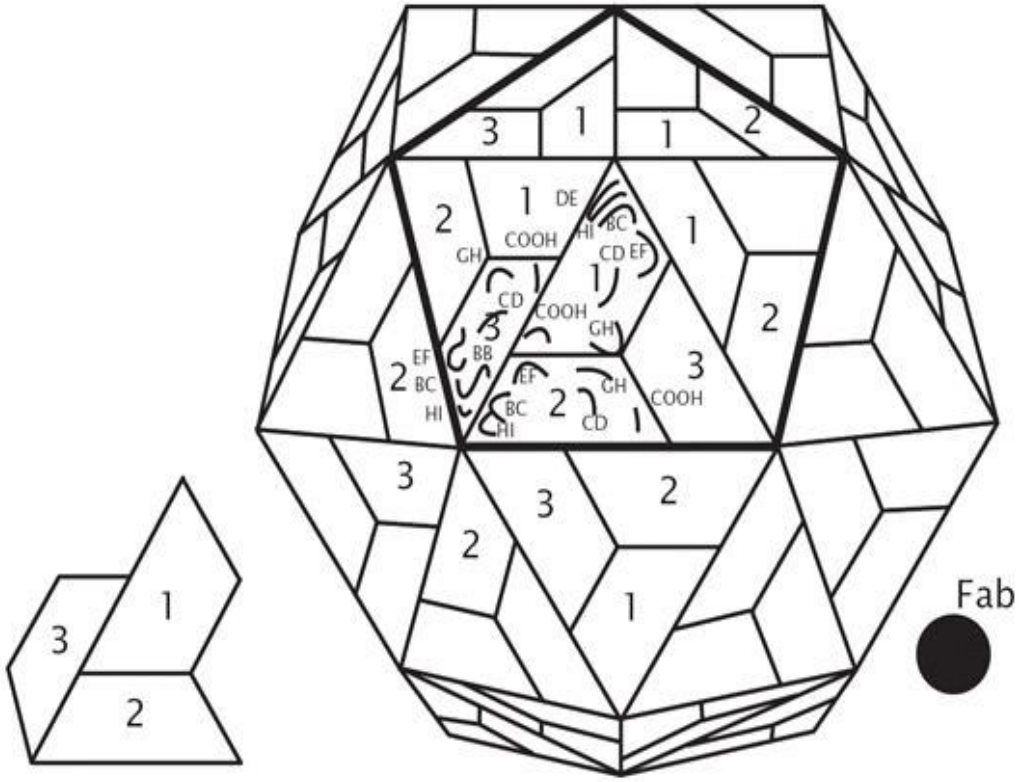

Figure 1: Icosahedral capsid structure of FMDV made up of sixty copies of each of Viral Proteins (VP1, VP2, VP3 and VP4). Source: Frank, (2002).

2.2 History of foot-and-mouth disease

The history of FMD may be traced to era of Hieronymus Franstorious, a monk who described the disease outbreak in 1546 AD that occurred in cattle near Verona Italy as was reported by Fracastorius 1546 and cited by Longjam *et al.* (2011). The disease was recognized as significant epidemic disease threatening the cattle industry since 16thC and till to- date it is the major global animal health problem. Foot-and-mouth disease is endemic in Southern and Eastern Africa countries with six out of seven serotypes (A, O, C, SAT1, SAT2 and SAT3) and numerous topotypes occurring for eachserotype on the continent. This provides challenges in control of the disease, sinceinfection with one serotype does not confer immunity against another (OIE, 2012).

2.3 Geographical distribution

Foot-and-mouth disease occurs in parts of Africa, Asia, the Middle East, and South America. In parts of Africa virus persistence in wild buffalo makes eradication unfeasible in some parts of the world North America, New Zealand Australia Greenland and most of Europe are free of this disease. Serotypes present in Africa are six out of seven which are A, O, C and SAT 1-3.

Foot-and-mouth disease virus in endemic setting across the world have been categorized into six pools (Fig. 2) each comprising a different geographical location with different predominant serotypes. The FMDV pools include pool1 in East Asia (A, O and Asia1), pool 2 in Central Asia (O, A and Asia 1), pool 3 in Europe and South Asia (O, A and Asia 1) and Horn of Africa (A, O ,SAT 1, 2 and 3) pool 5 Western Africa (O, A, SAT 1and 2) and pool 6 in Southern African (SAT 1, 2 and 3) (Kasanga *et al*., 2012).Tanzania links East Africa and Southern Africa in the region that overlaps between pool 4 and pool 6 (Kasanga *et al*.,2012).This means the country is affected by five serotypes among seven which are O, A, SAT 1-3.

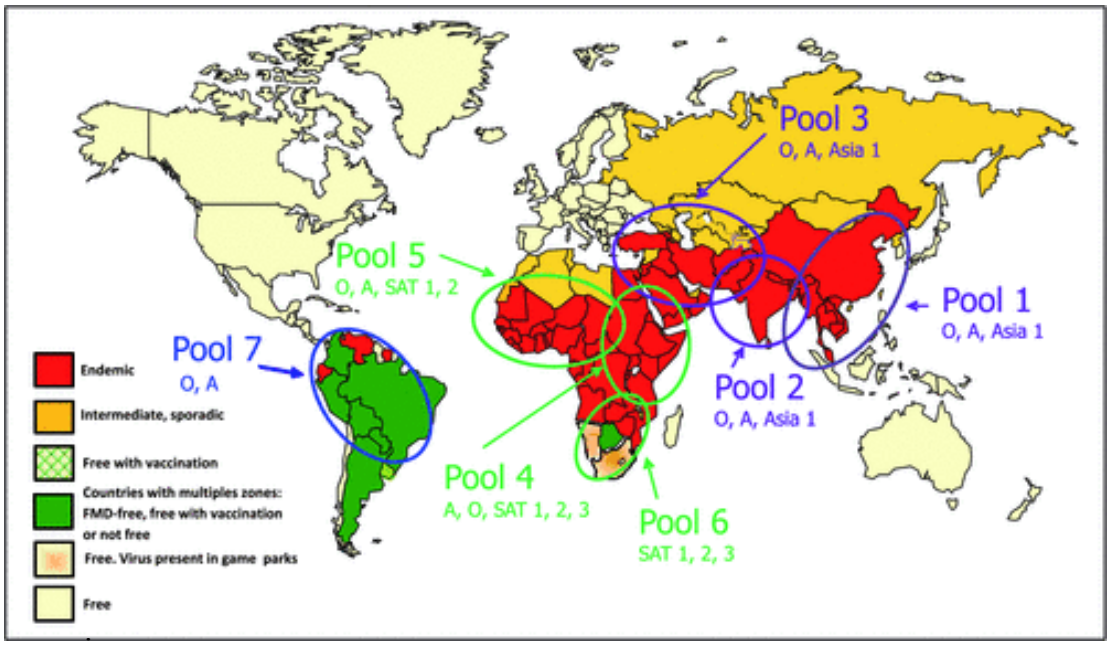

Figure 2: Map showing FMDV pools world-wide. Source: Kasanga *et al*. (2012).

2.4 Foot-and-mouth disease in Africa

Six of the seven serotypes of FMDV occur on the African continent and numerous topotypes occur for each serotype. The disease is endemic to most countries in sub-Sahara (Vosloo *et al.,* 2002) and may not be eradicated from southern and East Africa while infected buffalo are present. In sub-Saharan Africa, two cycles of FMD occur, one where virus circulates between wildlife hosts and domestic animal and the other where the virus spread among domestic animals, without the involvement of wildlife (Vosloo and Thomson, 2004). In Southern Africa and to larger extent Eastern Africa, the cycle between wildlife and domestic animal occurs while in West Africa the disease is maintained among domestic animals due to low numbers of wildlife.

2.5 Foot-and-mouth disease virus

2.5.1 Classification

The FMDV is classified within genus *Aphthovirus* in the family *Picornaviridae*. The virus exists in form of seven serologically distinguished types namely A, O, C, SAT 1, SAT 2, SAT 3 and Asia 1. There is high variation in structural protein among these serotypes, therefore, infection with one serotype does not confer immunity against another serotype.

2.5.2 Genome structure

Foot-and-mouth disease virus is single stranded (ss) positive sense RNA with 8.4Kbp in size including highly structured 5' and 3'untranslated region. The genome is polyadenylated at 3' end and carries a small covalently linked protein, VPg at 5', end. The 5' UTR contains a short fragment, a poly (C) tract followed by large (L) fragment of over 700 bases. Functionally, the genome can be categorized into three main regions which are 5' non coding regulatory region, polyprotein coding region (subdivided in L, P1, P2 and P3) and 3'non coding regulatory region as indicated in Fig. 3.

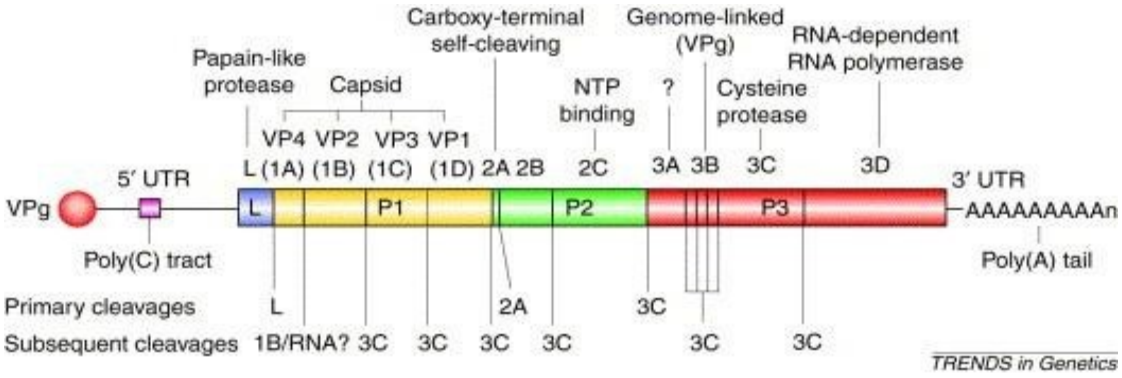

Figure 3: The complete genome orientation of foot-and-mouth disease virus. Source: Longjam *et al.* (2011).

2.5.3 Genome translation and antigenicity of viral protein

The viral genome is translated as single polyprotein which is post transcriptional cleaved by viral protease into four structural proteins (VP1, VP2, VP3 and VP4) and several non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D). The P1 region of the genome encodes the four structural proteins VP1, VP2, VP3 andVP4 encoded by 1D, 1B, 1C and 1A genomic region respectively. Among the four structural polypeptides VP1 is the most immunogenic protein of FMDV having its G-H loops protrude from the surface and is maximally exposed on the capsid surface forming large part of 54% of viral surface (Morrel *et al.,* 1987). The VP1 plays an important role in virus attachment, protective immunity and serotype specificity.

Serological studies show that different serotypes of FMDV shared the highly variable region of VP1 comprising residue 135 to145 as one of the major antigenic sites of the virus (Longjam *et al.,* 2011). Though the genome of FMDV is small it has high and spontaneous mutation rate. In FMDV structural protein are more variable than non-structural protein. Mutation or deletion may help FMDV to evade an immune response produced by the host (Li-na Ma *et al*., 2011). Mutation and recombination of the virus facilitate the emergence of new FMDV strains resulting into seven serotypes (A, O, C, SAT1, SAT 2, SAT 3, and Asian1).

2.6 Transmission

Foot-and-mouth disease is a highly contagious disease that can be transmitted in different ways leading to very rapid spread of the disease within and between the farms. The primary transmission modes are direct contact between infected animals

and susceptible animals, mechanical transmission by indirect means, e.g. contaminated human clothing and animal feeds. Aerosol spread, including long distance wind-borne transmission under exceptional epidemiological and environmental conditions (Alexandersen *et al*., 2002). Infected animals release virus in exhaled air, all excretions and secretions, and from ruptured vesicles. As excretion may commence up to four days before the appearance of clinical signs, the movement of animals that are incubating the disease is of great epidemiological significance (Donaldson and Alexandersen, 2002). The disease may spread extremely rapidly in intensive farming areas because of high stocking density and the level of challenge both from infected animals and the environment. Conversely, disease spread in extensive grazing areas in hotter climates can be more insidious (Bannet and Cox, 1999).

2.7 Pathogenesis

In the cattle infected via the respiratory tract, the virus initially replicates in the pharynx from where it proceeds towards the epithelium of the mucosa associated lymphoid tissue of the nasopharynx mucous membrane of oral cavity and invades the basal layer of the stratified epithelium of the tongue and produce primary lesions (Alexandersen *et al*., 2003).

Foot-and-mouth disease vesicles are formed due to virus multiplication in the stratum spinosum layer where cytolysis takes place and hence giving rise to small cavities in the epithelial layer. The process continues for 24 h and huge quantities of the virus are produced in the vesicles of the tongue from primary lesions.

Viruses also invade the lymphatics and enter into the blood stream. Foot-and-mouth disease virus also infect other organs and tissues such as epithelia of mouth, dental pad, coronary band, interdigital space of hoof, mammary gland, teats (cattle) and snout (pigs) where the secondary lesions develop .One to two days after infection, fever and viraemia may be observed (Alexandersen *et al*.,2003).

In advanced and unaddressed cases, secondary bacterial infection may set up extensive damage of the tissue. Damage of feet may lead to loss of the horny covering and sloughing of the hoofs. Udder lesions may lead to mastitis due to secondary infections. Heart muscles of young animals may show acute degeneration of the myocardial fibers (tiger heart). Distribution of the virus via the lymphatics to replication sites in epithelium of different organs as mouth and muzzle, interdigitalspaces of feet, and teats. Sometime involvement of damaged skin may be observed in pigs that are kept on concrete. Generally, the vesicular lesions appear at these sites (parts of knees and hocks) and within 48hit ruptures. The viremia persists for about 3 days (Chakraborty *et al.,* 2014).

2.8 Laboratory diagnosis of foot-and-mouth disease virus

Foot-and-mouth disease has a great impact in livestock sector in Tanzania, thus developing rapid diagnosis method that can help in making early intervention thereby reducing loss of livestock due to disease. As the result, this reduces poverty among livestock keepers with an ultimate increase of the national income.

In order to prevent the spread of FMDV there is need to have a laboratory diagnostic technique which offers rapid result in order to prevent the spread of the virus before, during and after the outbreak.

Conventional techniques such as complement fixation test (CFT), serum neutralization test (SNS), enzyme linked immunosorbant assay (ELISA) are still of use for routine detection of FMDV in clinical sample (Longjam *et al*., 2011).

Other molecular techniques such as polymerase chain reaction (PCR) and real time reverse transcription polymerase chain reaction (qRT-PCR) also can be used for detection of FMDV. Current methods for diagnosis of FMD in the laboratory are very laborious, expensive, requires highly specialized and experienced laboratory expertise to obtain results. In developing countries, where there is limited laboratory resources it will be difficult to control the disease if there focus on development and adaptation of the methods which can easily be deployed in the field.

2.9Loop-mediated isothermal amplification

Loop-mediated isothermal amplification technique is an established nucleic acid amplification method offering, rapid, accurate and cost effective diagnosis of infectious diseases (Mori and Notomi, 2009). It is a one step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions. Loop-mediated isothermal amplification technology is very simple and requires only one temperature for amplification of target DNA. Comparing to other methods including nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA) they can amplify target DNA to a similar magnitude, all with detection limit of less than 10 copies but they require either precision instruments for amplification or elaborate method for detection of amplified product.

Different assay for detection of FMDV including those which focus on highly conserved region 3D region, serotype specific LAMP assay and of the current multiplex RT-LAMP have been developed (Yamazaki *et al.,* 2013). Loop-mediated isothermal amplification provide rapid and sensitive diagnosis of FMD that can be deployed *in situ* without transferring the samples to central laboratory (Dukes *et al.,* 2006).

Sensitivity and specificity of the LAMP has been reported to be very high, and LAMP is capable of detecting as little as six copies of starting temperate (Nagamine *et al.*, 2002a; Dukes *et al.*, 2006; Kasanga *et al.,* 2012; Waters *et al.,* 2014) and has been shown to be 10 times more sensitive than end point RT PCR (Nagamine *et al.,*2002b).

Unlike PCR and RT- PCR, which are carried out in series of alternating temperatures, LAMP is carried out under isothermal temperature with no variation of temperature. This reduces cost of the test and makes it suitable for field deployment especially in endemic settings and resource limited laboratories.

The RT-LAMP assay can be of greatest value in endemic settings as it can use wide range of samples and wide range of heating equipment from thermal cyclers, heating blocks, water bath or disposable thermal devices. The possibility of using disposable equipment for these assays would overcome the difficulties of decontaminating apparatus that has to be transferred between premises (Dukes *et al.,* 2006).

Early detection of FMD is essential for recommendation of effective disease control measures. Simple, rapid, and non-invasive diagnostic tests are critical for FMD diagnosis Many FMD endemic countries in the developing world have relatively poor infrastructure and under-funded laboratory facilities, therefore, accurate, rapid and cheap diagnostic tests which are able to be implemented on farm would enable the majority of clinical cases suspected to be FMD to be confirmed (Water *et al*., 2014).

Distance from field to central laboratory is an issue of concern as it adds time and reduces sample integrity when sample preservation and cold chain is not properly followed especially where if distances involved are long (Waters *et al*., 2014). Ability of LAMP to be performed under field conditions with minimum equipment and minimum sample processing requirements make it more convenient to be used in developing countries where FMDV is endemic and most of the laboratories are resource-limited.

Loop-mediated isothermal amplification stands out to be a good and effective diagnostic test for empowering developing countries as it does not require sophisticated equipment and skilled personnel and proves to be cost effective (Dhama *et al.,* 2014). Since its invertion, LAMP has been used in diagnosis of number of pathogens both in animals and humans.

2.9.1.1 RT-LAMP requirement

2.9.1.2 Primers

Loop-mediated isothermal amplification requires minimum of four primers which are named as Forward outer (F3), Backward outer (B3) and Forward inner (FIB) and Backward inner (BIP).The F3 and B3 have their major role during strand displacement and called as strand displacing primers as shown in Fig 4. Forward inner and Backward inner have their function in loop formation this was reported by Parida (2008), and cited by Dhama *et al.* (2014). The two primers namely LF (loop forward) and LB (loop backward) can also be incorporated to accelerate the reaction. The primers are designed based on the eight target region present on the gene: F3c, F2c, F1c and FLP which are in 3'side and B1, B2, B3 and BLP which are in the 5'side

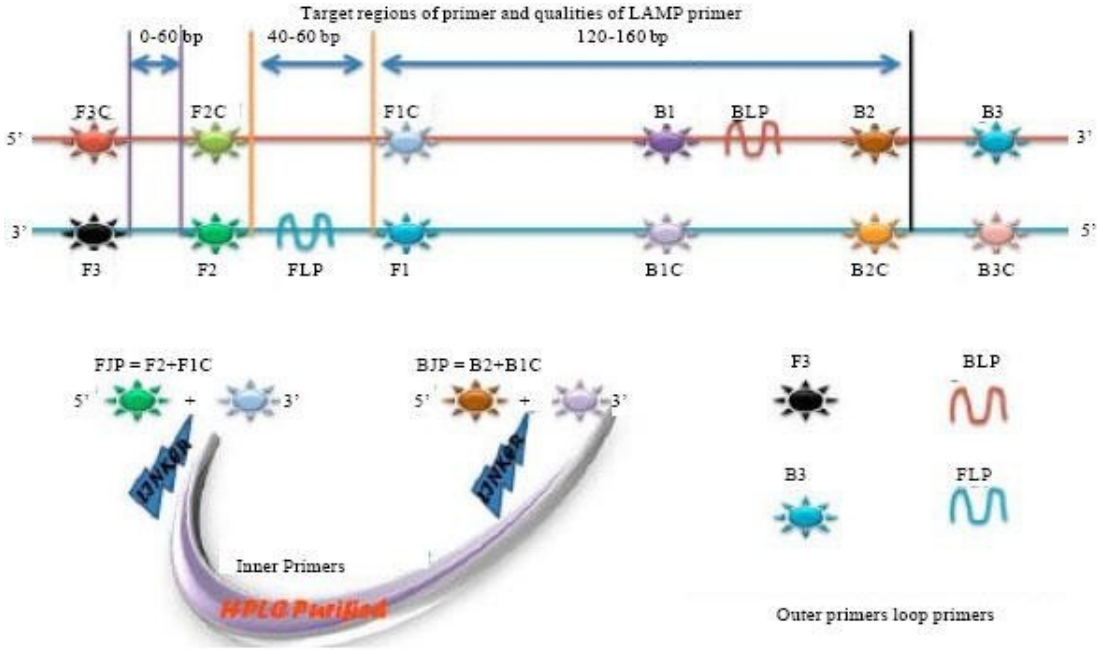

Figure 4: Image showing target regions and properties of LAMP primers. Source: Dhama *et al*. (2014).

The LAMP primers are designed by special software, the most commonly used is Primer Explorer which is available online. In order for these primers to function well they need to have certain characteristics which include (i) Inner primers should not have AT rich sequence at both ends, (ii) GC content should be about 50-60%, (iii) In case of GC rich sequence the melting temperature should be 60-65% and for AT rich sequence it should be within 55-60%, (iv) designed primers should have not any secondary structure formation, (v) Distance between 5 end and F2 and B2 should be 120-180bp and that of F2 and F3 is 0-20. The same features can be considered for B2 and B3 (Dhama *et al*., 2014).

2.9.1.3 Enzymes

Enzymes are the most important component of LAMP as they have ability for DNA polymerization and strand displacement which enables continuous, rapid isothermal amplification of LAMP reaction. The most commonly used enzymes are *Bst*polymerase isolated from *Bacillus stearothermophilus* and*Bsm*polymerase isolated from *Bacillus smithii*. Both enzymes have a unique property of strand displacement and can catalyze 5'-3' DNA polymerization but they don't have exonuclease activity as reported Nagamine *et al.* (2001) cited by (Dhama *et al.,* 2014). The *Bst*polymerase has its enzyme activity till 66^0C and *Bsm*polymerase has its activity till 63^0C and best at60^0C.

These enzymes cannot work alone, they need some buffer and other components such as dNTPs for providing nucleotides in order for polymerization process to occur. Magnesium sulphate is another component added in LAMP reaction, during DNA polymerization, pyrophosphate ions are produced yielding white precipitate of

magnesium pyrophosphate in reaction mixture and these enable to visualization the results based on the turbidity formed.

Betaine is the chemical used to stabilize the AT and GC content and finally buffer which contains tween, Tris-HCl with a pH 8.8, $(NH4)2SO4$, $MgSO4$ and KCl are commonly used (Dhama *et al.,* 2014).

2.9.1.4 Template preparation

There is no need for preparation of template DNA or RNA for amplification when LAMP assay is to be performed. Omission of DNA/RNA extraction during LAMPreduces time and cost of the assay. LAMP assay can work well with moderately prepared template or the sample as such can be used (Kaneto *et al*., 2007). In FMDV detection epithelial sample can be diluted and tested directly. This adds advantage of the test for field deployment in endemic settings of Africa.

2.9.1.5 Versions of LAMP

2.9.1.6 Micro LAMP

Loop-mediated isothermal amplification carried out on microfluidic chip for easy read out of results just by visualizing the precipitation or can be measured on optic sensor (Dhama *et al.,* 2014).

2.9.1.7 Micro- fluidic electrochemical quantitative (MEQ)

Micro-fluidic electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP) system an integrated micro fluidic platform for the rapid, sensitive, and quantitative detection of pathogenic DNA.

2.9.1.8 LAMP combined with lateral flow device (LFD)

This is the combination of both LAMP and LFD. In this detection of dual labeled LAMP by product is detected using molecular LFDs (Waters *et al.,* 2014).

2.9.1.9 Lympholized LAMP

Lympholized LAMP mixture makes the reaction much easier as requires only sample and primers for specific pathogen to be detected. Use of lyophilized LAMP in FMDV detection which is heat stable increases credibility for this technique especially in endemic settings of Africa where electricity supply is not reliable.

Advantages of using RT-LAMP in diagnosis

i. RT-LAMP is an ideal technique to be used in developing countries as the amplification of DNA/RNA does not require highly specialized equipment for thermo cycling hence making the assay suitable for field deployment (Dhama *et al.,* 2014).
ii. The diagnosis of FMDV with RT-LAMP is very rapid and it takes only 30 min to an hour since extraction procedure and post-amplification processes are omitted and the results can be visualized directly by adding colour developing dyes or turbidity.
iii. RT-LAMP works at constant temperature and therefore no need of expensive thermal cyclers (Dukes *et al.,* 2006).
iv. RT-LAMP is both sensitive and highly specific assay for genome detection (Kasanga *et al*., 2014).

Field deployment of LAMP assay

Loop-mediated isothermal amplification has been reported to be used for both diagnosis of animal and human pathogens as shown in Table 1. Its ability to be used in diagnosis with minimum equipment and reagents makes it a more suitable DNA/RNA amplification method for developing countries where laboratory infrastructures are relatively poor and underfunded. Ability of LAMP to be performed on pen-side or at the field with minimum cost and at the same time having high sensitivity and specificity makes the assay to be user friendly and highly beneficial in the control of highly infectious animal diseases such as FMD.

Table 1: The LAMP assays that have been developed and used for detection of both animal and human pathogens Source: Dhama*et al.* (2014).

	LAMP test for	**Microbe target**
Viral	Foot-and-mouth disease	Ruminants
	Severe Acute Respiratory Syndrome	Human
	Hepatitis B Virus	Human
	Human papilloma virus type 6, 11,16and 18	Human
	Coronavirus	Ruminants
	West Nile virus	Poultry
	Highly pathogenic avian influenza	Poultry
	Infectious bronchitis(IBV)	Poultry
	Chicken anaemia virus	Poultry
	Classical swine fever	Pigs
	Porcine cytomegalo virus	Pigs
	Camel pox	Camel
	Porcine circo virus2	Pigs
	Newcastle	Poultry
Bacterial	Leptospirosis	Human
	Staphylococcus aureus	Human
	Listeria monocytogenes	Human
	Tuberculosis	Animal/human
	Streptococcus suis	Pigs
	Shigella and Enteroinvasive E.coli	Pigs
	Yerseniaenterocolitica	Pigs
	Staphylococcus strain	Animals
	Brucella species	Animals
	Riemerellaanatipesifer	Ducks
	Edwardsiellaictaluri	Cat fish
Rickettisial	*Anaplasmaphagocytophilum*	Dogs
	Anaplasmaovis	Small ruminants
Mycotic	*Pneumocyst pneumoniae*	Human
	Candidiosis	Human
	Paracoccidiodesbraziliensis	Human
Parasitic	Cysticercosis	Human
	Giardiaduodenalis	Human
	Brugaliafilaliasis	Human
	Babesiagibsoni	Dogs
	Plasmodium species	Animals
	*Leishmania*species	Animals
	*Thelieria*infection	Ovine

2.10Virus neutralization test

The virus neutralization test is currently considered as the gold standard for detection of antibodies to structural proteins of FMDV. However, as various primary cells and cell lines with variable degrees of sensitivities are used in the VNTs, they are more prone to variability than other serological tests. Furthermore, VNT is slower, subject to contamination and requires restrictive bio-containment facilities in contrast to other serological tests which can use inactivated viruses as antigens (OIE, 2012). This method is not reliable in endemic settings where infrastructure is very poor and laboratory facilities are not well established.

2.11Virus isolation

Virus isolation is done using Primary cell cultures (such as bovine thyroid cells and porcine or ovine kidney cells) or cell lines (such as BHK or IBRS2) are considered to be generally suitable for isolation of FMDV (OIE,2012).

However, the production of consistent quality, ready-for-use primary cells is laborious, time-consuming and expensive. This test requires the presence of infectious virus, which depends on sample quality. Up to four days may be required to demonstrate the presence of virus, especially when the levels of virus are low (OIE, 2012).

Moreover, some FMDVs fail to grow in a specific cell type. Thus the absence of apparent growth does not guarantee absence of the virus and, therefore, samples collected from a suspected case of FMD should be subjected to further investigations, using other diagnostic assays. Additional disadvantages include the problems associated with obtaining and maintaining a regular supply of cells compounded by

possibility of contamination of cell cultures and the necessity to confirm any apparent virus growth by ELISA. These may delay the initiation of control measures to contain outbreaks (Jamal and Belsham, 2013).

2.12 PCR and RT-PCR

Polymerase chain reaction assays amplify specific nucleotide sequences from few copies to thousands to millions of copies. These assays rely on thermal cycling consisting of repeated heating and cooling process which involve enzymatic replication of DNA. These assays are widely used in detection of FMDV in the laboratory and non-laboratory conditions (mobile PCR). Several assays have been developed to detect FMDV that use 5'nuclease assay (Taq Man®) systems to detect PCR amplicons (Callhan*et al.,*2002; Oem*et al.,* 2005; Reid *et al.,* 2002). Also the use of modified minor groove binder (MGB) probe (Moruwa*et al*., 2007), hybridazation probe (Moonen*et al*., 2003), Primer probe energy transfer (PriPro ET: Rasmussen *et al.,* 2003) and RT-PCR-liner after the exponential PCR LATE PCR; (Reid *et al.,* 2002). Initially conventional PCR was used for detection of FMDV where gel electrophoresis was used to detect PCR products. This method is labor intensive and very prone to contamination. Currently, RT-PCR assays are widely used over conversional PCR. These assays are fluorescent-based which are highly sensitive enabling amplification and quantification of specific nucleotides (Hoffmann *et al.,* 2009). PCR assay are very suitable for detection and identification of many infectious agents including FMD virus. These assays can use wide range of different samples that is blood tissue, swabs esophageal pharyngeal (OP), scraping feacal sample and milk (King *et al.*, 2012).

2.13 Enzyme linked immunosorbent assay (ELISA)

Complement fixation test (CFT) was the test of choice for diagnosis of FMD and virus typing until the 1970s and is still used in some endemic areas (Jamal and Belsham, 2013). However, in order to overcome the problems of its low sensitivity and difficulty in interpretation of its results due to pro- and anti-complement activities, ELISA for antigen detection and virus typing were desired. Establishment of these suitable assays by using high titre antisera raised in rabbits and guinea pigs against purified 146S FMDV particles for antigen capture and detection, respectively.

The assays were found to be 125 times more sensitive than the CFT and are still routinely used for the diagnosis of FMD and for virus typing. The ELISA, however, gives positive results with only about 70-80% of epithelial suspensions that contain virus due to a lack of sensitivity. Thus the virus may have to be propagated in tissue culture and subsequently tested in ELISA to detect the virus and ascertain the serotype.

Monoclonal antibody (MAb)-based ELISAs have also been developed for diagnosis of FMD and virus typing. Recently, a sandwich ELISA using recombinant integrin (a receptor for FMDV) for virus capture and serotype-specific monoclonal antibodies as detecting reagents was compared with the conventional polyclonal antibody-based sandwich ELISAs for the identification and serotyping of all the seven types of FMDV (OIE, 2012). The integrin/MAb ELISA recognized FMDVs of wide antigenic and molecular diversity from all seven serotypes. Although some FMDVs could not be detected, the assay showed greater specificity than theconventional polyclonal ELISA while retaining test sensitivity (Jamal and Belsham, 2013).

2.14 Lateral flow devices assay

A simple lateral flow device assay (LFDA) based on monoclonal antibodies was developed for detection of FMD in the laboratory and under field conditions. It is also called immunochromato-graphic strip test uses monoclonal antibody format similar to sandwich capture ELISA. The positive test signal is generated by diffusion of colored antibody coated latex beads or colloidal gold particle through a membrane towards an immobilizing band of trapping antibody (King *et al.,* 2012).

Currently, LFD assay for detection of all seven FMDV serotypes have been already developed this assist in detection of FMDV in field conditions. Lateral flow device has many advantages, it require minimum laboratory facilities, trained personnel and special equipment with minimum sample preparation. Low cost of testing device result interpretation is an added advantage of LFD. Foot-and-mouth disease virus is endemic in resource limited countries like Tanzania. Diagnosis of FMDV under field condition is very critical in order to initiate appropriate control strategy. Development of these pen-side diagnostic assays can be useful for rapid detection of circulating virus during outbreak. These assays also increase sample integrity by reducing time for transportation of sample from field to reference laboratory.

Although it is reported that LFD has similar sensitivity with ELISA, it takes only 30 minutes with LFD assay to get results under minimum laboratory conditions, facilities and trained personnel.

Sensitivity and specificity of LFD assay was done by comparing the result of all seven FMDV serotypes with Ag ELISA. The overall sensitivity and specificity of the test were similar at 86.9% and 98.8%, respectively to those obtained by Ag ELISA 87.7% and 100%, respectively (Oem *et al.,* 2009).

The LFD assays can be of the greatest value in endemic settings where the issue of multiple serotype infection, lack of laboratory facilities, trained personnel and limited resources for laboratory diagnosis are the major problem.

However, only a limited number of sample types can be tested using the LFD which must contain large amount of viral antigen in order to generate positive results. These factors restrict the usefulness of this test to acute clinical phase of FMD where diseased epithelial is collectable (up to 3-4days after the onset of the lesion) and contain large amounts of intact FMDV antigen (Waters *et al.,* 2014).

CHAPTER THREE

3.0 MATERIALS ANDMETHODS

3.1 Study area

This study was carried out in three regions of Morogoro, Dar es Salaam and Mara in Tanzania. Geographically Tanzania is among the sub-Saharan countries located in East Africa. The country covers an area of 945,000km^2 whose climate varies from tropical to temperate (Picado *et al.,* 2010).It is located between latitude and longitude of -6.369 and 34.8888 respectively. Tanzania borders with Burundi Rwanda and Democratic Republic of Congo (DRC) on the west, Kenya and Uganda in the north, Mozambique Malawi and Zambia in the south and Indian Ocean in the east.

3.2 Study design

The design of this research was laboratory based experimental study which involved testing and analysis of FMD-suspected samples in the Centre of infectious disease and biotechnology laboratory.

3.3 Sampling site

Tissue epithelial samples from FMD suspected cattle were collected from different parts of Tanzania as shown in Fig 5, through Veterinary Investigation Centers (VIC) following the official reporting of FMD outbreak in particular location.

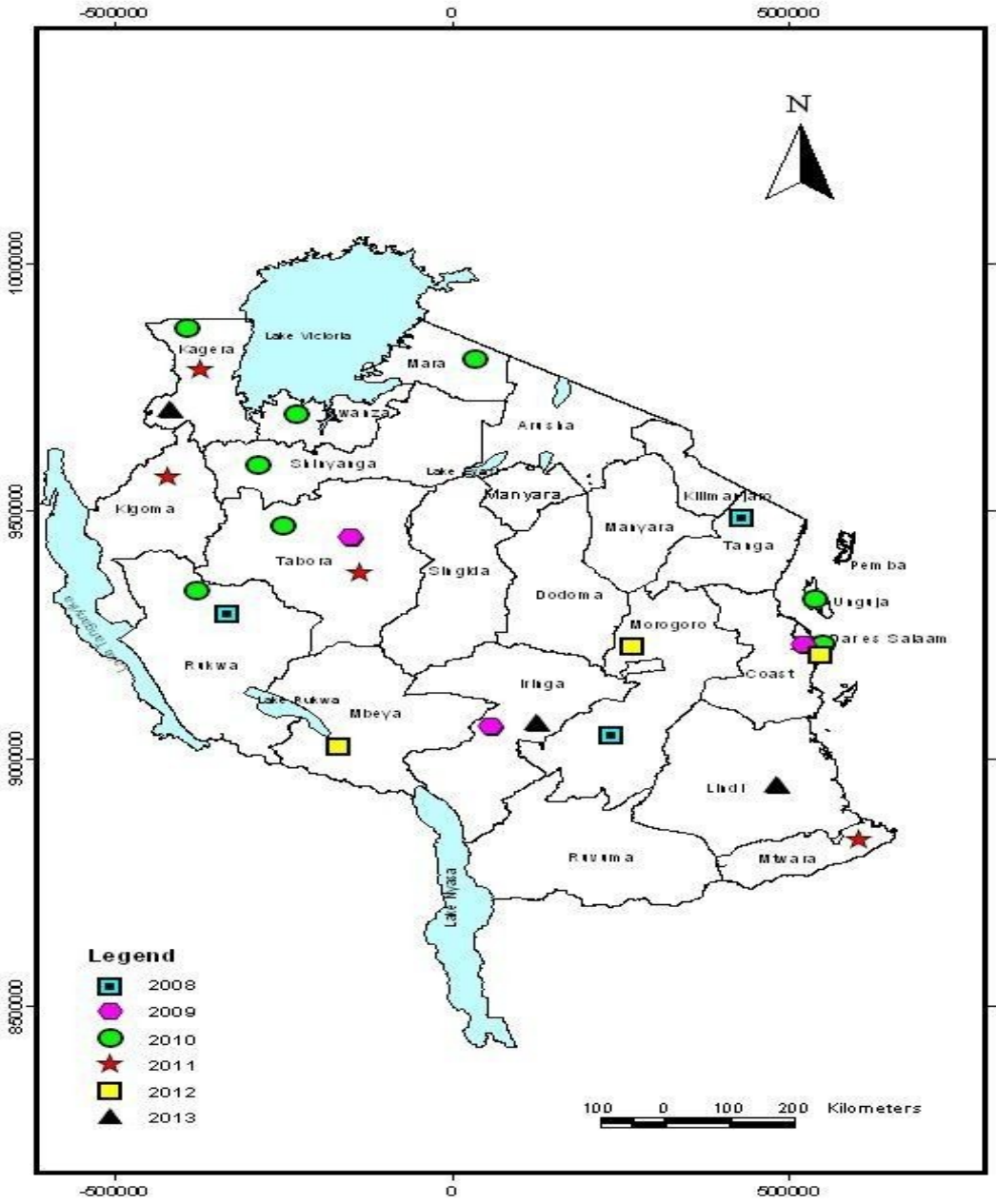

Figure 5: Map of Tanzania showing specific sites and years when samples were collected. Source: TVLA Official Report (2013).

3.4 Samples and sampling approach

Tissue epithelial samples were collected from lesions on the mouth, teats and feet of animals that revealed FMD clinical signs. The sample collection exercise was

organized through the District Veterinary Officers (DVO). The collected samples weretransported to the TVLA laboratory and stored before analysis. In this study only epithelial samples were used.Epithelial tissues were collected following physical examination of lesions on suspected animals. Animals were restrained by rope and samples were collected by the aid of forceps and scissors. Approximately $2cm^2$ were collected from unruptured vesicles or fresh ruptured vesicles. Samples were placed in cryo vials and labeled with identificationnumberoftheanimalseartagandnameofthevillagewhereanimalislocated. Immediately, samples were placed in liquid nitrogen for transportation. Then samples were transported to TVLA laboratory and stored in -80^0C until analysis.

3.5 Sample transportation

All samples collected from FMDV infected and suspected animals were transported to TVLA and stored at -80^0C prior to laboratory analysis.

3.6 Laboratory analysis of samples

3.6.1 Sample preparation

All samples were given laboratory numbers prior to analysis. Using bio-safety cabinet class II, samples were removed from transport media then weighed out one gram and placed in sterile mortar. The remaining tissues were stored at -20^0C. Tissues were then ground witha small amount of sterile sand and phosphate buffered saline (PBS) tomake 10% (w/v) suspension. Then the grounded sample were transferred to two cryo vials of which one was stored at -80^0C until further laboratory use.

3.6.2 Sample analysis

3.6.3 Preparation of sample for analysis

Epithelial suspension samples were thawed and then diluted to 1:5 dilutions. This was done by adding 20µl of epithelium suspension to 80µl in RNAse free water and mixed by pipeting before sample analysis.

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

Loop mediated isothermal amplification was conducted in using lyophilized reagents with un labeled primers (Table 2). Briefly, 5µl of nuclease free water were added in re-suspension buffer then 15 µl of re-suspension buffer were added in lyophilized RT-LAMP reagent from OptiGene Company. Five microliter of sample which prior diluted in 1:5 were added onto there-suspended reagent and then run into the machine for 30minutes. Reverse transcription-loop-mediated isothermal amplification was performed according to manufacturer instruction to determine the presence of FMD viral RNA. Genie II platform (OptiGene., Horsham, UK) was used during sample analysis.

Table 2: Sequence of primers used for RT-LAMP designed for amplification of the 3D polymerase gene of FMD virus. Source: Dukes *et al*. (2006).

Primer Name	**Sequence5'-3'**
F3	CATGGACTATGGAACTGGGT
B3	GGCCCTGGAAAGGCTCA
FIB	CACGGCGTGCAAAGGAGAGGATTTTAC AAACCTGTGATGGCTTCG
BIP	GGAGAAGTTGATCTCCGTGGCATTTTAA GAGACGCCGGTACTCG
F LOOP	TAG CCT CGT GGG TCT TCG
B LOOP	GGA CTC GCC GTC CAC TCT

3.7 Reverse transcriptase loop-mediated isothermal amplification lateral flow device

To determine the presence of FMD viral RNA, RT-LAMP was performed according to manufacturer instruction. Six set of primers were used which are FIB/BIP 50µM, F3/B3 25µM, FLP/BLP5µM.

Loop-mediated isothermal amplification was conducted using labeled FIB/BIP primers. Briefly, 5 µl of nuclease free water were added in re-suspension buffer then 15 µl of re-suspension buffer were added in lyophilized reagent. Five microliter of sample re-diluted in 1:5 were added onto re-suspended reagent to make total volume of 25µl per reaction then run into the Genie II (OptiGene., Horsham, UK) platform for30 minutes. Post amplification samples analysed with labeled primers were then

examined for positivity or negativity using molecular LFDs. Briefly, 200µl of buffer were measured in sterile cryo vial, this were mixed with 20 µl of amplification product. After thoroughly mixing then 75 µl of suspension were transferred in well labeled molecular LFD for detection of viral RNA in the samples.

3.7.1 Serotype-specific RT-LAMP

3.7.2 Primer preparation

Serotype specific primers were reconstructed to 100µM by adding required amount of nuclease-free water as per the synthesis report sheet. The 100µl solution/re-suspension was then stored as stock solution.

Table 3: Sequence of the RT-LAMP primers designed to detect the VP1 gene of FMDV Serotype A. Source: International Atomic Energy Agency (IAEA).

Primer	**Sequence 5'-3'**
FIP	TGC GAC TGT CCC TAG GTC ACT TTT TAA C
BIP	GCC CAA CTT CCT GCC TCT TTC ATT TTC T
FLP	CAA GTA CTC CGC GGC CAG TG
BLP	GGT GCA ATC AAG GAC G
F3	CTA CAC TGC GCC TAA CCG
B3	TGG GGC AGT AGA GTT CGG

Table 4: Sequence of the RT-LAMP primers designed to detect the VP1 gene of FMDV Serotype O. Source: International Atomic Energy Agency (IAEA).

Primer Name	**Sequence 5'-3'**
FIB	GGA AGT GTT CGG TCC GCT CAC TTT TCC C
BIP	CAG AGT TGT GCA GGC AGA ACG GTT TTA A
F3	CAT CCT CAC CAC CCG TAA C
B3	GAC ACC TTT GTG GTC GGT C
FLP	GGA GTC ACA TAC GGG TAC G
BLP	CAC CTC GTC ACA TAC GGG TAC G

These primers for serotype-specific assays were developed and supplied in courtesy of Herman Unger of the International Atomic Energy Agency (IAEA).

3.7.3 Preparation of working solution

The respective primers were diluted to working concentration before analysis of samples. In this study, the working solutions were diluted to the concentration of 50µM for (FIB/BIP), 25µM for (F3/B3) and 5µM (FLP/BLP).

3.7.4 Preparation of master mix

Master mix lyophilized reagent without primers was prepared by adding re-suspension buffer and serotype specific primers in lyophilized preparation. Re-suspension buffer 1 was diluted by adding 5µl of nuclease free water. Then, 15µl of diluted buffer were added to the lyophilized reagent and 3µl of serotype specific primers were added.

3.7.5 Serotype-specific assay for FMDV serotypes A and O

To determine serotype specificity of the positive samples, RT-LAMP assays using serotype-specific primers were used. Following dilution and addition of serotype-specific primers, 5µl sample re-diluted in nuclease free water in 1:5 dilution were added .This make total volume 25µl per reaction .Serotype specific RT-LAMP was run at 65 ^{0}C for 30 minutes on Genie II platform (OptiGene , Horsham, UK).

3.7.6 Determination of RT-LAMP post amplification

In this study two sets of primers were used which are unlabeled and labeled primers. For unlabeled primers amplification was determined in real time and by end-point analysis through gel electrophoresis (figure not shown). For labeled primers molecular LFDs were used to determine the presence or absence of amplification as presented in Figure 12.

CHAPTER FOUR

4.0 RESULTS ANDDISCUSSION

4.1 Results

4.1.1 Clinical examination

A total of 150 cattle of different age groups were physically examined in different geographic areas. Of these 23% (n=35) showed clinical signs suggestive of FMD. The principle clinical signs were salvation and lameness, foot lesions comprised of erosion in inter-digital space and coronary bands. Of the positive samples 40% (n=14) were positive from different regions and zones. Most were from Iringa 29% (n=4), Morogoro 14.2% (n=2) whereas Kilimanjaro, Mara, Tanga, Tabora, Mtwara, Kagera, Dar es Salaam and Mwanza had 7.1% (n=1).

4.1.2 Pan-serotypic reverse transcription loop- mediated isothermal amplification

Of the 35 positive samples, 40% (n=14) were positive with Tp value ranging from 10-30 minutes. Whereas 21 (60%) of the samples examined showed no Tp value hence were considered as negative. The amplification plots and anneal derivatives for RT-LAMP of selected samples are shown in Figures 6 and 7, Figure 8 and 9 shows amplification plots and anneal derivatives of RT-LAMP serotype A samples and Figure 10 and 11 shows amplification plots and anneal derivatives of RT-LAMP serotype O.

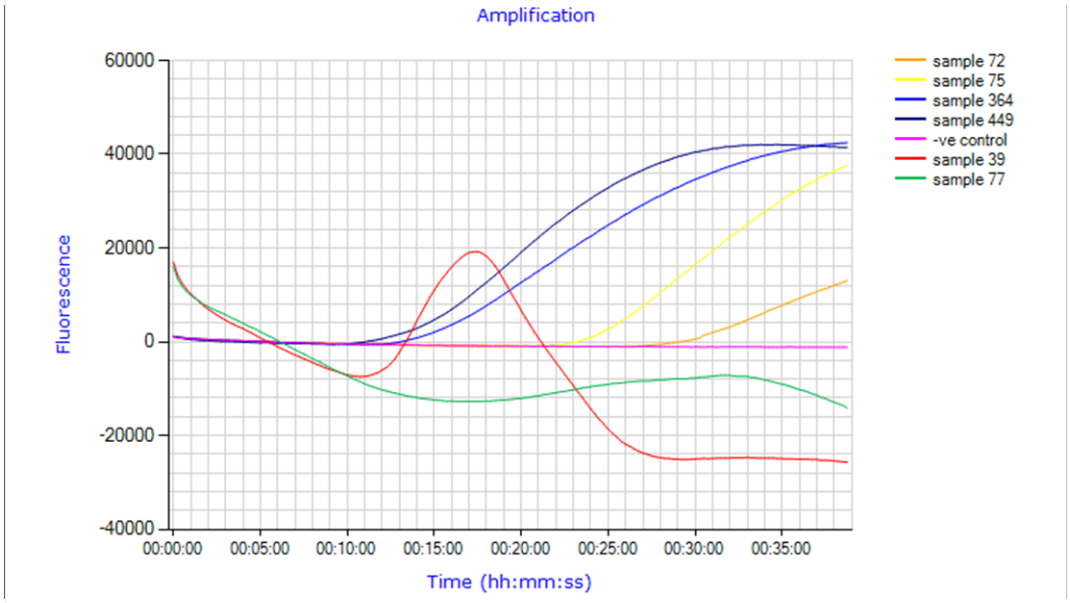

Figure 6: Graph showing amplification plot of Pan- serotypic RT-LAMP for sample collected from different regions in Tanzania.

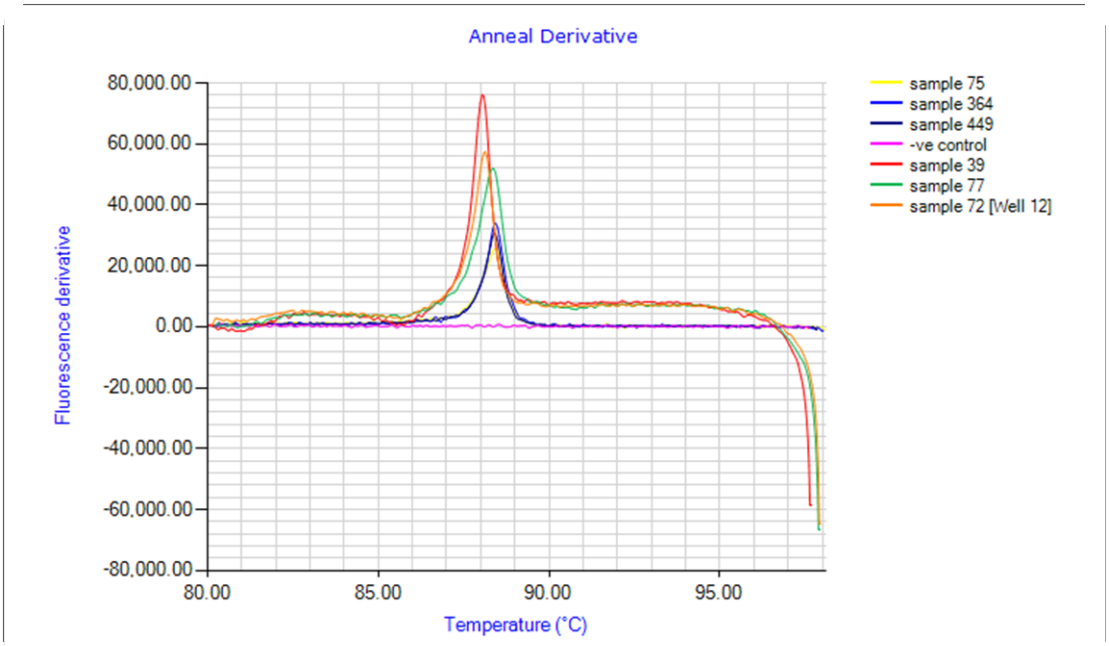

Figure 7: Graph showing anneal derivation of RT- LAMP positive samples.

4.1.3 Serotype-specific RT-LAMP

4.1.3.1 FMD virus serotype A

Amplification plot of FMDV serotype A during RT-LAMP analysis.

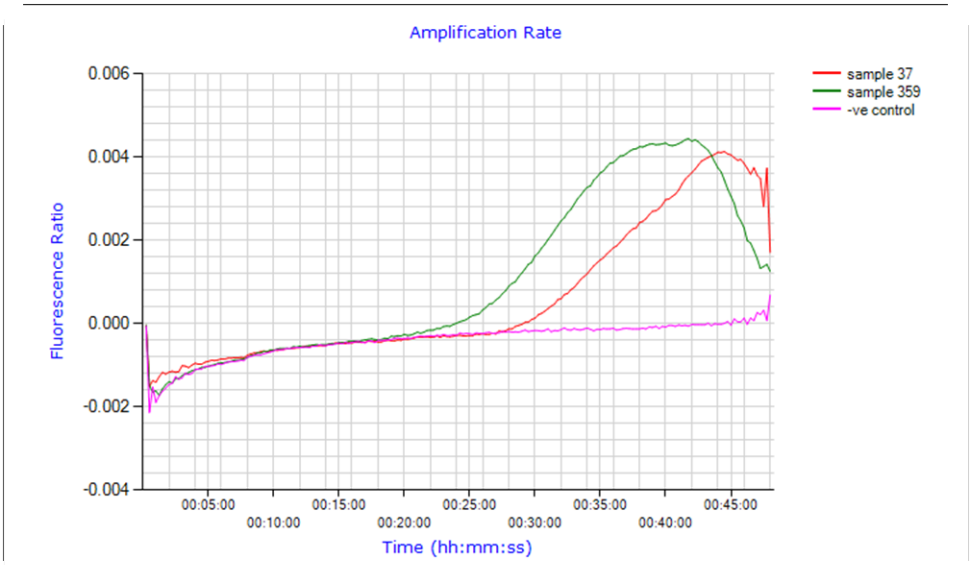

Figure 8: Graph showing amplification plot of FMDV serotype A RT- LAMP positive samples

Anneal derivation serotype A RT-LAMP analysis.

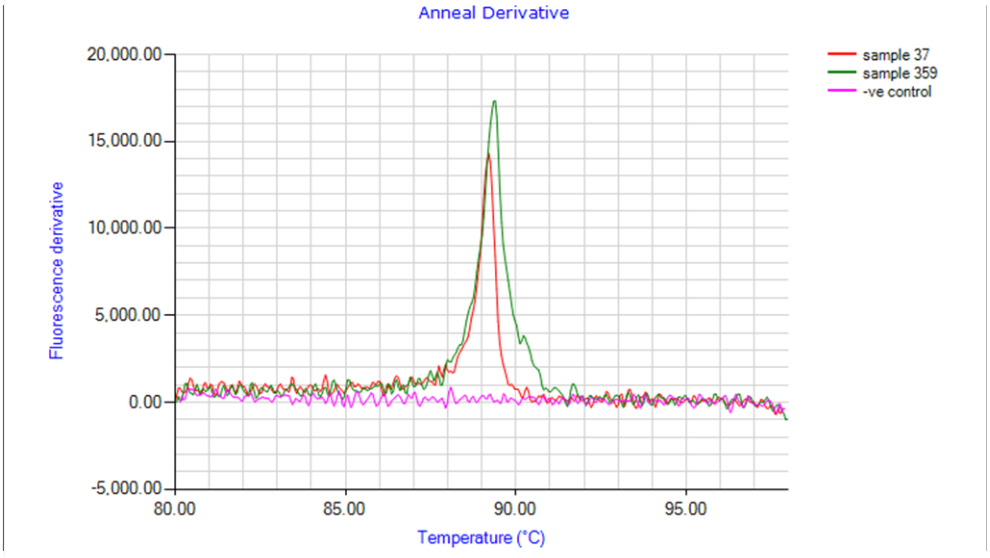

Figure 9: A graph showing anneal derivation of serotype A samples following RT-LAMP analysis.

4.1.3.2 FMD virus serotype O

Amplification plot of serotype O RT-LAMP analysis

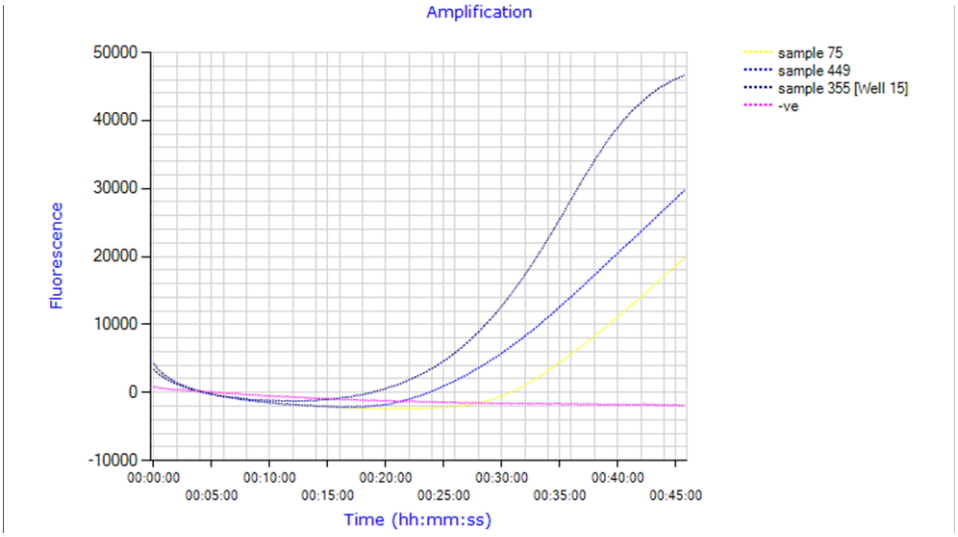

Figure 10: A graph showing amplification plot of FMDV serotype O following RT-LAMP.

Anneal derivation serotype O RT-LAMP analysis

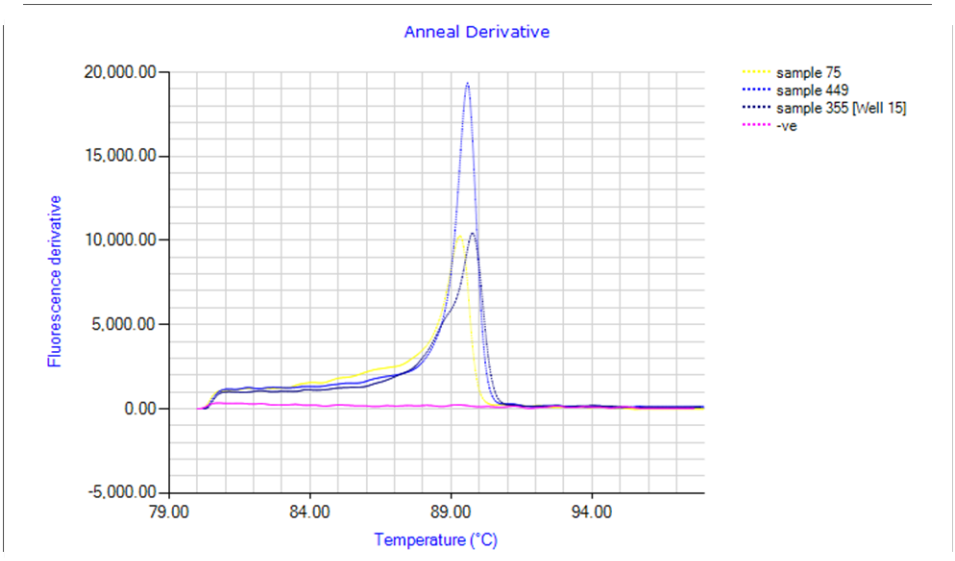

Figure 11: A graph showing anneal derivation of FMDV serotype O samples

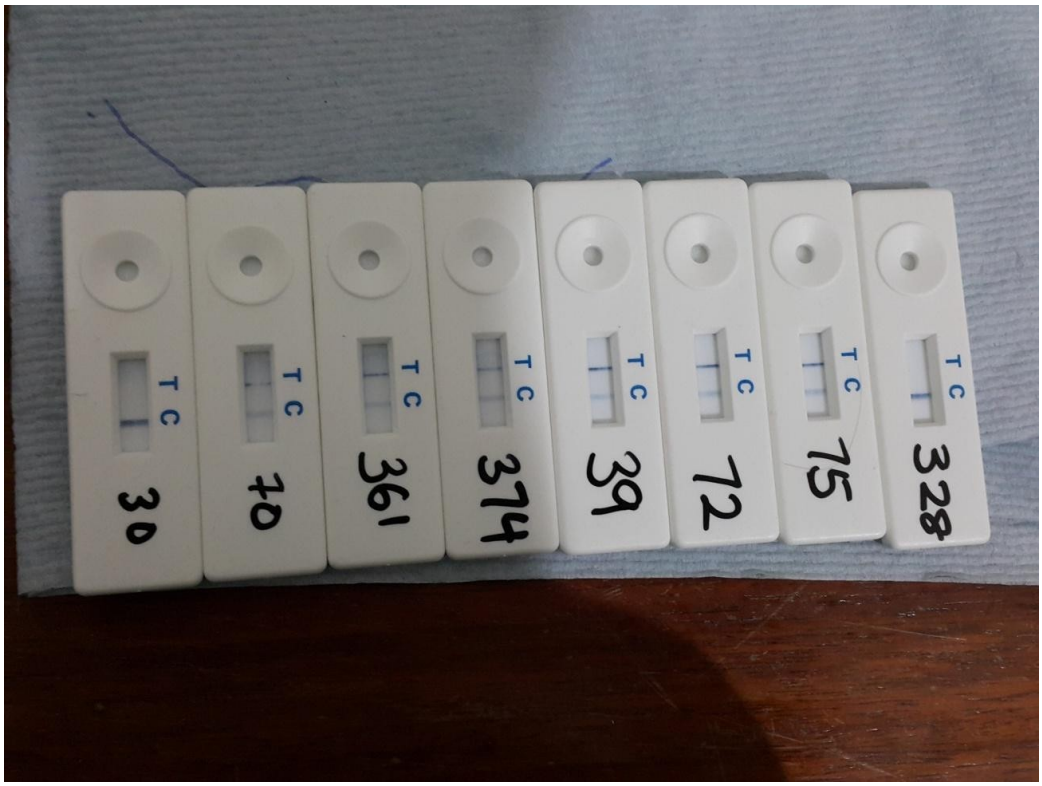

Figure 12: Images of molecular lateral flow devices representing post amplification detection of FMDV by using labeled primers.

Legend for Lateral Flow Devices: Numbers = Indicate sample ID:

T=Test; and C=Control line.

Table 5: Results of RT-LAMP analysis

Serial No.	Sample ID	Location	Year of Outbreak	RT-LAMP Time of positivity	Anneal derivatives for RT-LAMP	RT-PCR Cut-off values	Antigen-ELISA
1	37	Tabora	2010	12	88.34	14	O
2	271	Rufigi	2011				
3	355	Morogoro	2012			13	
4	62	Tanga	2011			24	
5	326	Longido	2012			16	
6	325	Longido	2012			22	
7	287	Tabora	2011			19	A
8	12	Musoma	2010			24	
9	29	Kasulu				24	
10	38	Kasulu				28	
11	82	Morogoro	2012			19	
12	139	Mabuki		21	88.75		
13	142	Mabuki				27	
14	359	Kimara		28.15	89.25		
15	70	Tanga	2011	15.15	89.24	20	
16	30	Tabora	2009	26	88.94	18	O
17	125	Mtwara	2011			21	A
18	282	Kasulu	2011			24	
19	374	Karagwe	2013	27	88.94	15	A
20	361	Mafinga	2013	30	89.92	14	O
21	362	Mafinga	2013			21	O
22	39	Musoma	2010	17	88.04	18	O
23	72	Mtwara	2011	30	88.29	26	O
24	75	Iringa	2012	28.25	88.41	20	O
25	77	Iringa	2012	27	88.09	18	SAT2
26	328	Longido	2012			25	
27	364	Mafinga	2013	20	88.44	21	
28	449	Mwika	2013	19.05	88.39	39	NEG
29	43	Iringa	2011			23	
30	321	Mbeya	2012			21	
31	355	Morogoro	2012	18	89.05	13	SAT1
32	357	Morogoro	2012	30	89.39	18	O
33	363	Iringa	2013			32	O
34	378	Muleba	2013			16	
35	376	Muleba	2013			16	

Table 6: Results of FMDV serotype-specific RT-LAMP.

Serial No.	Sample ID	ELISA	Serotype A	Serotype O
1	37	O	Positive	Negative
2	359	SAT 1	Positive	Positive
3	355	SAT 1	Positive	Positive
4	357	O	Positive	Negative
5	30	O	Negative	Positive
6	449	NA	NA	NA
7	75	O	Negative	Positive

Table 7: Table indicating time for positivity in relationship to zone where samples were collected

Zones	**Time of positivity for different samples in minutes**		
East	18	30	30
West			
North			
Southern highlands		30	12
Central		26	12
Lake zone		21	27

4.2 DISCUSSION

Foot-and-mouth disease is a highly contagious disease of livestock, affecting cloven-hoofed animals. Diagnosis of the disease is critical in initiating effective control strategy. The aim of this study was to use LAMP for rapid detection of FMDV using serotype-specific primers under field conditions. Foot-and-mouth disease is endemic in most sub-Saharan Africa with six (A, O, C, SAT1, SAT2 and SAT3) out of seven serotypes circulating worldwide. In Tanzania four serotypes (A, O, SAT1 and SAT 2) and many topotypes cause disease outbreaks each year in different parts of the country. In this study a targeted RT-LAMP assay was evaluated using pan-serotype primers and used in diagnosis of FMD in field samples.

In this study the validated pan-serotypic RT-LAMP assay was used in field deployment for testing FMD suspected samples. All samples (n=35) were tested for FMDV genome of which 40% (n=40) were positive with time of positivity ranging from 12 to 30 (Table 4). These results were similar to those obtained in a parallel study conducted using 3D real time RT-PCR at Centre of Infectious Disease and Biotechnology (CIDB) laboratory which indicated that the assay was working optimally. Samples used were all epithelial cells which were pre diluted 1:5 and analyzed. The observed Tp values were ranging from 12 to 30 in all zones and there was no significant variation on Tp values from different zones (Table5).

These results are in agreement with previous study by Duke *et al*. (2006) that demonstrated the sensitivity and specificity of RT-LAMP assay when applied for diagnosis under field condition.

However, 46 % (n=16) samples which were positive in real-time RT-PCR were not detected by RT-LAMP. The failure of RT-LAMP assay to detect certain isolates could be ascribed to (i) high concentration of RNA inhibitors in the epithelial samples, (ii) sequence changes at specific sites within FMD virus genome, (iii) low concentration of RNA in the tested samples, and (iv) sequence mismatch at $3^{'\,end}$ F1 and/or 5'-endof the primers B1. Furthermore, discordance of these results can be explainedbydifficulties in maintenance of cold chain during transportation and storage of samples that could contribute to denaturation of RNA.

These observations indicate that RT-PCR was superior to RT-LAMP for detection of FMDV genome from tissue epithelial samples. Studies conducted by Kasanga *et al.*(2014), Dukes *et al.*(2006), Parida *et al.*(2008) and Ding *et al.*(2014) revealed that RT-LAMP was superior to RT-PCR. The current findings indicate that RT-PCR is superior to RT-LAMP. The variation of the current findings compared to the results reported in previous studies could be ascribed to (i) different nature of the sample used in the experiments, (ii) different FMDV serotype(s) that were examined using different primers per specific serotype, and (iii) sample denaturation due long storage condition.

The validated assay condition for pan-serotype RT-LAMP were used to test FMDV serotype-specific primers for A and O. A total of 14 samples (100%) were tested by using primers for both serotypes A and O. In this case 28% (n=4) were FMDV serotype A, 36% (n=5) were FMDV serotype O whereas the remaining 36% (n=5) were neither serotype A nor type O. Since these samples were not tested for FMDV serotype SAT 1 and SAT 2, which are known to be circulating in Tanzania (Kasanga

et al., 2014), it raises the possibility that the negative samples for FMDV serotypes A and O could be either FMDV serotypes SAT1 or SAT 2 or true negatives.

A simultaneous experiment conducted at the laboratory using Ag-ELISA on four samples positive for type A by RT-LAMP indicated that 50% (n=2) were serotype O whereas another 50% (n = 2) had antibodies for SAT 1. The results obtained from serotype-specific assay were 100% discordant to Ag-ELISA. These variations could be due to cross reactivity of the primers since VP 1 region of FMDV genome is highly variable with high mutation rate which may result into false negative results.

A pilot parallel study conducted at CIDB using Ag-ELISA assay revealed that 42% (n=3) of the samples were serotype O whereas 28% (n=2) were SAT 1. These results show 29% discordance compared to those for RT-LAMP. Amplification of SAT 1 samples in both A and O primers indicate low specificity of the SAT1 serotype-specific primers, which ultimately lead to non-specific amplification of the target fragment. A similar observation has also been reported by Duke (2006) suggesting that high mutability of this region may cause false negative and could impact on the accuracy of serotype-specificity of RT-LAMP assay.

CHAPTER FIVE

5.0 CONCLUSIONS ANDRECOMMENDATIONS

5.1 CONCLUSIONS

This study detected FMDV genome in the samples collected from different zones in Tanzania. It was revealed that several outbreaks of FMD occurred in different zones since 2009-2013. The findings of this study have shown that:

(i) The standardized RT-LAMP can be used for field detection and identification of FMDV following suspected FMD outbreaks in Tanzania.

(ii) There is a high potential of using serotype-specific RT-LAMP for typing FMDV field strains, although further experiments might be required to validate serotype-specific assays.

5.2 RECOMMENDATIONS

(i) It is recommended that serotype-specific primers for detection of FMDV serotypes A and O viruses should be re-designed using the improved methods targeting the conserved VP1 coding regions of the recently sequenced viruses from the Tanzania and neighbouring countries.

(ii) Since there is current evidence for FMDV serotypes A, O, SAT 1 and SAT 2 circulation in Tanzania, it is recommended that the development of SATs 1-3-specific primers should be instituted to enhance the wide range typing of the FMDV strains under the field conditions.

(iii) Further experiments to validate the assays using different FMDV strains from the region should be conducted.

(iv) Further in-depth investigations to examine other serotype(s) from the tested samples are recommended to enhance the understanding of circulating serotypes in region.

REFERENCES

Alexandersen, S., Brotherhood, D. A. (2002). Natural aerosol transmission of Foot-and-mouth disease virus to pigs: Minimal infection dose for strain O1 Lausanne. *Journal of Epidemiology and Infection* 128: 301-312.

Alexandersen, S., Zhang, Z., Donaldson, A.I., and Garland, A.J.M. (2003). Review: The Pathogenesis and diagnosis of Foot-and-mouth Disease. *Journal of ComperativePathology*129: 1–36.

Bannett, P. V and Cox, S. J. (1999). The role of small ruminants in epidemiology of foot-and-mouth disease. *Veterinary Journal* 158: 6-13.

Callahan, J.D., Brown, F., Osorio, F.A., Sur, J.H., Kramer, E., Long, Lubroth, G.W.,Ellis, J., Shoulars, S.J., Gaffney, K.S., Rock, K.L., Nelson, D.L., (2002). Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal American Veterinary Medical Association* 220(11):1636–1642.

Chakraborty, S., Kumar, N., Dhama, K., Verma A.K., Tiwari, R., Kumar, A., Kapoor, S.S. (2014). Foot–and–Mouth disease, an economically important disease of animals. *Advances in Animal and Veterinary Science* 2(2S):1–18.

Dhama, K., Karthik, K., Chakraborty S., Tiwari R., Kapoor, S., Kumar, A., and Thomas, P., (2014). Loop-mediated Isothermal Amplification of DNA (LAMP): A New Diagnostic Tool Lights the World of Diagnosis of Animal and Human Pathogens: A Review. *Pakistan Journal of Biological Sciences* 17: 151-166.

Domingo, E., Mateu, M. G., Mart´ınez, M. A., Dopazo, J., Moya, A., and Sobrino, F., (1990). "Genetic variability and antigenic diversity of foot-and-mouth disease virus*,"* in *Applied Virology Research* Kurstak, E., Marusk R.G., Murphy, S.A., and Van Regenmorte M. H. V. l, Eds., vol. 2, pp. 233–266.

Donaldson, A.I and Alexandersen, S. (2002). Predicting the spread of Foot-and-mouth disease virus by airborne virus. *Review and Scientific technical Office International des Epizotes*21 :(3) 569-575.

Dukes, J. P., King, D.P., and Alexandersen, S. (2006).Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Archives of Virology* 151(6):1093-106.

Dukes, J.P., King, D.P., Alexandasen, S. (n.d). Rapid, simple, field deployable Foot-and- mouth disease virus detection. www.fao.org>doc>paphos>App47.

Frank, S.A. (2002). Experimental evolution; Foot-and-Mouth Disease Virus In: *Immunology and Evolution of Infectious Disease*. Princeton University press, Princeton New Jersey.358pp.

Guohui, L., Zhipeng C., Junfeng, W., Xiu-Feng, W., Lizhe, X. and Randy, G. (2008). Identifying a few foot-and-mouth disease virus signature nucleotide string for computational genotyping. *BCM Bioinformatics* 9:279 doi: 10.1186/1471-2105-9-279.

Hoffman, B., Beer, M., Reid, S.M., Mertens, P., Oura C.A.L., Van Rijn, P.A., Slomka M.J., Banks, J., Brown, H.I., Alexander, D.J., King D.P. (2009). A review of RT-PCR technologies vorology and disease control: Sensitive and specific diagnosis of five livestock disease notifiable to World Organisation for Animal health .*Veterinary Microbiology* 139:1-23.

Jamal, M. and Belsham, G.J. (2013). Foot-and-mouth disease. Past, present and future. *Veterinary Research* 44:116 doi10.1186/1297-9716-44-116.

Kaneko, H., T. Kawana, E., Fukushima and Suzutani, T. (2007).Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal Biochemical and Biophysical Methods* 70: 499-501.

Kasanga, C.J, Wataru, Y., Mioulet, V., King, D. P., Mulumba, M. Ranga, E., Deve, J., Mundia, C., Chikungwa, P., Joao, L ., Wambura, P. N., Rweyemamu M. M.(2014). Rapid, sensitive and effective diagnostic tools for foot-and-mouth disease virus in Africa. *Onderstepoort Journal Veterinary Research* 23; 81(2):E1-5.

Kasanga, C.J., Sallu, R., Kivaria, F., Mkama, M., Masambu, J., Yongolo, M., Das, S., Ngeleja, C.M., Wambura, P., King, D., Rweyemamu, M. (2012). Foot-and- mouth disease serotypes detected in Tanzania from 2003-2010: Conjecctured status and future prospects. *Onderstepoort Journal Veterinary Research* 79(2) 462 page 1-4.

King, D.P., Madi, M., Mioulet, V., Wadsworth, J., Wright, CF., Valdazo-González, B., Ferris, N.P., Knowles, N.J., Hammond, J. (2012). New technologies to diagnose and monitor infectious diseases of livestock: challenges for sub Saharan Africa. *Onderstepoort Journal Veterinary Research*79(2):456.

Kivaria, F. M (2003). Foot and mouth disease in Tanzania: An Overview of its national status. *Veterinary quarterly* 25(2)72-8. DOI: 10.1080/01652176.2003.9695147.

Li-na, M., Jie, Z., Hao-tai, C., Jian-hua, Z., Yao-zhong D., Yong-sheng L. (2011). An overview on ELISA techniques for FMD. *Virology Journal* 8:419.

Longjam, N., Rajib-Deb, A. K., Sarmah, T. T, Awachat, V. B, and Saxena, V. K. (2011).A Brief Review on Diagnosis of Foot-and-Mouth Disease of Livestock: Conventional to Molecular Tools. *Veterinary Medicine International* Article ID 905768, 17 pages, doi:10.4061/2011/905768.

Moniwa, M., Clavijo, A., Li, M., Collignon, B., Kitching, P.R. (2007). Performance of a foot-and-mouth disease virus reverse transcription–polymerase chain reaction with amplification controls between three real time instruments. *Journal of Veterinary Diagnostic Investigation* 19(1):9-20.

Moonen, P. Boostra, J., Honing H., Boonstra-Leendertse, C., Jacob, L., and Dekker,A. (2003). Validation of Light Cycler-based reverse transcriptase polymarase chain reaction for the detection of foot-and-mouth disease virus.*Journal of Virology Methods* 113: 35-41.

Mori, Y. and Notomi, T. (2009).Loop-mediated isothermal amplification (LAMP): A rapid, accurate and cost-effective diagnostic method for infectious diseases. *Journal Infectionand Chemotherapy*15: 62-69.

Morrell, D. J., Mellor E. J. C., Rowlands, D. J. and Brown, F. (1987). Surface structure and RNA-protein interactions of foot-and-mouth disease virus, *Journal of General Virology*68 (6):1649–1658.

Murphy, F. A., Gibbs, E.P.J., Horzinek, M.C. and Studdert, M.J. (1999). *Picornaviridae Veterinary Virology* 3rd edition. London, UK: Academic Press.

Mwiine, F.N., Ayebazibwe, W., .Olaho-Mukani, Alexandersen S., and Tjornehoj, K. (2010). Prevalence of Antibodies Against Foot-and-Mouth Disease Virus in Cattle in Kasese and Bushenyi Districts in Uganda. *International Journal of Animal and Veterinary Advance 2*(3): 89-96.

Nagamine, K., Hase T., and Notomi T. (2002a). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes 16*: 223-229.

Nagamine, K., Watabane, K., Ohtsuka, K., Hase, T., and Notomi, T. (2001). Loop mediated isothermal amplification reaction using non-denatured template. *Clinical Chemistry 47*: 1742-1743.

Nagamine, K., Y. Kuzuhara and Notomi, T. (2002b). Isolation of single-stranded DNA from loop-mediated isothermal amplification products. *Biochemical Biophysical Research Communication* 290:1195-1198.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watabane, K., Amino, N. and Hase, T. (2000). Loop-mediated isothermal amplification. *Nucleic AcidsResearch Volume* 28, No: 12 pE63.

Oem, K. J., Ferris, N.P., Lee, K. N., Joo, Y.S., Hyun, B. H., Park, J. H.(2009).Simple and Rapid Lateral-Flow Assay foe the detection of Foot-and-mouth disease Virus. *American Society for Microbiology*doi: 10.1128/CVI.00213-09p.1660-1664.

Oem, J.K., Kye, S.J., Lee, K.N., Kim, Y.J., Park, J.Y., Park, J.H., Joo, Y.S., Song,H.J. (2005). Development of a Light cycler-based reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus. *Journal Veterinary Science and Technology 6* (3): 207–212.

Office International des Epizooties (OIE). (2012). Foot-and-mouth disease. In: Manual of Standards for Diagnostic Tests and Vaccines for Terrestrial Animals, 7th edition. Paris, OIE. [http://www.oie.int/eng/normes/mmanual/200 8/pdf/2.1.05FMD.pdf.] Site visited on March 10, 2013

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.L.V. Morita, K. (2008). Loop mediated isothermal amplification (LAMP) A new generation innovative gene technique; perspective in clinical diagnosis of infectious diseases. *Review in Medical Virology* 18:407-421.

Picado, A., Speybroeck, N., Kivaria, F., Mosha, R. M., Sumaye, R. D., Casal, J. and Berkvens, D. (2010). Foot-and-Mouth Disease in Tanzania from 2001 to 2006.*Transboundary and Emerging Diseases* 58: 44–52.

Rasmussen, T.B., Uttenhal, A., de Stricker, K., Belak, S., and Storgaad, T. (2003). Development of novel quantitative real-time RT-PCR assays for simultaneous detection of all serotypes of foot-and-mouth disease virus. *Archives of Virology 148*: 2005-2021.

Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z., Belsham, G.J., Alexandersen, S., (2002). Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *Journal Virology Methods* 105 (1):67–80.

Vosloo, W., Bastos, A.D., Sangare, O., Hargreaves, S.K., and Thomson, G.R. (2002). Review of the status and control of Foot-and-mouth disease in sub-Saharan*Africa Revue Scientifique et Technique 21:437*-449.

Vosloo, W., Dwarka, R.M., Bastos, A.D.S., Esterhuysen, J.J., Sahle, M., Sangare, O. (n.d). Molecular epidemiological studies of Foot-and-Mouth disease virus in sub-Saharan Africa indicate the presence of large numbers of topotypes: implications for local and international control. *Appendix 22.*

Vosloo, W., and Thomson, G. R. (2004). Foot-and-mouth disease. In Natural Habitats in which Foot-and-Mouth Disease Viruses are Maintained, Foot-and-mouth disease pp.383–410. Edited by Domingo E., Sobrino F. Horizon Scientific Press.

Waters, R.A., Fowler, V.L., Armson, B. (2014). Preliminary Validation of Direct Detection of Foot-And-Mouth Disease Virus within Clinical Samples Using Reverse Transcription Loop-Mediated Isothermal Amplification Coupled with a Simple Lateral Flow Device for Detection. Menéndez-Arias L, ed. *PLoS ONE*. 2014; 9(8):e105630. doi:10.1371/journal.pone.0105630.

Yamazaki, W., Mioulet, V., Murray, L., Madi, M., Haga, T. Misawa, N. (2013). Development and evaluation of multiplex RT-LAMP assays for rapid and sensitive detection of foot-and-mouth disease virus. *Journal of Virological Methods* 192(1–2): 18–24.

Yang, X., Ying-shun, Z., Hong-ning W., Yi Z., Kun Wei, T.W. (2011). Isolation identification and complete genome sequence analysis of a strain of foot-and- mouth disease virus serotype Asia 1 from Pigs in southwest China. *Virology Journal* 8. 1. 175.