DEVELOPMENT AND EVALUATION OF A REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR RAPID TYPING OF SEROTYPE “O” FOOT-AND-MOUTH DISEASE VIRUS IN ENDEMIC SETTINGS OF TANZANIA.

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

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ABSTRACT

The foot-and-mouth disease (FMD) is an economically important transboundary animal disease (TADs) affecting all cloven-hoofed animals. It is caused by foot-and-mouth disease virus (FMDV), which has seven antigenically distinct serotypes. FMD is endemic in Tanzania, with outbreaks caused by predominantly five serotypes. In order to improve the control of this disease, for instance using serotype-specific vaccines, rapid detection and identification of circulating FMDV strains is of paramount importance. This study describes the development and evaluation of a reverse transcription loop- mediated isothermal amplification (RT-LAMP) assay for diagnosis of serotype 'O' FMDV in endemic settings in Tanzania. A retrospective study design was employed for this research whereby a total of forty-four (n=44) archived epithelial tissue samples were analyzed by RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), RT-LAMP and DNA sequencing. Primers for RT-LAMP targeting serotype 'O' FMDV isolates in Tanzania were developed and found to optimally amplify the targeted gene at 65.0˚C for 45 minutes in the presence of both Avian Myeloblastosis Virus (AMV) reverse transcriptase enzyme and loop primers. The results indicated that RT-LAMP assay could amplify the viral protein 1 (VP1) gene of serotype 'O' FMDV within a range of 13 to 26 minutes, with annealing temperatures of between 70.0 and 89.0°C. The findings indicate that RT-LAMP assay is highly specific as no cross- reactivity occurred between serotype 'O' primers with any of the other serotypes. The sensitivity as indicated by the detection limit of the assay was deduced to be 3.78×10^{-2} ng/µl. This study concludes that RT-LAMP assay could be used to rapidly and accurately detect VP1 gene of serotype 'O' FMDV from Tanzania. It is advisable that further studies

are required to evaluate the comparative sensitivity of the assay and check whether the assay is field deployable. Further evaluation is also needed to determine whether the assay would be useful for detecting serotype ‘O’ FMDV strains circulating in other regions in Tanzania.

DECLARATION

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

_____ _____

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The above declaration if confirmed

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DEDICATION

This work is dedicated to the late Mary Bahati. Rest in Peace Mother.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMV	Avian Myeloblastosis Virus
AT	Adenine-thymine
B1c	Backward inner complementary primer
B2	Backward internal primer
B3	Backward outer primer
BIP	Backward inner primer
BLASTN	Basic Local Alignment Search Tool Nucleotide
BLP	Backward loop primer
Bsm	*Bacillus smithii*
Bst	*Bacillus stearothermophilus*
CPE	Cytopathic effect
CFT	Complement Fixation Test
CVMBS	College of Veterinary Medicine and Biomedical Sciences
EA	East Africa
ELISA	Enzyme Linked Immunosorbent Assay
EURO-SA	Europe-South America
F1c	Forward inner complementary primer
F3	Forward external primer
FAO	Food and Agriculture Organization of the United Nations
FIP	Forward inner primer
FLP	Forward loop primer
FMD	Foot-and-mouth disease
FMDV A	Foot-and-mouth disease virus serotype A

FMDV O	Foot-and-mouth disease virus serotype O
FMDV SAT1	Foot-and-mouth disease virus serotype SAT1
FMDV SAT2	Foot-and-mouth disease virus serotype SAT2
FMDV SAT3	Foot-and-mouth disease virus serotype SAT3
FMDV	Foot-and-mouth disease virus
GF-TAD	Global Framework for Transboundary Animal Diseases
HIV	Human Immunodeficiency Virus
IGAD	Intergovernmental Authority on Development
ISA	Indonesia
LAMP	Loop Mediated Isothermal Amplification
LFD	Lateral flow device
LPBE	liquid phase blocking ELISA
MAbs	Monoclonal antibodies
ME-SA	Middle East-South Asia
NC	Negative control
NCBI	National Center for Biotechnology Information
NSP	Non-structural protein
OIE	World Organization for Animal Health
ORF	Open reading frame
PAbs	Polyclonal antibodies
PAUSTI	Pan African University Institute for Basic Sciences, Technology and Innovation
PBS	Phosphate Buffered Saline
PC	Positive control
PCP	Progressive Control Pathway

PCR	Polymerase Chain Reaction
RGD	Tripeptide arginine-glycine-aspartic acid
rRT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
RT	Reverse transcription
RT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
RT-LAMP	Reverse Transcription-Loop Mediated Isothermal Amplification
SACIDS-ACE	Southern African Centre for Infectious Diseases Surveillance-African Centre of Excellence
SADC	Southern African Development Community
SAT	Southern African Territories
SEA	Southeast Asia
SK1	Tanzania FMDV serotype O LAMP primers
SPCE	Solid phase competition ELISA
SUA	Sokoine University of Agriculture
TAD	Transboundary Animal Disease(s)
TBE	Tris-borate EDT
TTR	Time to Result
TTT	Time to Threshold
U	Unit(s) of enzyme
USDA	United States Department of Agriculture
UTR	Untranslated Region
VI	Virus isolation

VNT	Virus Neutralization Test
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3
VP4	Viral Protein 4
WA	West Africa
WRLFMD	World Reference Laboratory for Foot-and-Mouth Disease

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Foot-and-mouth disease (FMD) is an economically important transboundary animal disease (TADs) affecting all cloven-hoofed animals. This viral disease threatens sustainable food production leading to food insecurity. Food security is one of the drivers towards achieving zero hunger within the global Goals for Sustainable Development and the African Union Agenda 2063. Achievement of these goals are constrained by several factors including foot-and-mouth disease (OIE and FAO, 2018a). The burden of FMD on developing countries cannot be underestimated as it threatens food security and economic growth in countries where the disease is endemic (OIE and FAO, 2018b). FMD is a trans-boundary, highly contagious viral disease of all cloven-hoofed wildlife and livestock animals. It is caused by the Foot-and-mouth disease virus (FMDV), a small, naked, single-stranded, positive-sense RNA genome virus. The virus belongs to the genus *Aphthovirus* of the family *Picornaviridae* and exists as seven immunologically distinct serotypes (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3) (Kasanga *et al.*, 2014; Bastos *et al*., 1998; Belsham *et al.*,2005; Grubman and Baxt 2004). Within each serotype, genotypes and/or topotypes with significant antigenic variability exist. This ensues from diverse genetic characteristics that is enhanced by its high replication process (Domingo *et al.,* 1997). Protection from one serotype does not confer immunity against the other circulating serotypes in a given geographical area (Balinda *et al.*, 2010). The high antigenic diversity nature yields FMDV variants making the disease difficult to control.

FMD exhibit a broad host spectrum as it affects more than 70 different livestock and wildlife species (Thomson *et al.,* 2003b) notably cattle, swine, goats and sheep. The African buffalo (*Syncerus caffer*) acts as the main reservoir for Southern African Territories (SAT) serotypes in African continent. For infection to occur, FMDV requires low infectious dose and a substantial level of viral excretion (Alexandersen *et al.,* 2003b).

Transmission can occur via several routes mostly through direct contact as seen in pigs or aerosol droplets as in the case of ruminants. For an infected animal, the FMDV is shed into in all secretions and excretions, hence transmission is facilitated via direct contact between infected and susceptible animals. Indirect transmission is evidenced through fomites, animal products such as milk and untreated meat products from an infected animal (Alexandersen *et al.,* 2003a) and in other cases wind has been implicated. Reports claim that FMDV infections can spread over long distances by wind, especially when favored by cold and humid conditions (Donaldson *et al.,*1972). In light of this, FMDV virulence and degree of transmission may be attributed to the difference in viral strain, type of infected animal and the extent in which they excrete the virus (Alexandersen *et al.,* 2003b; Kitching *et al.,* 2002).

FMD is evidenced mostly in pigs and cattle but slightly present itself in small ruminants. Clinically the disease manifests as vesicles (feet, mouth, tongue, mammary glands and interdigital space), lameness, excessive salivation and anorexia. The disease is highly infectious with low mortality rate in adult animals and notably high mortalities in young stock due to myocarditis (Alexandersen *et al.,* 2003a). Direct repercussions of this disease are evidenced in death (primarily in

young livestock), weight loss, abortion and drop in milk production. Indirect effects include loss of draught power for crop production, and the costs incurred in controlling the disease and trade sanctions. FMD incursion into countries known to be disease-free causes massive losses, one of such incidences was seen during the 2001 outbreak in the United Kingdom where losses of billions of pounds ensued (Knight-jones and Rushton, 2013).

FMDV has an RNA genome of 8.5 kb that encodes a polyprotein which undergoes cleavage to 4 structural proteins (VP1–VP4) which form the virus capsid and 8 non-structural proteins vital for protein processing and for virus replication. All structural proteins are exposed to the surface with the exception of VP4 (Belsham *et al.*, 2005; Grubman and Baxt, 2004). The VP1 coding region sequence has been extensively used to give insight on the genetic, evolutionary dynamics and diagnosis of FMDV which is vital in understanding and inferring the epidemiological patterns (such as tracing the origins of outbreaks) of these viruses and determine appropriate strategies for FMD control (Bastos *et al.*, 2003; Knowles and Samuel, 2003; Kasanga *et al*., 2014; Valdazo-González *et al.*, 2011)

Foot-and-mouth disease is endemic in most of sub-Saharan countries. In southern Africa, SAT 1, 2 and 3 serotypes are prevalent. In East Africa FMDV is classified under pool 4 with outbreaks in cattle being attributed to five serotypes. Serotypes A, O, SAT1, SAT2 and SAT3 are predominantly prevalent (Vosloo *et al.,* 2002). SAT 3 was recorded once in Uganda in 1970 while serotype C was last reported in 2004, Kenya (Sangula *et al.,* 2011). The disease spreads within domestic animals or from wildlife to domestic animals, the latter being observed in southern Africa countries (Vosloo and Thompson, 2004).

Foot-and-mouth disease is endemic in Tanzania and outbreaks occur throughout the year in different geographic locations. Currently, serotypes O, A, SAT 1 and SAT 2 are in circulation in the country (Kasanga *et al.*, 2014). Serotype 'O' is among those with the highest distribution in Tanzania (Kasanga et al., 2014; Knowles and Samuel, 2003). The serotype is typically divided into 11 topotypes with Tanzania topotypes lying within EA-2 in the broader category of East Arica 1 to 4 (EA-1 to -4). This serotype has a greater genetic diversity that allows the classification of many distinct lineages, in contrast, the antigenic variation is not wide and relatively few vaccine strains suffice in protecting against most field outbreaks (Knowles and Samuel, 2003).

The socio-economic implications of FMD cannot be underestimated neither can it be measured in monetary value. The disease is known to cause economic strain on Tanzania's livestock sector, due to trade embargo on livestock and their products, overall, this affects national development goals (Kivaria *et al.*, 2003; Maree *et al.*, 2014; Rweyemamu *et al.*,1984; OIE and FAO, 2018b). The persistence nature of FMDV serotype 'O' in some of the susceptible hosts, surveillance and timely diagnosis is implicated as the main hindrance to the control and elimination of the disease. Due to the infectious nature and economic implications of the disease, rapid and sensitive diagnostic tests for identifying FMDV serotype causing the disease outbreak(s) is imperative (Jamal and Belsham, 2013; Kasanga *et al.*, 2012).

Diagnosis of the disease is based on clinical signs, such as profuse salivation, vesicular lesions (e.g., on tongue) etc. Nevertheless, clinical manifestation of FMD resembles other vesicular diseases, such as vesicular stomatitis, swine vesicular disease and vesicular exanthema of swine. This resemblance pose constrains on early confirmation of field outbreaks (Jamal and Belsham, 2013; Kasanga *et al.*,

2012). Furthermore, diagnosis involves a pipeline that usually begins with recognition of clinical sign of the disease, followed by collection of samples, transportation of the specimens to national laboratories, sample analysis using available diagnostic tests and lastly reporting of results (Howson *et al.*, 2017). However, transportation of the samples from the field to the laboratory requires effective cold-chain, takes times and access to remote areas might prove to be a challenge. This subsequently might affect the integrity of the sample and increase the turn-around time (time between sample collection and results dispatch) which is vital for decision making hence controlling the disease in the event of an outbreak. Laboratory based diagnosis of FMD can be confirmed by employing test such as enzyme-linked immunosorbent assay (ELISA), virus neutralization test (VNT) and complement fixation test (CFT). These diagnostic tests consume time, are labor intensive and less sensitive (Jamal and Belsham, 2013; Kasanga *et al.,* 2012; Maryam *et al.,* 2017).

Recent molecular approaches such as polymerase chain reaction (PCR) have been extensively acknowledged for the quantification and detection of FMDV, notably diagnostic rRT-PCR assays have been developed for detection of FMDV serotypes across various sample types (Callahan *et al.,* 2002; Reid *et al.,* 2002). PCR assays has been employed in FMDV serotyping but require sophisticated equipment like a thermal cycler, costly reagents and gel documenting unit (Yamazaki *et al.,* 2013). Additionally, these techniques require trained personnel and well-equipped laboratories. In limited resource settings, these limitations affect the diagnosis of FMD which is vital and initial step in controlling the disease. This warrants the development of FMD diagnostic assay that is field deployable and is rapid, accurate, cost-effective and simple (Maryam *et al.,* 2017; Madhanmohan *et al.,* 2013; Kasanga *et al.,* 2012; Farooq *et al.,* 2015; Howson *et al.,* 2017).

Since its development and inception in 2000 (Notomi *et al.,* 2000), loop-mediated isothermal amplification (LAMP) has been an instrumental tool for detection of both animal and human diseases. LAMP has high specificity and sensitivity, simplicity and rapidity (Dhama *et al.,*2014; Mori and Notomi, 2009). Amplification takes place at a single temperature and it is rapid; typically less than 30 minutes, utilizes DNA with high strand displacement activity and can detect fewer DNA or RNA copies. LAMP can be adapted for RNA templates by addition of reverse transcriptase (RT) or by using an enzyme with both DNA polymerase and RT activities (Notomi *et al.,* 2000; Madhanmohan *et al.,* 2013; Hong *et al.,* 2004). An RT-LAMP assay for the detection of FMDV was first developed by Dukes *et al.,* (2006). Several RT-LAMP assays been later developed for detecting the seven serotypes of FMDV (Shao *et al.,* 2010; Chen *et al.,* 2011; Guan *et al.,* 2013; Yamazaki *et al.,* 2013; Waters *et al.,* 2014; Farooq *et al.,* 2015; Lim *et al.,* 2018). Serotype-specific RT-LAMP for diagnosing individual FMDV serotypes O, A, C, and Asia 1 have been developed (Madhanmohan *et al.,* 2013; Maryam *et al*., 2017; Lim *et al.,* 2018; Chen *et al.,* 2011; Ding *et al.,* 2014)

Rapid FMDV serotyping is imperative under field condition to understand the nature of outbreak various serotypes in Tanzania, hence rational of the disease control. The most common FMDV serotype in outbreaks is serotype ‘O’, hence an RT-LAMP assay for specific detection of this virus is required (Brito *et al.*, 2017; Jamal and Belsham, 2013).

However, the only serotype ‘O’ FMDV specific RT-LAMP assay is reported to have been developed by Madhanmohan *et al.,* (2013), India, Maryam *et al.,* (2017), Pakistan, and Lim *et al*., (2018), South Korea. These assays have been developed specifically for isolates prevalent in these geographical areas. However, the

suitability of these assays for the diagnosis of FMDV serotype ‘O’ circulating in other countries, particularly sub-Saharan Africa has not yet been verified. This study aims to develop, an RT-LAMP assay using newly designed primers for the detection of serotype ‘O’ FMDVs circulating in the Tanzania and to verify the suitability of RT-LAMP assay from Pakistan (Maryam *et al.,* 2017) on Tanzania FMDV serotype ‘O’. This is the first serotype specific RT-LAMP assay that has been developed in sub-Saharan Africa for diagnosis of FMD. The information gathered from this study will be vital in contributing to adoption of appropriate control measures and surveillance of FMD in Tanzania.

1.2 Problem Statement and Justification of the Study

Foot and mouth disease is a disease of economic importance with severe constraint to national, regional and international trade on livestock and livestock products (Grubman and Baxt, 2004; OIE and FAO, 2018b). FMD is endemic in Tanzania, with outbreaks occurring throughout the year. Currently, serotypes O, A, SAT 1 and SAT 2 are circulating in the country with serotype ‘O’ among those with highest distribution (Kasanga *et al.,* 2015). Timely diagnosis is implicated as a hindrance to the control and elimination of the disease. Laboratory based diagnosis of FMD are laborious and consumes time, whereas recent and novel molecular approaches like RT-PCR require specialized conditions, expertise and a well-equipped laboratory; limitations that make these techniques less attractive in limited resource settings (Kasanga *et al.,* 2014; Jamal and Belsham, 2013). FMDV serotype-specific RT-LAMP assay has not been developed in Tanzania.

Owing to these reasons, it is essential to develop a rational diagnostic method which is prompt, sensitive, specific, simple, cost effective for typing the serotype ‘O’ FMDV. Due to genetic diversity of serotype ‘O’ FMDV, RT-LAMP assay has to be specific to FMDV strains circulating in specific geographical areas so as to improve the diagnostic performance of the assay. In this study, an RT-LAMP assay using newly designed primers was developed for the detection of serotype ‘O’ FMDVs circulating in Tanzania. This is the first serotype specific RT-LAMP assay that has been developed in sub-Saharan Africa for diagnosis of FMD.

The key research questions of the current study were:

1. What is/are the optimum condition(s) for RT- LAMP assay in diagnosis of FMDV serotype ‘O’ in Tanzania?
2. What are the chances that RT-LAMP assay will successfully and rapidly discriminate serotype ‘O’ FMDV in Tanzania?
3. What is the sensitivity and specificity of RT-LAMP assay in detecting serotype ‘O’ FMDV?

1.3 Objectives

1.3.1 General Objective

To develop and evaluate the performance of RT-LAMP assay for diagnosis of serotype ‘O’ in endemic settings in Tanzania for appropriate control measures of FMD in the region.

1.3.2 Specific Objectives

1.2.1.1 To determine the appropriate and optimum conditions for RT-LAMP assay for typing the serotype ‘O’ FMDV strains

1.2.1.2 To examine the presence of serotype ‘O’ FMDV strains from FMD-suspected samples using the optimized RT-LAMP assay

1.2.1.3 To determine the sensitivity and specificity of RT-LAMP assay in typing the serotype ‘O’ FMDV strains

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Foot-and-Mouth Disease

2.1.1 History and Overview of the Disease

FMD is a trans-boundary, highly contagious viral disease of all cloven-hooved wildlife and livestock animals that dates back to 1514. FMD was first observed in cattle and recorded by Fracastorius in 1514, Italy (Fracastorius e*t al.,* 1546). Similar observation was reported 4 centuries that a filterable agent caused FMD (Loeffler and Frosch, 1897). Additionally, similar reports on FMD from Hieronymi Fracastorii were made (Wright *et al.*, 1930), followed later by other documentations of the disease in various countries on a global scale. The disease is caused by foot-and-mouth disease virus (FMDV), a small, naked, single-stranded, positive-sense RNA genome virus. The virus belongs to the genus *Aphthovirus* of the family *Picornaviridae* and exists as seven immunologically distinct serotypes; O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 (Bastos *et al.,* 1998; Kasanga *et al.,* 2015). FMDV is genetically and antigenically diversified.

Within each serotype, topotypes with significant antigenic variability, ensuing from diverse genetic characteristics that is enhanced by its high replication process exist (Domingo *et al.,* 1997). Protection from one serotype does not confer immunity against the other circulating serotypes (Balinda *et al.,* 2010). FMD exhibit a broad host spectrum as it affects more than 70 different livestock and wildlife species (Thomson *et al.,* 2003a). The African buffalo (*Syncerus caffer)* acts as the main reservoir for SATs serotypes in African continent. Transmission of FMDV occurs mostly via direct contact or aerosol droplets and clinically manifest as vesicles

(feet, mouth, tongue etc.), lameness, excessive salivation and anorexia. The disease is highly infectious with low mortality rate in adult animals and notably high mortalities in young stock due to myocarditis (Alexandersen *et al.*, 2003a).

2.1.2 Global Distribution of FMD

Foot and mouth disease is endemic and is currently known to circulate in various continents (Africa, Asia and South America). Continents such as, North and Central America, Europe, Greenland, New Zealand and Australia are currently epidemiologically clustered as FMD-free without vaccination (Fig.1) (Di Nardo *et al.*, 2011).

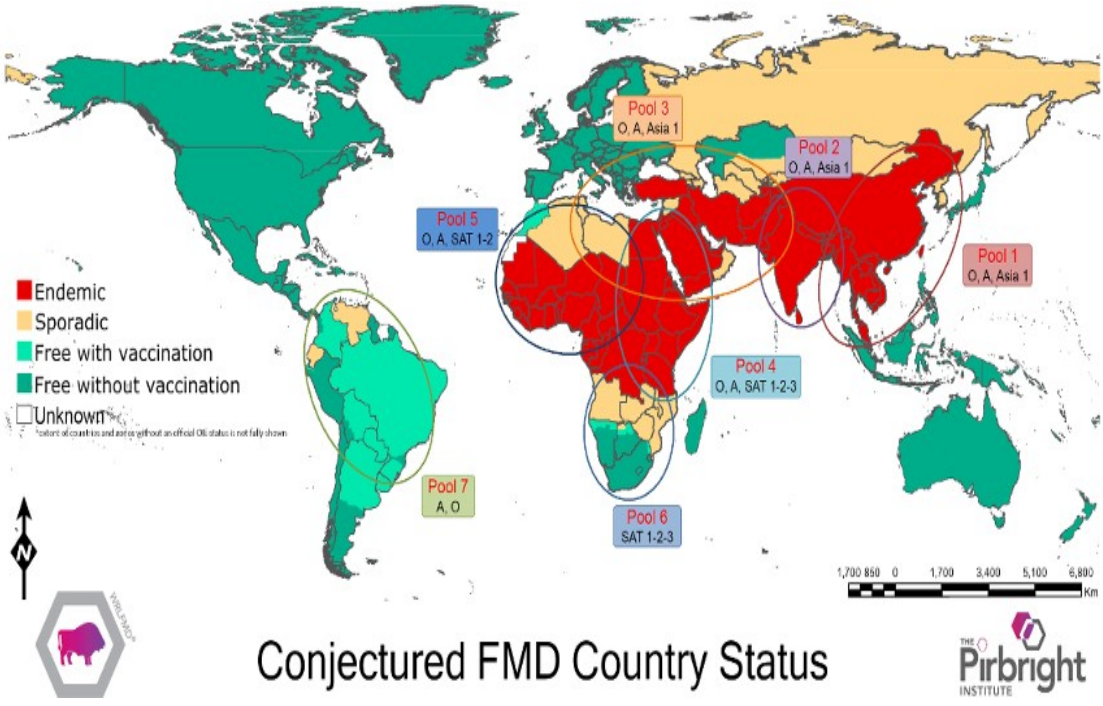

Figure 1: Conjectured status of FMD worldwide. The disease is circulating in South America, Asia and Africa and is further subdivided into seven regional pools. Source: Di Nardo *et al.*, (2011).

Despite being accorded this status, these countries are at risk of occasional incursions as evidenced during the FMD outbreak that was reported in the UK in (Samuel and Knowles, 2001; Cottam *et al.,* 2008). Serotypes O and A have widest distribution and have been implicated to cause outbreaks in various parts of the world. SAT and Asia 1 viruses are normally confined in sub-Saharan Africa and Asia respectively while serotype C was last isolated in Kenya (Knowles and Samuel, 2003; Sangula e*t al., 2*011). FMDV serotypes cluster into type-specific subtypes when nucleotide or amino acid sequences are compared. Serotypes are further subdivided into topotypes based on 15-20% variation in the genetic sequence of VP1 region (Samuel and Knowles, 2001; Knowles and Samuel, 2003; Kasanga *et al.,* 2014)

The antigenic diversity differs between serotypes, with SAT 2 and A being particularly diverse (Bastos e*t al.*, 2003; Reeve *et al.,* 2016). Based on genetic and antigenic analyses, FMDVs circulating globally subdivide into seven regional pools (Fig. 1) (Di Nardo *et al.,* 2011). Virus is not restricted to pools as certain countries share viruses belonging to two different pools, for example, Egypt and Libya. The FMDV pools include pool 1 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 Africa (SAT 1, 2 and 3) (Di Nardo *et al.,* 2011; Paton *et al.,* 2009; Sumption *et al.,* 2012). Tanzania FMDV is classified under pool 6, however due to spill over of serotypes O and A, from pool 4, it is classified under both pools. It is therefore affected by five serotypes O, A, SAT 1, 2 and 3 (Kasanga *et al.,* 2011; Kasanga *et al.,* 2014) .

2.1.3 Epidemiological Patterns of FMDV in Africa

In Africa, the FMDV serotypes are not evenly distributed, which ascribes each serotype different epidemiological patterns (Maree *et al.*, 2014). Six of the seven serotypes of FMDV (O, A, C, SAT1, SAT2, and SAT3) have occurred in Africa. Genetic characterization of the virus and antigenic relationship of FMDV in Africa have been used to yield information that distributes the virus into three virus pools: such as pool 4 which covers East and North Africa (serotypes A, O, SAT1, and SAT 2) pool 5 confined to West and northern Africa (serotypes O, A, SAT1, and SAT2) and pool 6 restricted mainly to South Africa (SAT 1-3 serotypes). The virus pools in Africa are further divided into eight epidemiological clusters as proposed by Rweyemamu *et al.*, (2008) (Table 1).

Table 1: Eight epidemiological clusters of FMD virus pools in Africa

Virus pools clusters	Countries	Serotypes and FMD status
Indian Ocean Island cluster	Mauritius, Madagascar and Seychelles	FMD free without vaccination
Southern African Development Community (SADC) cluster	Namibia, South Africa, Swaziland, Lesotho, Botswana, southern and western part of Zimbabwe, and the southern part of Mozambique	Free from FMD without vaccination with exclusion of Zimbabwe and Mozambique Most outbreaks are due to SAT 2 serotype (Vosloo and Thomson, 2004)
North SADC cluster	Northern part of Malawi, Zimbabwe, Zambia, northern Mozambique, and southern Tanzania	Serotypes A, O, SAT1, and SAT2, with SAT 3 sometimes being reported.
Angola cluster	Angola and western Democratic Republic of Congo (DRC)	Angola lacks official information on FMDV since 1974 hence little is known.
East African Community cluster	Uganda, Kenya, Tanzania, Burundi, Rwanda, and the eastern part of the DRC	Serotypes A, O, SAT1, and SAT 2
Intergovernmental Authority on Development (IGAD) cluster	Sudan, South Sudan, Ethiopia, Somali, Eritrea, northern Kenya, and northern Uganda	Serotypes A, O, SAT1, and SAT2
Soudan/Sahel cluster	Western Sudan, Chad, Mali, Senegal, Niger, Burkina Faso, northern Nigeria, and Mauritania	Serotypes A, O, SAT 1, and SAT 2
North Africa/Maghreb cluster	Tunisia, Algeria, Morocco, Egypt and Libya	Tunisia, Algeria and Morocco, have not reported FMD since 1999. Egypt and Libya experience sporadic FMD. SAT 2, O and A.

2.1.4 FMD in Tanzania

FMD is the most important viral transboundary animal disease (TAD) in Tanzania. The disease is endemic with its first documentation dating 1927 and first isolation of the virus in 1954 (Kasanga *et al.,* 2014). Henceforth, serotypes (O, A, SAT1 and SAT2) causing outbreaks in various regions in Tanzania have been identified and reported by Rweyemamu and Loretu, 1972; Vosloo *et al.,* 2002; Rweyemamu *et al.,* 2008; Swai *et al.,* 2009: Kasanga *et al.,* 2012 and Kasanga *et al.,* 2014. Existence of serotypes O and SAT 2 in Tanzania dates back to 1950s while SAT 1 was first identified in 1971 (Rweyemamu and Loretu, 1972; Rweyemamu and Loretu, 1973). FMDV serotypes O, A, SAT1 and SAT2 are prevailing in most of the regions of the country (Kasanga *et al.,* 2012; Kasanga *et al.,* 2014). Serotype 'O' FMDV is prevalent in the northern, southern, western and eastern zones. The serotypes are further subdivided into genotypes/topotypes which have the following distribution; of serotype 'O' (VI), A (III), SAT1 (III) and SAT2 (IV) respectively (Rweyemamu *et al.,* 2008; Thomson *et al.,* 2003b; Kasanga *et al.,* 2014). The epidemiology within FMD-endemic regions is further intricately linked to Agro-pastoral from unrestricted human and animal movements (Kivaria *et al.,* 2003) and the presence of wildlife which serves as potential FMD reservoirs.

2.2 FMD Transmission and Clinical Signs

For infection to occur, FMDV requires low infectious dose and a substantial level of viral excretion. Transmission can occur mostly through direct contact as seen in pigs or aerosol droplets e.g., ruminants (Fig. 2). For an infected animal, the FMDV is shed into in all secretions and excretions, hence transmission is facilitated via direct contact between infected and susceptible animals. Indirect transmission is evidenced through fomites, animal products such as milk and untreated meat

products from an infected animal (Alexandersen *et al.,* 2003; Alexandersen *et al.,* 2003b) and in other cases wind has been implicated. Reports claim that FMDV infections can spread over long distances by wind, especially when favored by cold and humid conditions (Donaldson *et al.,* 1972). In light of this, FMDV virulence and degree of transmission may be ascribed to differences in viral strain, type of animal infected and the extend in which they excrete the virus (Alexandersen *et al.,* 2003b; Kitching *et al.,* 2002).

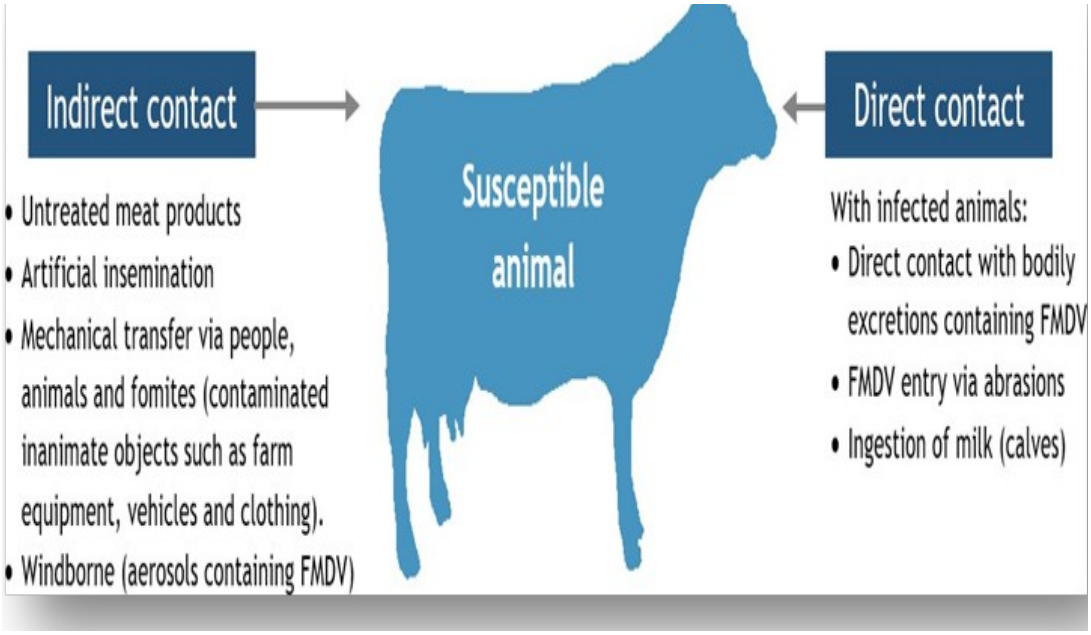

Figure 2: Transmission pathways of foot-and-mouth disease virus. Source: OIE, 2012.

FMD is evidenced mostly in pigs and cattle but slightly present itself in small ruminants. Clinically, the disease manifests as vesicles (feet, mouth, tongue, mammary glands and interdigital space), lameness, excessive salivation and anorexia. The disease is highly infectious with low mortality rate in adult animals and notably high mortalities in young stock due to myocarditis (Alexandersen *et al.,* 2003a; Alexandersen *et al.,* 2003b).

2.3 Foot-and-Mouth Disease Impact and Control

In regions where FMD is endemic the disease destabilizes food security, livelihoods and economic development at all production systems (OIE and FAO, 2018). The disease is considered one of the most economically devastating diseases of animals worldwide (Sumption *et al.,* 2012). FMD is highly infectious with less than 5% mortality rate in adult animals and notably high mortalities of about 50% in young stock due to myocarditis (Alexandersen *et al.,* 2003a). The impacts of FMD can be categorized either as direct (impact of the disease on production), or indirect effects. Direct repercussion is evidenced in death (primarily in young livestock), weight loss, abortion and a drop in milk production (Lyons *et al.,* 2015; OIE and FAO, 2018). Indirect effects include loss of draught power for crop production, and the costs incurred in controlling the disease and trade sanctions. FMD incursion into countries known to be diseased-free causes massive losses, one of such incidences was witnessed during the 2001 FMD outbreak in the United Kingdom where losses amounting to billions of pounds ensued. Cost associated with maintaining FMD-free status (mainly through intensive vaccinations), or regaining FMD-free status significantly varies. In both occurrences, the use of strong measures to prevent the introduction of the FMD virus or to control the disease at source is justified (Knight and Rushton, 2013; OIE and FAO, 2018) especially in developing countries. Taking into account the global burden of FMD, reducing the disease in endemic countries by a coordinated control strategy at the both global and regional level is vital.

The global FMD strategy was founded on biodefense approach to reinforce mechanisms; for detecting and reporting outbreaks promptly as they occur, harmonized control measures across the borders, managing risk of virus transmission and proper containment of the virus in laboratories. This strategy aims to lessen the burden of FMD on animal production in developing countries, and to

uphold the FMD-free status accorded to countries that have effectively eradicated the disease. The strategy is composed of; (a) improving the global FMD control (b) strengthening the Veterinary Services and (c) controlling other TADs. Strengthening Veterinary Services is inextricably linked the other two components as it improves effective implementation of the strategy as well as increase the capacity to fight other important livestock diseases. Reports indicate that FMD control has progressed following successful implementation of this strategy. Another indicator of progress is evidenced by the adoption and advancement of the Progressive Control Pathway (PCP). The PCP is the FMD control strategy proposed by OIE and FAO to control and eventually eradicate FMD from endemic countries (Fig.3). Different countries in sub-Saharan Africa are at different stages of the PCP (OIE and FAO, 2018b; OIE and FAO, 2018a).

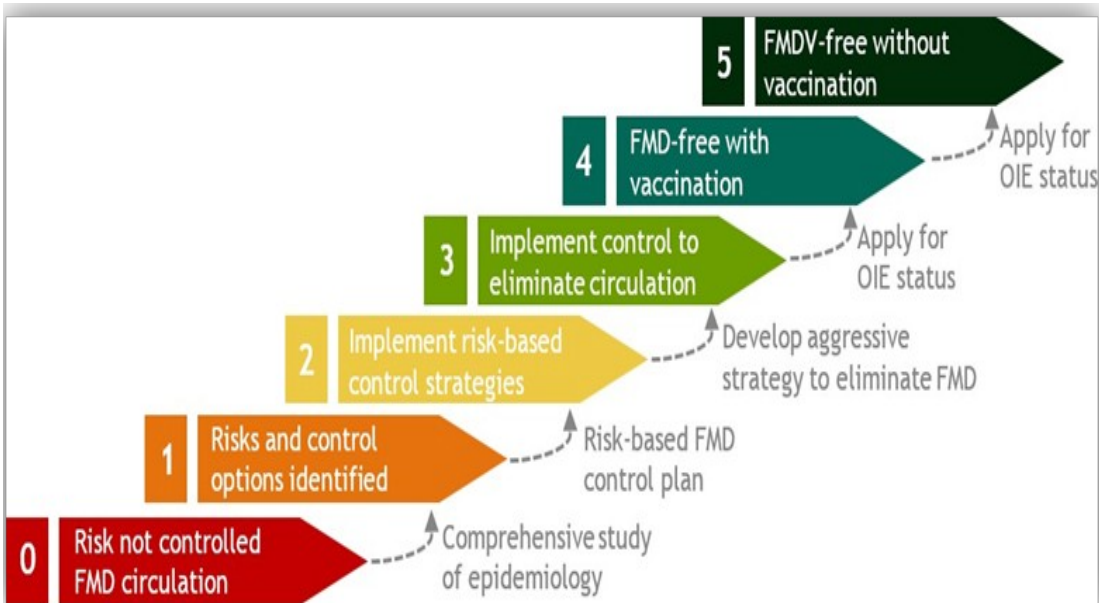

Figure 3: FAO-OIE FMD-PCP stages. The FMD PCP consists of six stages ranging from zero to five. The PCP recognized by OIE fall under only three categories with regard to FMD; (1) countries not free from FMD (PCP stages 0–3), (2) countries free from FMD or practicing vaccination (PCP stage 4), lastly (3) countries free from FMD without practicing vaccination. Source: Maree *et al.*, (2014).

Based on GF-TAD SADC report 2017, (OIE and FAO, 2017) Tanzania is currently at stage 3 (Fig. 3) and is expected (based on estimates) to maintain stage 3 of the PCP by year 2025 (Table 2). Tanzania control strategies involve; vaccination, mandatory livestock identification in the whole country, enforcement to remove livestock from game parks and harmonized cross border and import control between Tanzania and its neighboring countries (OIE and FAO, 2017). Control measures in non-endemic areas include; quarantine where farmers are advised to keep movements of susceptible animals, people and vehicle to an absolute minimum, border and import controls of animals and the products from endemic regions (Maree *et al.,* 2014; Jamal and Belsham, 2013).

Table 2: The provisional PCP- FMD roadmap for Tanzania for 2014 to 2025.

Country	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025
Estimation in 2012	2	2	2	2	3	3	3	3	3	4	4	4
Estimation in 2018	2	2	2	2	2	2	3	3	3	3	3	3

Source: GF-TAD SADC, 2017 report

2.4 Foot-and-Mouth Disease Virus

2.4.1 Virus Structure and Genome Organization

Foot-and-mouth disease virus (FMDV) is a small, non-enveloped, single-stranded, positive-sense RNA genome virus. The virus belongs to the genus *Aphthovirus* of the family *Picornaviridae* and has an RNA genome of 8.5 kb that encodes a polyprotein that undergoes post-translational proteolytic cleavages to generate structural and non-structural proteins. FMD viral genome contains; 5`-UTR (~ 1300 nucleotide long), a

single long open reading frame (ORF; ~7000 nucleotide long), 3`-untranslated region (UTR; ~100 nucleotide long) and a poly (A) tail as indicated in (Fig. 4). The structural proteins (each 60 copies) are designated as 1D, 1B, 1C, 1A (VP1–VP4) form the virus capsid are exposed to the surface with the exception of VP4 (1D). The 8 non-structural proteins are vital for protein processing and virus replication (Belsham *et al.*, 2005; Grubman and Baxt, 2004).

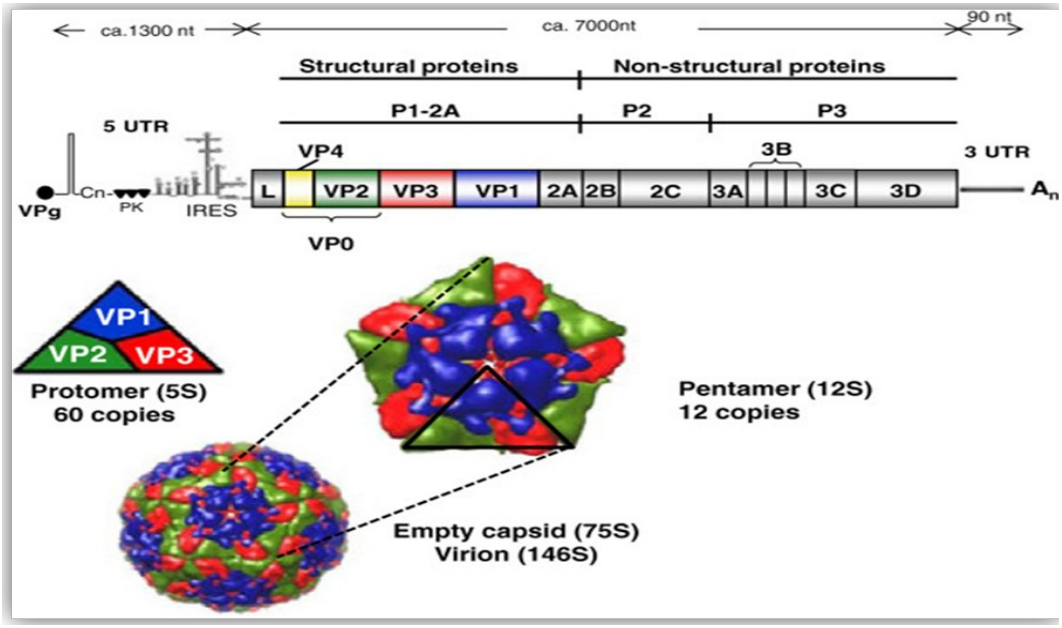

Figure 4: Structure and genome organization of FMDV. Source: Jamal and Belsham, (2005).

2.4.2 Genetic Diversity and Epidemiological Distribution of FMDV

Serotypes are grouped based on the structural protein coding region particularly the sequence for VP1. The VP1 varies significantly between serotype and strains hence many studies on; molecular characterization, antigenicity and immunity, and evolutionary relationships between strains and serotypes are based on the coding sequence of VP1. These studies have grouped the serotype into epidemiologically and genetically distinct regional clusters known as topotypes (genotypes) and lineages in agreement with intra-lineage (≥92.5%) and intra-topotype/genotype

nucleotide identity (≥85%) (Samuel and Knowles, 2001; Knowles and Samuel, 2003). Additionally, since topotypes are related to geographical region of the disease occurrence, characterizations studies help identify the source of new outbreaks and elucidate on geographical movement of FMDV and (Knowles and Samuel, 2003).

Genetic diversity is also attributed to quasi-species dynamics, persistent infection and recombination (Domingo *et al* ., 2001). FMDV exists as seven distinct serotypes; O, A, C, Asia and Southern African territories [SAT] types 1-3 whose distribution is uneven with serotype 'O' being dominant globally. Serotype 'O' has been implicated for causing outbreaks with drastic economic impact (Samuel and Knowles, 2001). It is further categorized into 11 topotypes namely; East Africa 1 to 4 (EA 1-4), West Africa (WA), Southeast Asia (SEA), Europe-South America (EURO-SA), Indonesia-1 and -2 (ISA 1-2), Cathay and Middle East-South Asia (ME-SA). Similarly, East Africa EA 1-4 circulate in these regions as shown in Figure 5; EA-1 (Kenya, and Uganda), EA-2 (Kenya, Tanzania and Uganda), EA-3 (Kenya, Sudan and Ethiopia), and EA-4 (Kenya, Uganda and Ethiopia). (Balinda *et al.*, 2010; Wekesa *et al.*, 2015; Namatovu *et al.*, 2015; Kasanga *et al.*, 2014; Ayelet *et al.*, 2009; Samuel and Knowles, 2001)

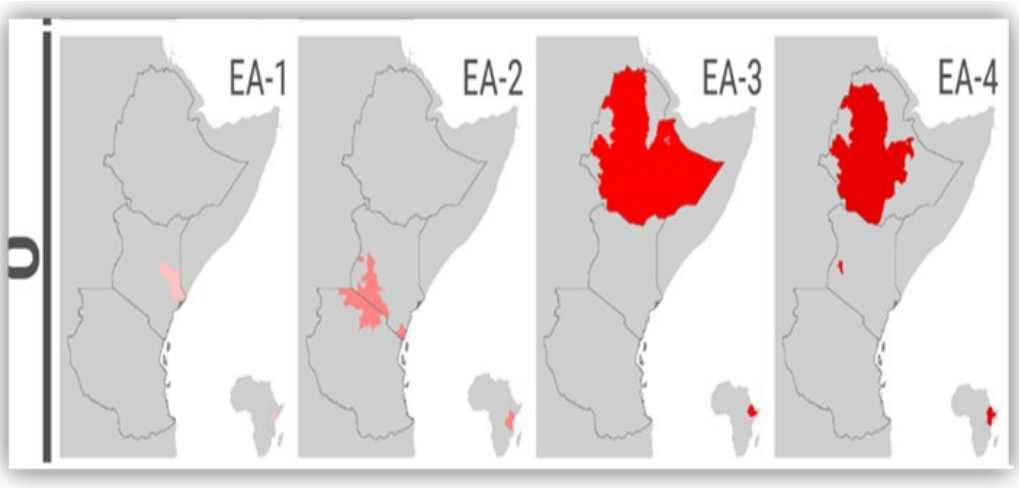

Figure 5: Occurrences of FMD viral serotypes and lineages within Kenya,

Tanzania and Ethiopia (2010-2017). Source: Molecular epidemiology reports produced by FAO and WRLFMD.

2.4.3 Antigenic Diversity

FMDV exhibits a high degree of antigenic variation ascribed to the error-prone nature of RNA polymerase during replication of viral RNA, and the inadequacy of a proof-reading mechanism linked with the viral replicase (Domingo et *al.*,1997; Steinhauer and Holland, 1987). Additionally, it can be due to reassortment or recombination between genetic materials of related strains during co-infections (Mumford *et al.,* 2007).The degree of antigenic diversity varies across the members of *Piconaviridae* family (Domingo *et al.*, 2001) and immunity to one serotype does not confer protection against the other (Balinda *et al.,* 2010). VP1 region contains; the major antigenic determinants of the virus, elicits neutralizing antibodies, two G-H loop and the C-terminus region. Antigenic variation of the virus is due to variation within the G-H loop and C-terminus regions (Sobrino *et al.,* 2001). Additionally, VP1 contains Arginine-Glycine-Aspartate (RGD), a highly conserved sequence involved in cell attachment (Leippert *et al.,* 1997).

2.5 Diagnosis of FMD

Given the infectious nature of FMD and the devastating economic implications arising from outbreaks, it is vital to rapidly, conduct specific and sensitive laboratory or field diagnosis to identify the serotype causing the FMD outbreak. Timely diagnosis is instrumental when designing control measures (Jamal and Belsham, 2013; King *et al., 2*012; Kasanga *et al.,* 2014). Diagnosis of the disease is based on clinical signs, such as profuse salivation, vesicular lesions (e.g., on tongue) etc. Nevertheless, clinical manifestation of FMD resembles other vesicular diseases, such as vesicular stomatitis, swine vesicular disease and swine vesicular exanthema. This resemblance pose constrains on early confirmation of field outbreaks. Therefore, there is a need of rapid and sensitive diagnostic tool for the

detection in order to control the disease (Kasanga *et al.,* 2014; Jamal and Belsham, 2013).

2.5.1 Laboratory Diagnosis

Laboratory diagnosis of FMD aims to check the presence FMDV genome which is the causative agent for the disease. Diagnosis can be performed in laboratories which meet the criteria and requirements for handling highly infectious agents. Methods such as virus isolation or ELISA are used to diagnose FMD. These methods aim to demonstrate the presence of FMD viral antigen in the samples or detect antibodies to viral non-structural proteins, The OIE recommends a manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2012). Diagnostic assays conducted are influenced by the type of sample received, and requires trained personnel to interpret the results. Laboratory diagnosis of FMD might be constrained due to samples (mainly epithelial tissues and probang) of low integrity owing to ineffective cold-chain and long transport periods. Globally methods such as; RT-PCR, ELISA and molecular detection of the viral agent have been employed in diagnosing FMD (Howson *et al.,* 2017; Jamal and Belsham, 2013; Madhanmohan *et al.,* 2013). In sub-Saharan Africa, laboratory diagnosis of FMD is only carried out at specialized laboratory.

2.5.2 Virus Isolation

Diagnosis of FMDV infection can be achieved by isolating the virus using primary cell cultures (e.g., bovine thyroid cells) or susceptible cell lines (e.g., Baby Hamster Kidney-21) (OIE, 2012). After 48 hours, the cell cultures are observed for cytopathic effect (CPE) as viable virus results to CPE (Fig. 6). The pros to this method are enormous; it consumes time, takes up to four days to generate results, requires fresh tissue for cell suspensions preparation, contamination, laborious,

expensive, requires the presence of infectious virus and some FMDV isolates might fail to grow or grow poorly in some cell types (OIE, 2012). Consequently, this may delay prompt response in controlling the disease in the event of an outbreak (Jamal and Belsham, 2013). Figure 6 shows isolation results; (A) negative (B) showing signs of cytopathic effect (C) positive, showing clear cytopathic effect.

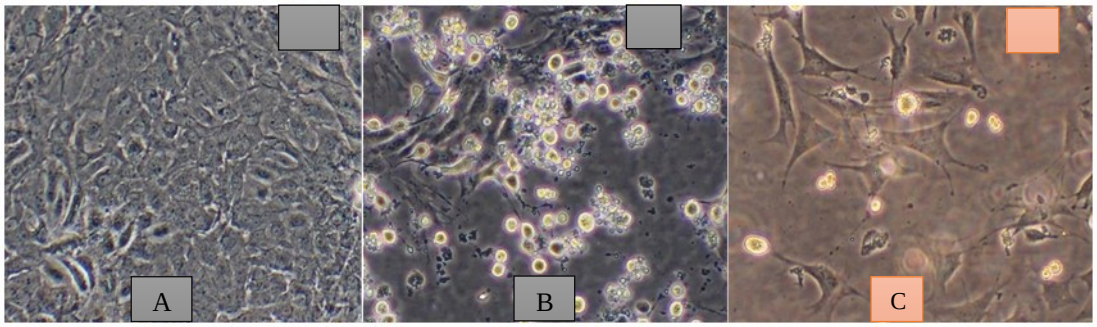

Figure 6: Foot-and-mouth disease virus growth in bovine thyroid cells. Source: World Reference Laboratory for Foot-and-Mouth Disease, 2016 (WRLFMD)

2.5.3 Virus Neutralization Test (VNT)

Currently considered as the "gold standard" for detection of antibodies to structural proteins of FMDV, virus neutralization test (VNT) is also the recommended test for trade of animal/animal (OIE, 2012). Inoculation is performed in cell culture etc. and the viruses are neutralized by specific antibodies found in serum. VNT employs various primary cells and cell lines which vary in sensitivity, hence are subject to variation than other serological tests (Jamal and Belsham, 2013). Although it was found to be 100% sensitive for the detection of FMDV (serotypes O, A, C and Asia-1) in epithelial suspensions tested, this method is slower and requires specialized conditions (high containment) comparative to other methods like ELISA.

2.5.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a serological assay that detects viral antigen or serum antibody in

samples using a specific antibody or antigen. These tests are competition- or blocking -based assays that employ serotype-specific monoclonal or polyclonal antibodies (MAbs or (PAbs). ELISA has been used to detect FMD viral antigen for clinical diagnosis and for the identification of viral serotypes (Ferris and Donaldson, 1992; Roeder and Le Blanc Smith, 1987). ELISA is further categorized into solid phase competition ELISA (SPCE) and liquid phase blocking ELISA (LPBE). SPCE is an indirect sandwich test that targets FMDV viral proteins (structural proteins) in which different rows in multi-well plates are coated with rabbit antisera to each of the seven serotypes of FMDV. LPBE are pan-specific assays and detect antibodies to non-structural proteins (2C, 3AB and 3ABC-NSPs) which are involved in viral replication (Shen *et al.,* 1999; Bergmann *et al.,* 2000).

2.5.5 RT-PCR

Conventional reverse transcription-polymerase chain reaction (RT-PCR) assays has been developed for both detection and serotyping of FMD (Reid *et al., 1*999). Targeting the 5’ untranslated region conserved region Reid *et al.*, (2000), employed reverse transcription PCR to detect amplified products. Real-time RT-PCR (rRT-PCR) assays have now largely replaced assays that employ agarose gel. Several assays that employ 5’-nuclease assay (TaqMan®) system to detect PCR amplicons have been developed for the detection of FMDV (Callahan *et al.,* 2002; Reid *et al.,* 2002). Due to this characteristics, real-time quantitative RT-PCR (*q*RT-PCR), are currently used for routine diagnosis of FMDV as recommended by (OIE, 2016). In detecting FMDV serotypes, RT-PCR uses serotype specific primers. Despite the high sensitivity and specificity of this technique, carrying out a RT-PCR assay is laborious, the process takes 3-4 hours to yield results, requires skilled personnel, sophisticated equipment and specialized conditions making it expensive in limited

resource setting (Dhama *et al.,* 2014). A more friendly technique called loop-mediated isothermal amplification (LAMP) serves to eliminate this constrain.

2.5.6 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technique that was developed by Notomi *et al.,* (2000). Since its inception in 2000, LAMP has been an instrumental tool for detection of both animal and human diseases. It is simple, rapid, has proven to be highly specific and sensitive. Amplification takes place at a single temperature hence isothermal and the reaction is rapid (factorial contrary to exponential in PCR); takes typically less than 30 minutes to yield results with a high product yield (Notomi *et al.,* 2000; Mori and Notomi, 2009; Dhama *et al.,* 2014). RT-LAMP requirements are discussed below.

2.5.6.1 RT- LAMP Enzymes

LAMP utilizes DNA polymerase with high strand displacement activity compared to Taq polymerase, and can detect fewer DNA or RNA copies in a test sample (Notomi *et al.,* 2000; Madhanmohan *et al.,* 2013). LAMP can be adapted for RNA templates by addition of reverse transcriptase (Hong *et al.,* 2004) or by using an enzyme which contains both DNA polymerase and reverse transcriptase activities. Other protocol employs enzymes such as Bst and Bsm polymerase which are isolated from *Bacillus stearothermophilus* and *Bacillus smithii* respectively. These enzymes amplify by strand displacement and are able to catalyze 5'-3' DNA polymerization, however they lack the 5'-3' exonuclease activity (Nagamine *et al.*, 2001; Dhama *et al*., 2014). They work optimally at a temperature range of 60 to 66 degrees Celsius.

Additionally, they require other components such as; reaction buffer, dNTPs (nucleotides used in polymerization), magnesium sulphate, betaine (chemical that

stabilizes the GC and AT content), Avian Myeloblastosis Virus (AMV) reverse transcriptase used for increasing the rate of converting RNA to cDNA, hydroxynaphthol blue (used in detection; a positive reaction indicates color change from violet to blue, no color change indicates negative reaction) and pyrophosphate ions (added when a turbidimeter is used as means for detection; these ions yields white precipitate of magnesium pyrophosphate in the reaction to facilitate visualization of the result (Notomi *et al.,* 2000; Mori and Notomi, 2009; Nagamine *et al.,* 2001; Dhama *et al.,* 2014; Mori *et al.,* 2001).

2.5.6.2 RT-LAMP Methods

RT-LAMP can be carried out using different methods such as; heating block (Shao *et al.,* 2010), thermal cycler (Lim *et al.,* 2018) and water bath (Maryam *et al.,* 2017; Farooq *et al.,* 2015). Other methods use instruments that require one to purchase reagents for amplification such as, Genie II/III® instrument (OptiGene Ltd, UK) and Loopamp real-time turbidimeter (LA-500; Eiken Chemical, Tokyo, Japan). Genie II/III® instrument are real-time devices which offer real time monitoring of the amplification process as detection is fluorescent-based (Howson *et al.,* 2017; Waters *et al.,* 2014). Loopamp real-time turbidimeter monitors the amplification by measuring turbidity (Kasanga *et al.,* 2014; Yamazaki *et al.,* 2013; Madhanmohan *et al.,* 2013)

2.5.6.3 Visualization of RT-LAMP Products

Visualization of LAMP products can be done by fluorescent dye, real time detection, lateral flow assay kit and gel electrophoresis. LAMP is amenable to visual detection, successful DNA amplification can be assessed based on turbidity because a positive LAMP reaction causes the solution to become cloudy due to the formation of the

magnesium pyrophosphate by product (Mori *et al.,* 2001; Madhanmohan *et al.,* 2013). The increase in the turbidity correlates with the quantity of RNA synthesized by the LAMP reaction (Parida *et al.,* 2006). Fluorescent detection methods are more sensitive e.g., commercially available fluorimeter Genie ® II/ III (OptiGene Ltd.). Time to Result (TTR) or Time to Threshold (TTT) is used to quantify assay performance. These detection methods can be adopted as real-time measurement or end-point assay (e.g., molecular lateral-flow devices (LFDs) (Howson *et al.,* 2017).

2.5.6.4 Deployment of RT-LAMP in Field Setting

LAMP assay is also tolerant to sample matrix inhibitors contrast to PCR which is sensitive, this and numerous advantages makes it field deployable. LAMP does not require sophisticated instruments like a thermal cycler, or specialized laboratory. RT-LAMP can efficiently amplify the gene of interest without the RNA extraction step, hence requires minimum reagents, making it feasible to diagnose infectious diseases at the outbreak site. This has proven to be useful in developing countries where diagnostic capacity is hindered due to limited resources (Howson *et al., 2017*).

2.5.6.5 LAMP Assays in Diagnosis of FMDV and other Pathogens

The original method by Notomi has been improved on and used to diagnose FMD viral genome. An RT-LAMP assay for the detection of FMDV was first developed by (Dukes *et al.,* 2006). Several RT-LAMP assays that focus on highly conserved region 3D region, been later developed for detecting the seven serotypes of FMDV (Shao *et al.,* 2010; Chen *et al.,* 2011a; Guan *et al.,* 2013; Yamazaki *et al.,* 2013; Waters *et al.,* 2014; Farooq *et al.,* 2015; Maryam *et al.,* 2017; Lim e*t al.,* 2018). Serotype-specific RT-LAMP for diagnosing individual FMDV serotypes O, A, C,

or Asia 1 have been developed (Chen *et al.,* 2011b; Ding *et al.,* 2014; Madhanmohan *et al.,* 2013; Maryam *et al.,* 2017; Lim *et al.,* 2018).

LAMP assays have been designed to detect the recent coronavirus (Augustine *et al.,* 2020; Yoshikawa *et al.,* 2020), influenza A virus, respiratory syndrome virus, African swine fever virus, swine fever virus, Japanese encephalitis virus, porcine reproductive, Hepatitis B Virus, dengue virus, Human papilloma viruses, human immunodeficiency virus (HIV), West Nile virus, Porcine cytomegalo virus, and Newcastle disease virus (Dhama *et al.,* 2014; Notomi *et al.,* 2000; Mori and Notomi, 2009; Parida *et al.,* 2006). Additionally, LAMP has been applied in detecting many kinds of pathogens that cause food-borne diseases such as; Listeria, Campylobacter, Salmonella, verotoxin-producing Escherichia coli, and Legionella (Mori and Notomi, 2009; Lukinmaa *et al.,* 2004)

2.5.6.6 RT- LAMP Primers

Unlike PCR which requires two sets of primers (forward and backward primers), LAMP requires six primers that targets eight regions in the sequence of interest. The use of these primers ensures sensitivity of the assay, albeit the assay can work effectively using four primers that target six regions in the target sequence. The design of LAMP primers is based on the six regions in the sequence, designated (Fig. 7) as F3 (forward external primer), FIP (Forward inner primer; consisting of the F2 sequence that is complementary to the F1c region), BIP (backward internal primer; consisting of the B2 sequence that is complementary to the B1c region), B3 (backward external primer), Forward loop primer (FLP) which is designed using the complementary strand that corresponds to the region between F1 and F2, while Backward loop primer (BLP) is designed using the complementary strand that corresponds to the region between B1 and B2 (Fig.7). The loop primers, reduce the

time for amplification and improve the specificity of LAMP assay although they are not an essential requirement for LAMP (Notomi *et al.*, 2000; Mori and Notomi, 2009).

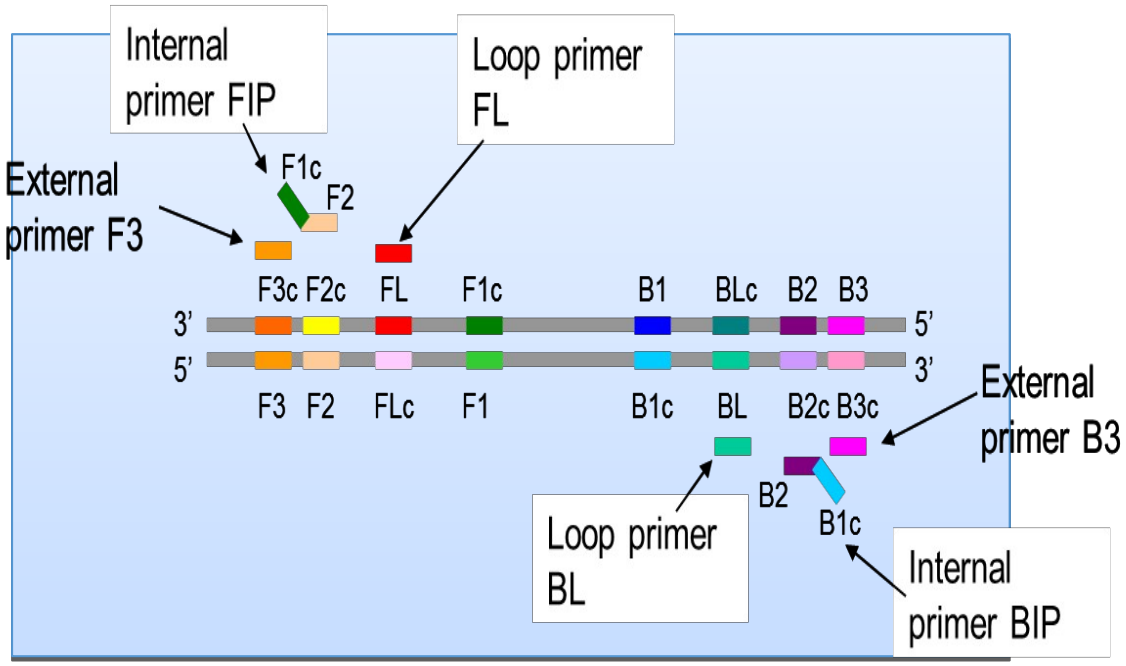

Figure 7: LAMP Primers. Source: Mori and Notomi, (2009)

2.5.6.7 RT- LAMP Primer Design

There are several LAMP primer design tools one of such is Primer Explorer V5 (version 5), a free online software developed Fujitsu Ltd. Primer Explorer provides an web-based interface as well as a guide for LAMP primer design (Fujistu Ltd, 2005). The most essential detail to capture prior primer design is the length of amplicon, which should be long so as to accommodate all the primers. During design, factors such as primer melting temperature, primer length and the distance between the primers should be cross checked against the stipulated set parameter; although the software has set these parameters as default settings (Table 3). Additionally, primers should be designed to contain GC content between 40% to

65%, although a GC content between 50% and 60% tend to give relatively good primers (Dhama *et al.*, 2014; Notomi *et al.*, 2000; Mori and Notomi, 2009).

Primer(s)	**Length (mer)**	**Tm (°C)**	**Distances between primers**	
F3/B3	15-25	59-61	5` F2 to 5` B2)	120-160nt
F2/B2	15-25	59-61	Loop (5`F2 to 5`F1c)	40-60nt
F1c/B1c	15-25	64-66	3`F3 to 5`F2	0-60nt
FLP/BLP	15-22	64-66	F1c-B1c	0-100nt

Table 3: Key factors to consider when designing LAMP primers

2.5.6.8 LAMP Amplification Process

The process of amplification initiates from strand invasion by one of the inner primers (F2), strand displacing DNA polymerase extends the primer and separates the target DNA duplex. The first product is then displaced by synthesis initiating from an outer primer (F3) which anneal to an upstream target region, as it is displaced, the end of the product forms a self-hybridizing loop structure due to inclusion of reverse complimentary sequence (F1c) in the inner primer sequence. This annealing and displacement cycle repeats on the upstream end of the target sequence (B2) resulting into a short dumbbell structure product which is used as a template for exponential LAMP amplification (Fig. 8). It also contains multiple sites for initiation of synthesis, from the 3` ends of the open loops and annealing sites for both the inner and loop primers. As amplification proceeds from these

multiple sites, the product grows and form long concatemers each with more sites for initiation resulting to rapid accumulation of double stranded DNA and amplification byproducts that can be detected using various methods (Fig. 9) (Notomi *et al.*, 2000: Mori and Notomi, 2009).

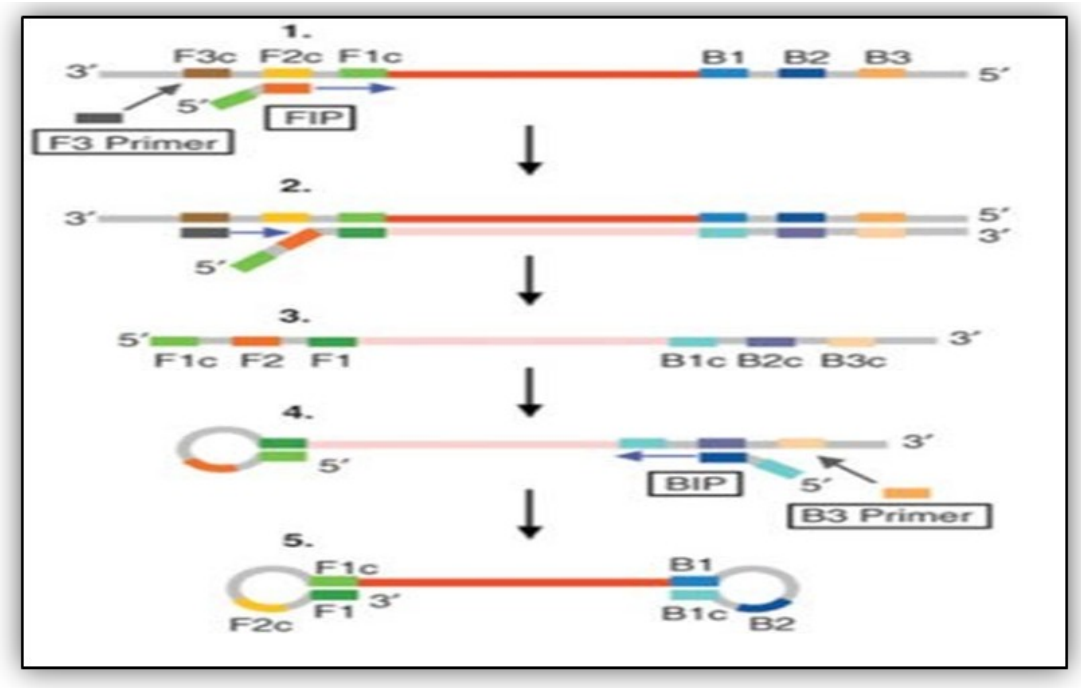

Figure 8: Amplification process; formation of stem-loop DNA structure. Source: Notomi *et al.*, (2000). http://loopamp. eiken.co.jp/e/ lamp/ principle. Html.

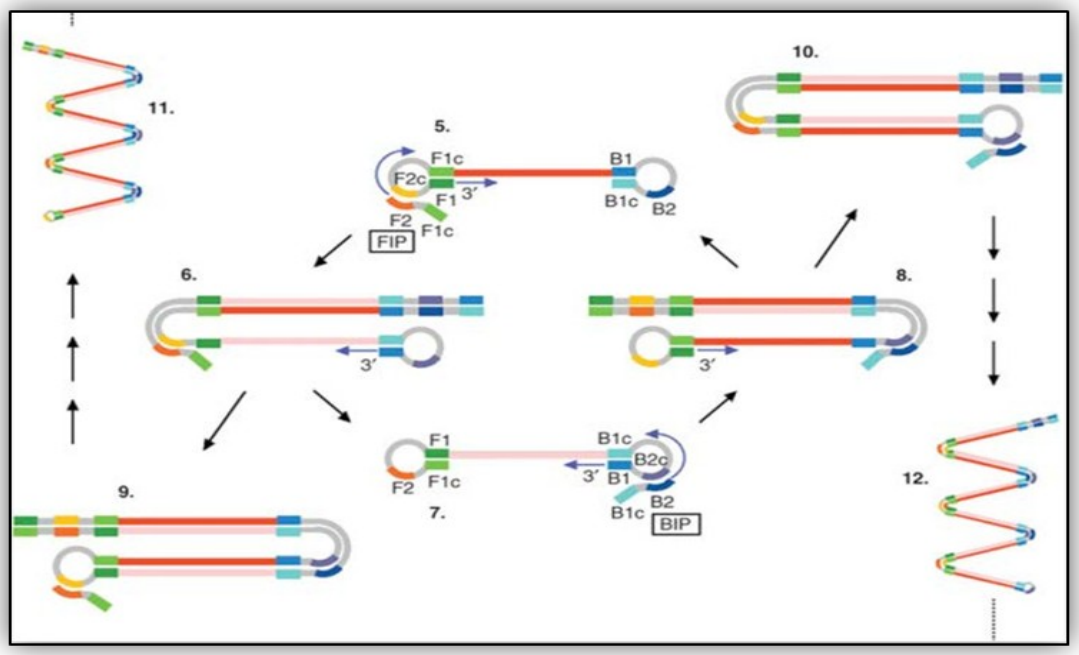

Figure 9: Cycling amplification steps and amplification products. Source: Notomi *et al.*, (2000). http://loopamp.eiken. Co.jp/e/ lamp/ principle. html.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This research study was conducted using samples that were collected from six regions in Tanzania (Kagera, Morogoro, Dar es Salaam and Pwani, Lindi and Mtwara,). Geographically Tanzania is located in East Africa region, between latitude and longitude of -6.369 and 34.8888 respectively, with an area coverage of 945,000km² and a climate that ranges from tropical to temperate depending on the region. This sub-Saharan country neighbors; Kenya and Uganda to the north; Malawi, Mozambique and Zambia to the south; Burundi, Rwanda, and Democratic Republic of the Congo to the west; and the Indian ocean to the east. It also harbors archipelago such as Zanzibar (FAO country report, 2003) .

Tissue epithelial samples (serotype ‘O’) from FMD suspected cattle were obtained from different regions of Tanzania (Fig. 10) following reports of FMD outbreak. Additionally, samples used in cross-reactivity studies (specificity evaluation) were serotype A and SAT 1 (Tanzania) and SAT 3 (Namibia and Zambia).

3.2 Study Design and Sampling Approach

The study design of this research was a retrospective laboratory and in-silico based that used archived samples. The sampling approach was purposive. The laboratory work was performed at the College of Veterinary Medicine and Biomedical Sciences (CVMBS) at the Molecular Biology and Biotechnology laboratory.

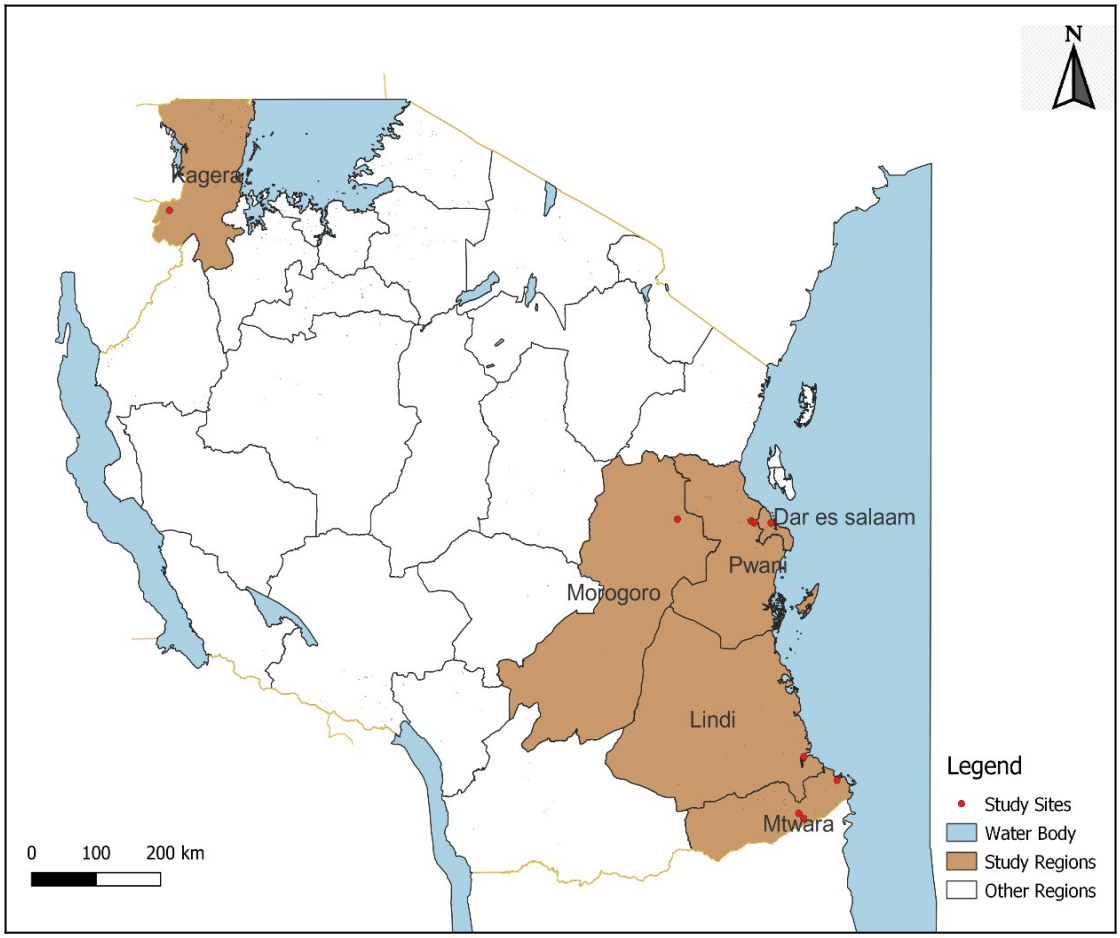

Figure 10: Sampling regions and sites for serotype ‘O’ FMDV in Tanzania. Red points denote the location of the sampling sites.

3.3 Samples and Storage

This study was conducted using archived samples from previous experimental studies. Epithelial samples from FMD suspected cattle, was collected following FMDV outbreaks by field or veterinary officers among others. The samples were preserved in transport media, transported to the laboratory and stored at -80^{o}C prior to analysis. Relevant information regarding the year and region was obtained from the laboratory archived data. Characterized samples (sequenced samples that have been confirmed to be serotype O in previous studies) were selected for the both development and evaluation of the assay. The same criteria were adopted in cross-reactivity study where characterized samples were used.

3.4 Sample Preparation

The selected samples for the study were assigned the same number as that of the laboratory (numbers assigned prior storage from sample collection in the field). The Samples were thawed and using bio-safety cabinet class II (Telstar life science solutions), were removed from transport media and placed in sterile mortar and the remaining epithelial tissues stored at -80°C. Using 1000 µl of phosphate buffered saline (PBS) and a pestle, the epithelial tissues were then ground to make epithelial suspension. The grounded sample were transferred to cryo vial and stored at -80°C until further use.

3.5 RNA Extraction

Total RNA was extracted from the epithelial suspensions using RNeasy® Mini-kit (Qiagen, Hildan, Germany) using protocol as described by the manufacturer. Additionally, positive control (one strong positive archived sample with a high viral concentration used in previous studies) and negative control (RNase free water used in place of the sample) was extracted.

3.6 Reverse-Transcription Loop-Mediated Isothermal Amplification (LAMP)

3.6.1 Design of Primers for RT-LAMP

Characterized data for serotype ‘O’ FMDV was used in designing LAMP primers. SeqMan Pro™ tool of Lasergene 8.0 software package (DNAStar Inc., Madison, WI, USA) was used to assemble the VP1 Nucleotide coding sequences of serotype ‘O’. Confirmation of VP1 nucleotide sequence was done using BLASTN search tool in GenBank, National Center for Biotechnology Information (NCBI).

This tool aimed to search for similarities between VP1 nucleotide sequences (obtained from sequenced samples from previous studies) intended for LAMP primer design with those in (NCBI-http://www.ncbi.nlm.nih.gov/). Primer Explorer V5 software (http://primerexplorer.jp/ lampv5e/ index.html, Fujitsu System Solutions, Ltd., Japan), was used to design the primers.

Several sequences were used to design serotype ‘O’ FMDV LAMP primers. Four RT-LAMP primers, two outer (F3 and B3) and two inner primers (FIP and BIP) from 20 sequences of FMDV serotype ‘O’ were designed that aimed to amplify six distinct regions of the target. Primer Explorer V5 generated only one loop primer (backwards loop primer). To address this problem, loop primers designed by Maryam *et al.*, (2017) were in cooperated in the study. The 4 primer sets designed were tested twice (run performed twice) in the RT-LAMP assay. Details for the designed LAMP primers have been provided (Table 4).

Table 4: Details of Tanzania FMDV serotype ‘O’ RT-LAMP primers.

Primer	Sequence (5'-3')
F3	CGTGACTCAGAACCAGAGAC
B3	CAAGTGAGGTTGCCCTCG

FIP	TGGTCTTGGGGCGTGACCTT-TCAACACACGGACGTTTCG-3	Current study
BIP	ATCCCTGCCCACACACTGGT-TGCTTCACTGCTACCTCCAA	
FLP	CATCCTCACCACCCGTAAC	Maryam *et al.* (2017)
BLP	GACACCTTTGTGGTCGGTC	

Source: Designed by this study

3.6.2 Primer Preparation

The designed primers were sent for synthesis in Macrogen, Korea. Lyophilized serotype 'O' primers were reconstituted to 100µM (concentration) by adding nuclease-free water as specified in the synthesis report sheet. 200µl (microlitres) was aliquoted to be used as working solution and the rest of the primer re-suspension stored at -20°C as stock solution.

3.6.3 Preparation of Primer Working Solution

LAMP primers were prepared/mixed in the ratio (F3/B3) 5 pmol: (FIP/BIP) 20 pmol and (FLP/BLP) 10 pmol to make a working concentration. 5 µl of this mixture was then used in the preparation of the master mix.

3.6.4 Mastermix Preparation and Addition of Sample

RT-LAMP tubes **(**OptiGene Ltd, Horsham, UK.**)** were used to prepare 20 µl of the total volume of master mix reaction mixture in a PCR work station (Bigneat Ltd, Hampshire, UK) as follows; 15 µl isothermal mastermix ISO-004 (OptiGene Ltd, Horsham, UK.), 5 µl primer mixture (from primer working solution) and Avian Myelo blastosis Virus (AMV) reverse transcriptase enzyme (New England Biolabs, MA, USA.). Five microlitres (5 µl) of extracted RNA samples (n=44) was mixed homogeneously with the master mix at the sample extraction hood. In addition, a known positive and negative control was included. Twenty-five (25 µl) reaction mixture of negative control was prepared using 5 µl nuclease free water (served as

a sample) in the PCR workstation to avoid contamination.

3.6.5 Optimization of RT-LAMP Assay for Serotype ‘O’ FMDV

RT-LAMP reaction conditions were optimized by performing test amplifications at varying reaction time (30, 45 and 60 minutes), temperatures (63°C, 64°C and 65°C), and AMV reverse transcriptase enzyme (0.2µl, 0.3µl and 0.4 µl) using one serotype ‘O’ sample. The experiments were repeated until optimum conditions were secured. Isothermal Master mix ISO-004 known to contain a proprietary DNA polymerase that contains reverse transcriptase activity was used, and evaluation of the RT-LAMP assay was performed with and in absence of AMV reverse transcriptase enzyme. The reaction was terminated by heating the RT-LAMP products to 98°C for one minute followed by cooling to 80°C ramping at 0.05°C.

3.6.6 Specificity and Cross-Reactivity of RT-LAMP Assay for Serotype ‘O’ FMDV

To assess the specificity of the FMDV serotype ‘O’ RT-LAMP assay, cross-reactivity studies was conducted with extracted RNA templates of three FMDV Isolates of serotypes; A (Tanzania), SAT1 (Tanzania), and SAT3 from Zambia and Namibia (Table 5). At the time of the study there was no characterized data of SAT 2 and SAT 3 in Tanzanian (no confirmed cases), hence available characterized data (confirmed samples) from Zambia and Namibia countries were incorporated in the study. The cross-reactivity studies were performed using six sets of primers against extracted RNA templates of serotypes; A, SAT 1 and SAT 3, and a positive control of serotype ‘O’ included. Other RT-LAMP requirements remained unchanged.

Table 5: FMDV archived clinical samples for evaluation of RT-LAMP assay specificity and cross reactivity studies.

Serotype	Sample ID	Location	Year
A	224	Malinyi-Ngomo, Tanzania	2017
	245	Kaole-Bagamoyo	2017
	279	Rwakalemela-Ngara	2017
	356	Bahija-Muleba	2017
	501	Chamwino-Dodoma	2017
	506	Singida	2017
	538	Ulanga	2017
	633	Iringa-Mwenda	2017
	634	Iringa-Nduge	2017
	635	Iringa-Hamisi	2017
	644	Kiwale	2017
SAT 1			
	128	Ulanga, Tanzania	2016
	130	Ulanga	2016
	140	Ulanga	2016
	167	Ulanga	2015
	171	Ulanga	2015
	173	Ulanga	2015
	198	Iringa-Kibedya-Gairo	2017
	205	Iringa-Kibedya-Gairo	2017
SAT 3	10	Zambia	2016
	25	Zambia	2016
	26	Zambia	2016
	27	Zambia	2016
	28	Zambia	2016
	2	Namibia	2019
	5	Namibia	2019
	6	Namibia	2019
	7	Namibia	2019
	8	Namibia	2019

3.6.7 Detection Limit (Sensitivity) of RT-LAMP Assay for Serotype 'O' FMDV

The detection limit of RT-LAMP assay was assessed by conducting serial ten-fold dilutions (10, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6,}$ and 10^{-7}) of the same viral RNA template with an initial concentration of 450.278 ng/µl. The diluted viral RNA concentration was quantified using Epoch microplate spectrophotometer (Biotek, USA). The diluted viral RNA was used in RT-LAMP assay.

3.6.8 Detection of FMD Viral Genome using RT-LAMP Assay

To determine the presence of FMD viral RNA, real time RT-LAMP (rRT-LAMP) assay was conducted using primers as indicated in (Table 6) following recommended incubation time of 30 minutes at 65°C on Genie® II (OptiGene Ltd, Horsham, UK). Samples were defined as positive if both amplification and annealing of the LAMP product occurred.

Primer	Sequence (5'-3')
F3	5' -CATGGACTATGGAACTGGGT-3'
B3	5'GGC CCT GGA AAG GCT CA-3'
FIP	5'CACGGCGTGCAAAGGAGAGGATT-TTACAAACCTGTGATGGCTTCG-3'
BIP	5'GGAGAAGTTGATCTCCGTGGCATT-TTAAGAGACGCCGGTACTCG-3'
FLP	5'TAGCCTCGAGGGTCTTCG-3'
BLP	5'GGACTCGCCGTCCACTCT-3'

Table 6: Details of RT-LAMP assay primer sets used for amplification of 3D polymerase gene of FMDV.

Source: Pirbright Institute, Surrey, UK.

3.6.9 RT-LAMP assay for Serotype ‘O’ FMDV using Designed Primers

Real time RT-LAMP (rRT-LAMP) assay for FMDV serotype ‘O’ was performed using the optimized conditions on Genie® II (OptiGene Ltd, Horsham, UK). 20µl master mix reaction mixture, primers as indicated (Table 4) was mixed with 5µl RNA template extracted from 14 samples (Table 7). The assay was run at 65°C for 45 minutes followed by annealing analysis that heats the RT- LAMP products to 98°C for 1 minute, followed by cooling to 80°C at a ramp rate of 0.05°C per second on Genie ® II (OptiGene Ltd. Horsham, UK). Time to positivity (fluorescence emitted at one minute interval) and annealing temperature (Ta) calculations were automated using Genie® Explorer v2.0.7.11 software (OptiGene Ltd.). Samples were defined as positive if both amplification and annealing of the LAMP product occurred. Annealing temperature of serotype ‘O’ FMDV VPI amplicon was to be deduced as a range in the study.

Table 7: FMDV serotype ‘O’ archived clinical samples used in RT-LAMP assay

Serotype	Sample ID	Location (Tanzania)	Year
O	19	Fulwe, Morogoro	2016
	184	Mtwara	2015
	254	Kibaha, Rehema	2017
	257	Kibaha, Rehema	2017
	259	Kibaha, Rehema	2017
	262	Kibaha, Rehema	2017
	265	Kibaha, TC	2017
	268	Kibaha, TC	2017
	272	Kibaha, TC	2017
	307	Kasulo, Ngara	2017
	365	Tandahimba, Mtwara	2017
	369	Tandahimba, Naputa	2017
	624	Kinondoni	2017
	625	Kinondoni	2017

3.6.10 RT-LAMP assay for Serotype ‘O’ FMDV using Pakistan Primers

Real time RT-LAMP (rRT-LAMP) assay for serotype ‘O’ FMDV was performed using the optimized conditions on Genie® II (OptiGene Ltd, Horsham, UK). RT-LAMP primers from Pakistan were used as provided (Table 8). This experiment served to check the suitability of Pakistan RT-LAMP primers in detection of serotype ‘O’ FMDV strains circulating in Tanzania. Other RT-LAMP reaction conditions remained unchanged (same protocol as above)

Table 8: Details of Pakistan FMDV serotype ‘O’ RT-LAMP primers.

Primer	Sequence (5'-3')
F3	CATCCTCACCACCCGTAAC
B3	GACACCTTTGTGGTCGGTC
FIP	GGAAGTGTTCGGTCCGCTCACTT-TTCCCAGTCAAGCGTTGGAG
BIP	CAGAGTTGTGCAGGCAGAACGGTT-TTAACGTCCGAATGAGTCACTG
FLP	GGAGTCACATACGGGTACG
BLP	CACCTCTTCGACTGGGTC

Source: Maryam et al., (2017).

3.6.11 Effect of AMV Reverse Transcriptase Enzyme and Loop Primers on RT-LAMP Assay

To evaluate how AMV reverse transcriptase enzyme and loop primers affected the RT-LAMP assay, the reaction mixture was carried out in presence and absence of loop primers. AMV added into the reaction mixture was varied as follows; 0.2 µl, 0.3 µl and 0.4 µl. The only RT-LAMP condition that was adjusted was, from the optimized 45 minutes to 60 minutes (to give enough time for amplification of reaction mixture that lacked loop primers). However, reaction temperature was maintained at 65°C.

3.6.12 Analysis of RT-LAMP Products

Determination of RT-LAMP post amplification was performed by agarose gel electrophoresis. Standard 1.5% agarose gel (Invitrogen life technologies) was prepared by dissolving 1.5g agarose in 100 ml 1x TBE buffer in Erlenmeyer flask and the mixture heated in microwave for 2 minutes (allows agarose dissolution and formation of the gel). Agarose gel was allowed to cool before adding 5µl of GelRed dye and poured onto a casting tray and combs fitted.

After 60 minutes, the combs were carefully removed and the casting tray immersed in an electrophoresis tank that contained 900 µl of 1 x TBE buffer. 5 µl of RT-LAMP products of each individual sample and DNA marker (New England BioLabs) was mixed with 1µl of gel loading dye 6× (Invitrogen, Thermo Fisher scientific) dye and then loaded into separate wells on the gel. The samples were run at 120 volts for 35minutes following which the gel was removed from the tank and visualized using E-Box imaging system (Vilber Lourmat)

CHAPTER FOUR

4.0 RESULTS

4.1 Optimization of RT-LAMP Assay for Serotype ‘O’ FMDV

Following the use of designed primers and standard procedures, the RT-LAMP assay optimally amplified the VP1 gene of serotype ‘O’ FMDV at 65°C for 45 minutes when AMV reverse transcriptase was added. The final reaction conditions adapted for RT-LAMP assay were; 15µl isothermal master mix, 5µl primer mixture (mixture of LAMP primers), AMV reverse transcriptase and 5µl RNA template. The optimized RT-LAMP assay was capable of reproducing the same results when repeated under the same conditions twice.

4.2 Specificity and Cross-reactivity of RT-LAMP Assay for Serotype ‘O’ FMDV

RT-LAMP assay was carried out using serotype ‘O’ LAMP primers on extracted RNA samples for serotypes A, SAT 1 and SAT 3. These primers were found to be highly specific for the VP1 gene of serotype ‘O’ FMDV as they did not show any cross-reactivity with these serotypes, by showing no amplification of the other serotypes as shown in Figures 11-14.

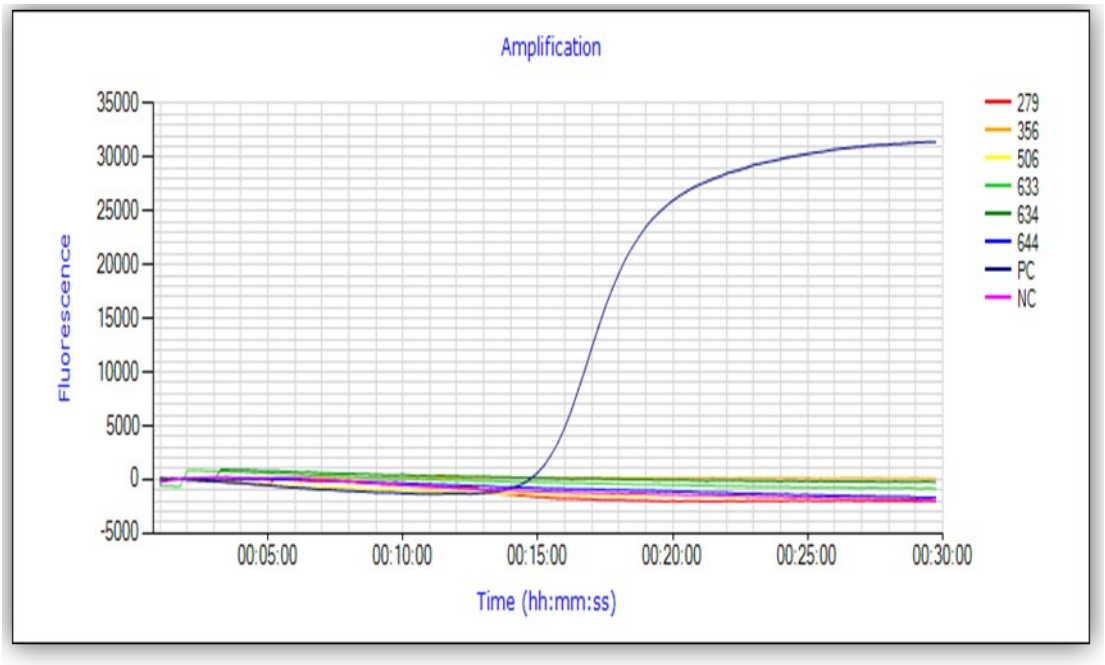

Figure 11: Amplification plot of serotype 'O' LAMP primers against FMDV serotype A samples collected in Tanzania. Legend: 279, 356, 506, 633, 634, and 644 are test samples of serotype A.

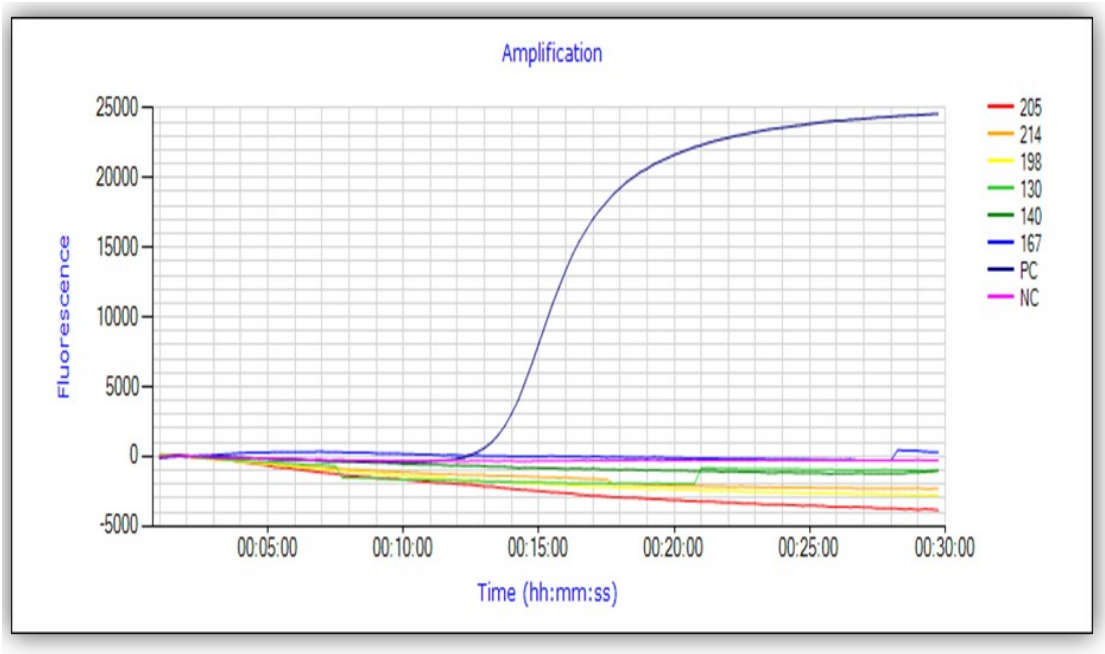

Figure 12: Amplification plot of serotype 'O' LAMP primers against FMDV SAT 1 samples collected in Tanzania. Legend: 205, 214, 198, 130, 140, and 167 are test samples of SAT 1 serotype.

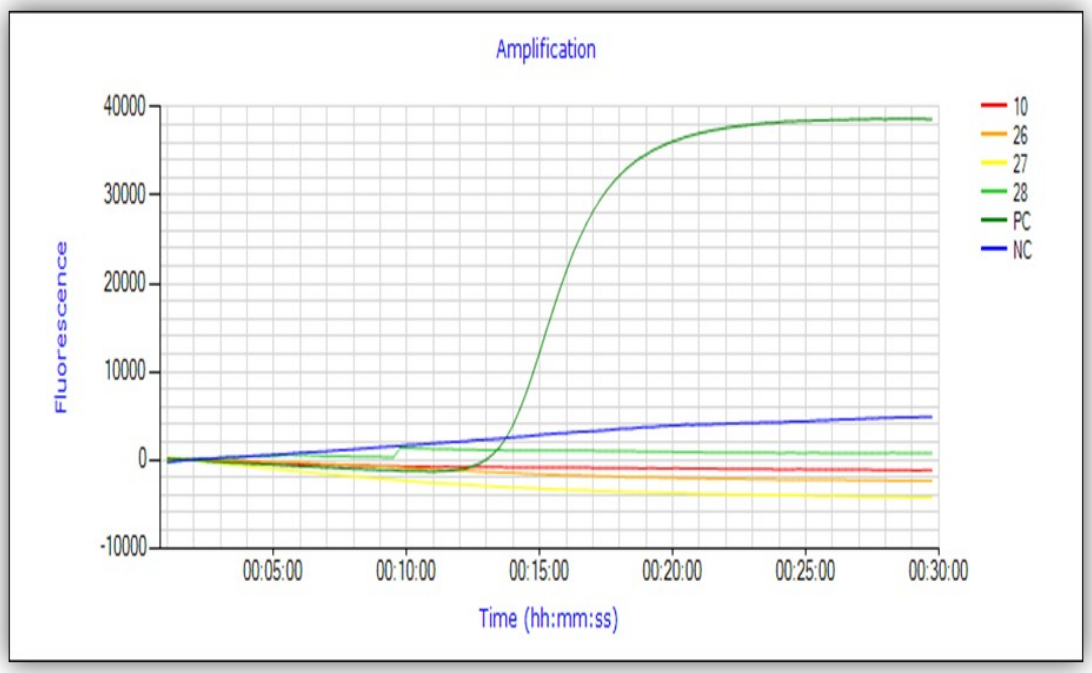

Figure 13: Amplification plot of serotype 'O' LAMP primers against FMDV SAT 3 samples collected from Zambia. Legend: 10, 26, 27 and 28 are test samples of SAT 3 serotype.

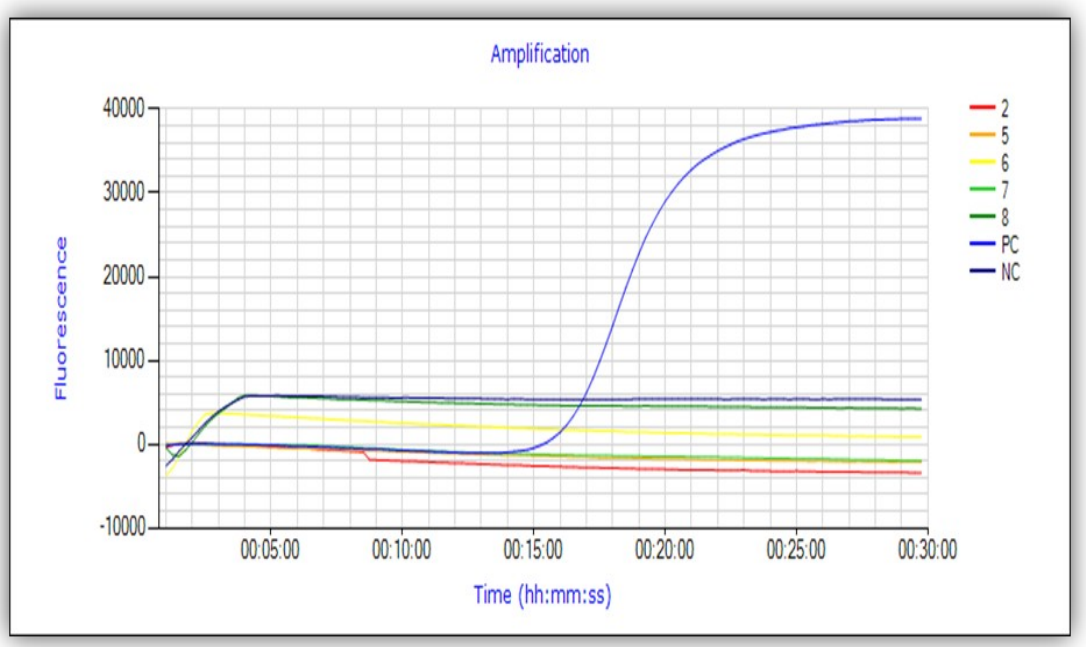

Figure 14: Amplification plot of serotype 'O' LAMP primers against FMDV SAT 3 samples collected from Namibia. Legend: 2, 5, 6, 7 and 8 are test samples of SAT 3 serotype.

4.3 Detection Limit (Sensitivity) of RT-LAMP Assay of Serotype 'O' FMDV

The detection limit of RT-LAMP assay was about 3.78 $\times 10^{-2}$ ng/µl at 10^{-2} dilution as shown in Fig. 15. The RNA template concentration before serial dilution was 450.278 ng/µl.

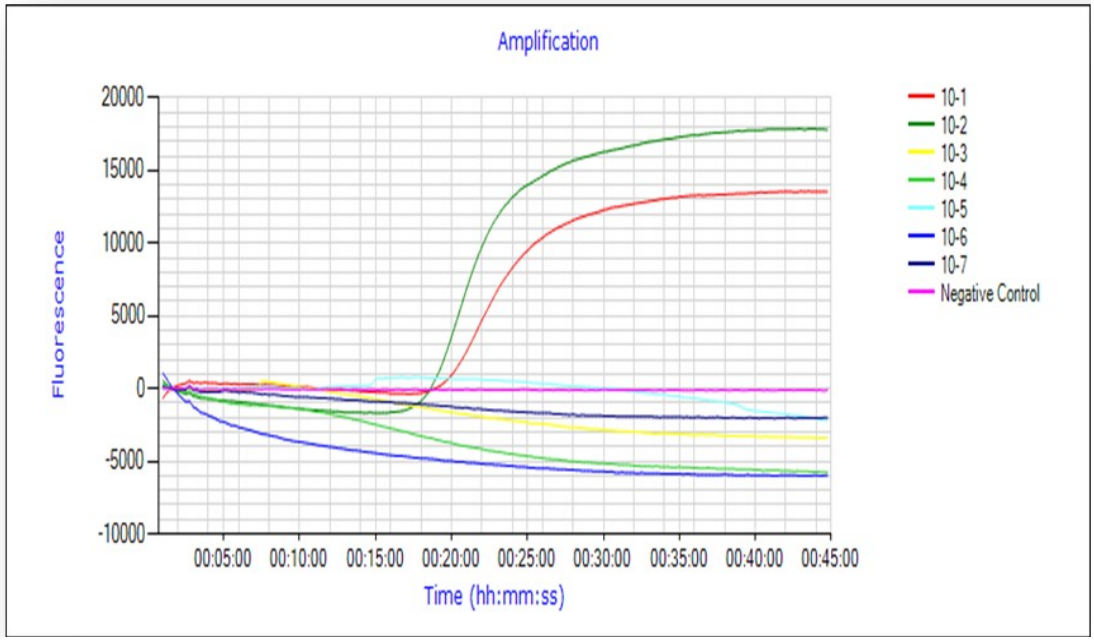

Figure 15: Amplification plot for evaluation of detection limit of RT-LAMP assay for serotype 'O' FMDV. The assay amplified serotype 'O' up to 10^{-2} dilution.

4.4 Detection of FMD Viral Genome using RT-LAMP

RT-LAMP assay was used for detection of FMDV genome from archived FMD suspected samples. Among the used 14 FMDV archived samples (n=14), RT-LAMP assay yielded positive results in all samples (n=14), detecting 100% of FMDV genome where both time of positivity (Tp) values ranged between 10-17 minutes and annealing temperature ranged from 86.1-87.8°C as shown in Table 9. Some of the amplification plots and annealing derivatives for detecting FMDV genome using RT-LAMP are shown in Fig. 16 and Fig. 17 respectively.

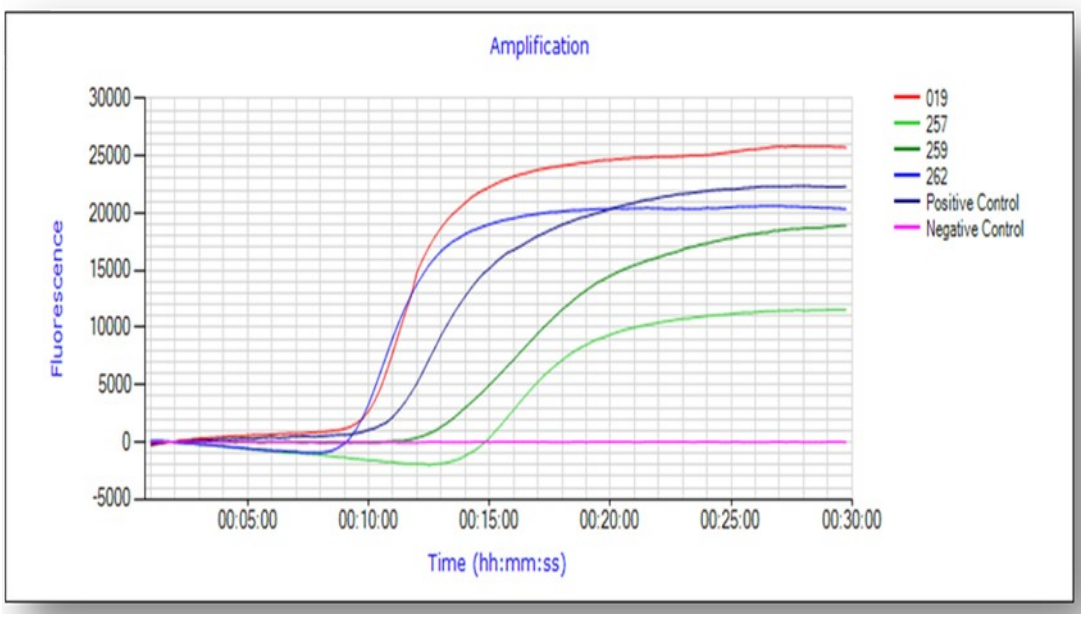

Figure 16: Amplification plot for detection of FMDV genome during RT-LAMP assay. Legend: 019, 257, 259 and 262, are test samples of serotype O.

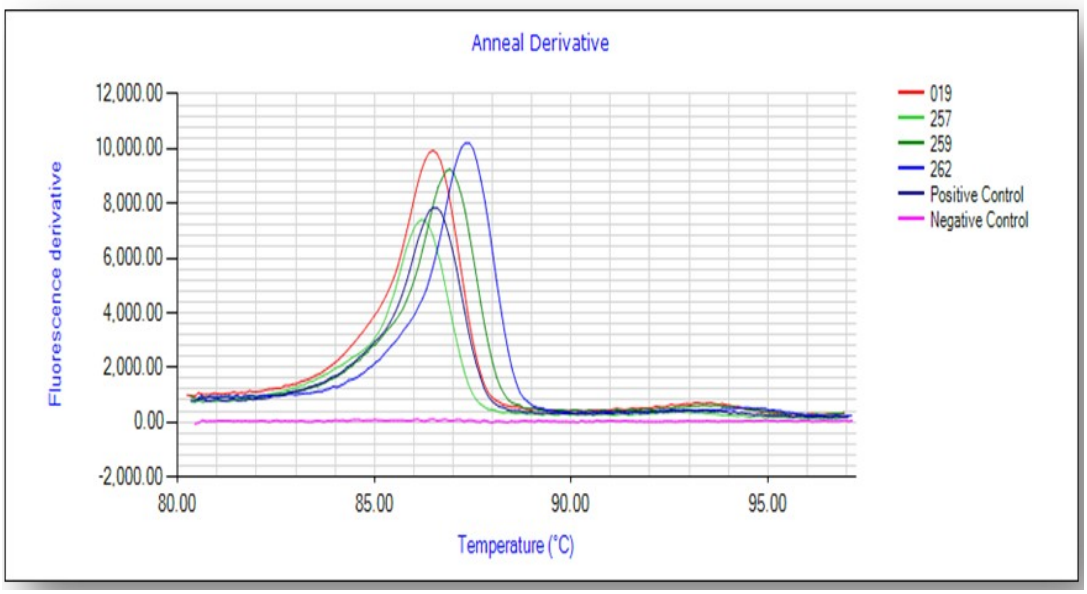

Figure 17: Annealing derivatives plot for detection of FMDV genome during RT-LAMP assay. Legend: 019, 257, 259 and 262, are test samples of serotype O.

Table 9: RT-LAMP assay results for detection and typing of serotype 'O'FMDV

Serotype	Sample ID	Location (Tanzania)	Year	RT-LAMP (PAN)		RT-LAMP SEROTYPING		Result
				T_P (min:sec)	T_A (°C)	T_P (min:sec)	T_A (°C)	
O	19	Fulwe, Morogoro	2016	11.00	86.49	14:45	88.28	+
	184	Mtwara	2017	17.00	87.39	15:30	87.78	+
	254	Kibaha, Rehema	2017	10.15	86.99	13:45	87.89	+
	257	Kibaha, Rehema	2017	16.00	86.19	15:00	87.88	+
	259	Kibaha, Rehema	2017	16.00	86.92	15:45	87.88	+
	262	Kibaha, Rehema	2017	10.30	87.41	10:45	88.28	+
	265	Kibaha, TC	2017	13.45	86.79	18:30	88.68	+
	268	Kibaha, TC	2017	10.15	87.38	16:30	88.08	+
	272	Kibaha, TC	2017	13.00	86.10	25:45	87.02	+
	307	Kasulo, Ngara	2017	13.00	86.79	16.00	87.39	+
	365	Tandahimba, Mtwara	2017	11.15	87.11	22:30	88.11	+
	369	Tandahimba, Naputa	2017	11.00	86.13	17.15	87.39	+
	624	Kinondoni	2017	12.00	87.19	17:15	88.29	+
	625	Kinondoni	2017	10.15	87.78	17:15	88.59	+

4.5 RT-LAMP Assay for Serotype 'O' FMDV using Designed Primers

The results of real time RT-LAMP assay for serotype 'O' were assessed by time to positivity (Tp) and annealing temperature (Ta). Archived clinical samples that showed both Tp and Ta were considered positive. RT-LAMP assay was able to amplify 100% of VP1 gene of serotype 'O' FMDV (n=14) as indicated (Table 9). The assay was able to detect the VP1 gene between 13 minutes to 26 minutes in the presence of AMV reverse transcriptase and recorded Ta values between 70.0-89.0°C (Table 9). The amplification plot (Figs. 18) and annealing derivative (Figs. 19) of the RT-LAMP reactions are shown below.

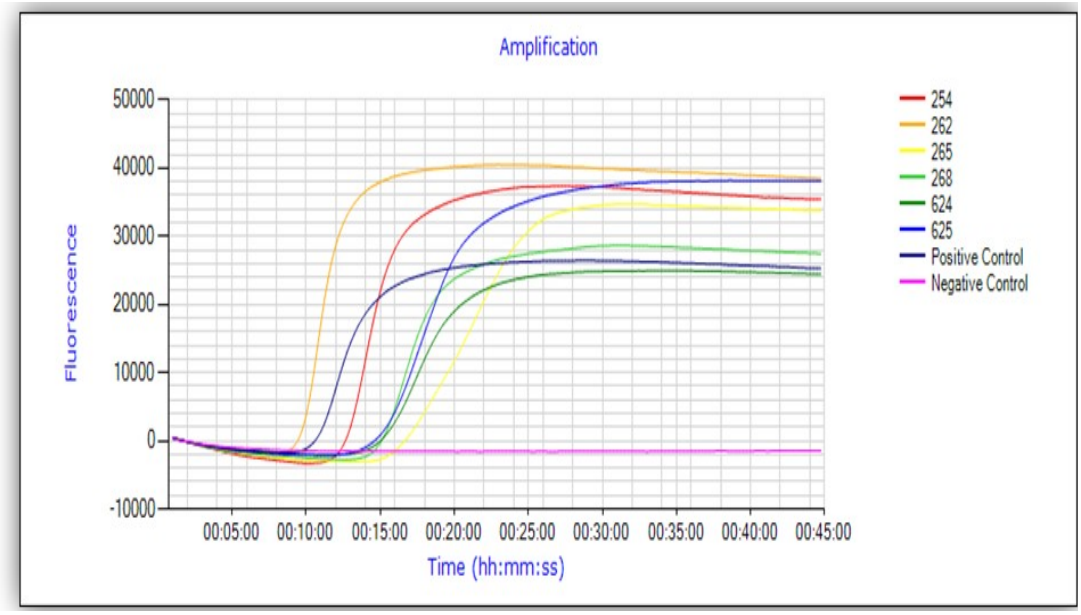

Figure 18: Amplification plot for FMDV serotype 'O' obtained during RT-LAMP Assay. Legend: 254, 262, 265, 268, 624, and 625 are test samples of serotype O.

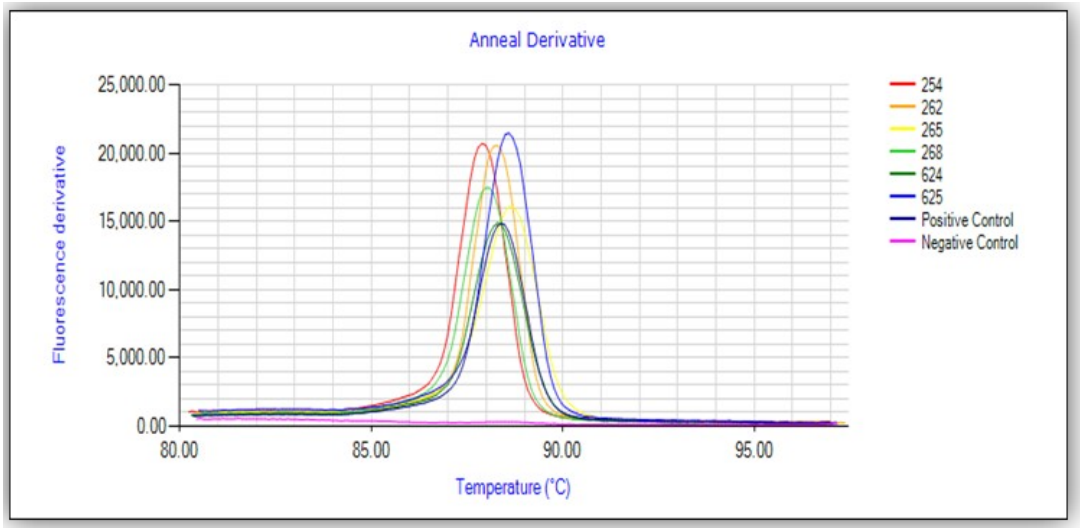

Figure 19: Annealing derivatives plot for FMDV serotype 'O' obtained during RT-LAMP Assay. Legend: 254, 262, 265, 268, 624, and 625 are test samples of serotype O.

4.6 RT-LAMP Assay for Serotype 'O' FMDV using Pakistan Primers

Detection (amplification) of VP1 gene did not occur on Tanzanian serotype 'O' FMDV isolates using Pakistan LAMP primers (Fig. 20). However, when the Pakistan LAMP loop primers were incorporated into four Tanzania LAMP primers (SK1 + Pakistan loop primers), the amplification rate was seen to increase as evidenced by halved time to positivity (Figs. 20 and 22).

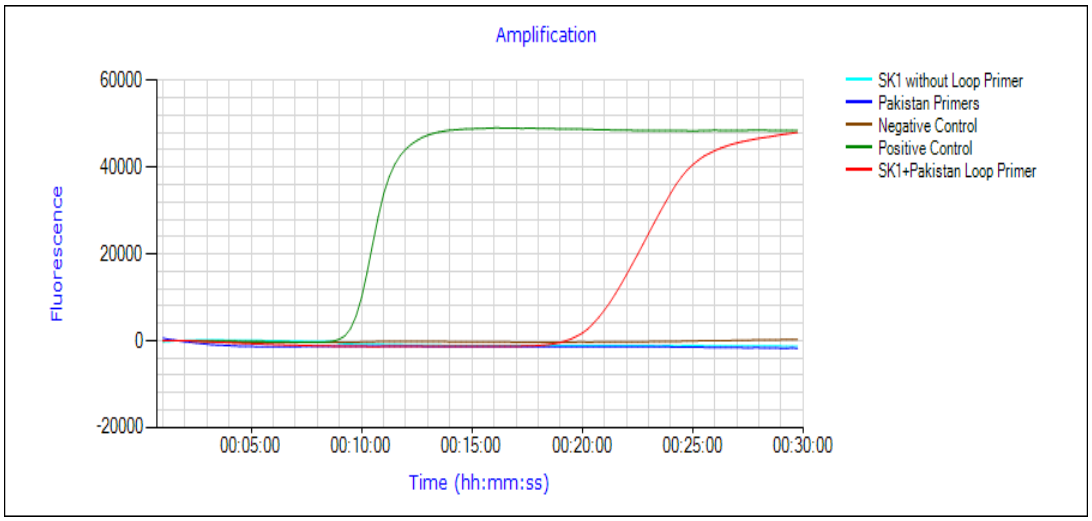

Figure 20: Amplification plot for FMDV serotype 'O' obtained during RT-LAMP assay using Pakistan primers. Legend: SK1 (4 Tanzania LAMP primers); Pakistan primers (No amplification occurred); SK1 + Pakistan loop primers resulted to amplification

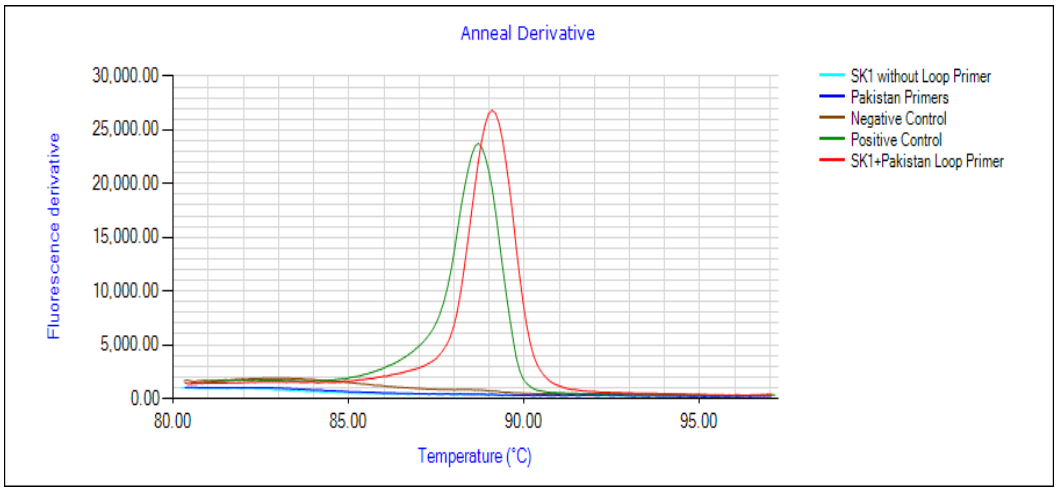

Figure 20: Amplification plot for serotype 'O' FMDV obtained during RT-LAMP assay using Pakistan primers. Legend: SK1 (4 Tanzania LAMP primers); Pakistan primers (No amplification occurred); SK1 + Pakistan loop primers resulted to amplification

4.7 Effect of AMV Reverse Transcriptase Enzyme and Loop Primers on RT-LAMP Assay

When AMV reverse transcriptase enzyme was added into the reaction mixture, it was noted that amplification rate increased (time for amplification reduced) (Fig. 22). Efficient amplification (less time) was evidenced when both AMV reverse transcriptase enzyme and loop primers (AMV reverse transcriptase enzyme, SK1 and loop primers) were present (Figs. 20 and 22)

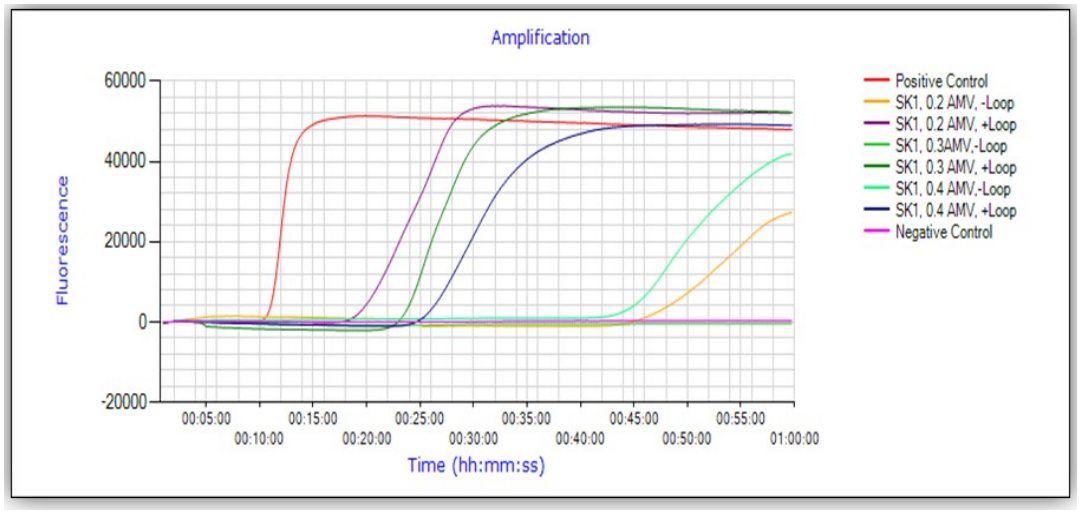

Figure 22: Amplification plot showing the effect of AMV reverse transcriptase and loop primers on RT-LAMP Assay. Legend: SK1, LAMP primers; - Loop, without loop primers; + loop, with loop primers.

4.8 Analysis of the RT-LAMP Products

Agarose gel electrophoresis was carried out on RT-LAMP assay products to verify whether amplification occurred. Bands, ladder-like patterns were observed which indicated positive amplification of VP1 gene of serotype ‘O’ FMDV in both RT-LAMP assay for serotype ‘O’ (Fig. 24) and limit of detection evaluation (Fig. 25). Ladder-like bands were also observed in RT-LAMP assay products indicating detection of the 3D Polymerase gene of the FMDV genome (Fig. 23).

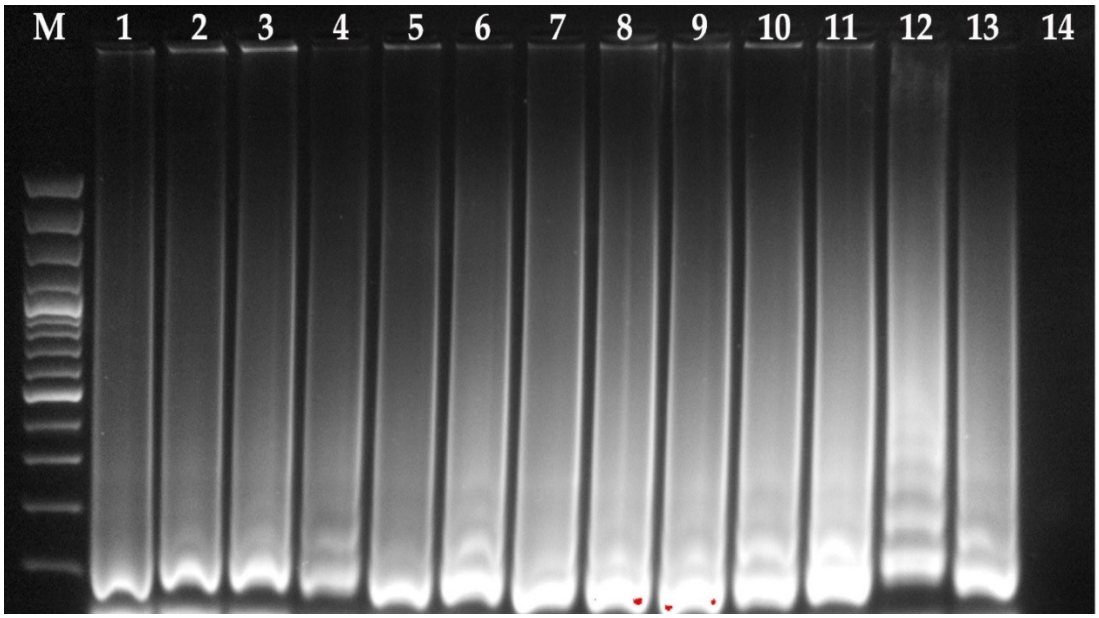

Figure 23: Agarose gel electrophoresis of FMDV detection RT-LAMP assay products on a 1.5% agarose gel. Legend: lane M, 100 bp DNA ladder (New England BioLabs); lane 1-12, test samples; lane 13, positive control; lane 14, negative control

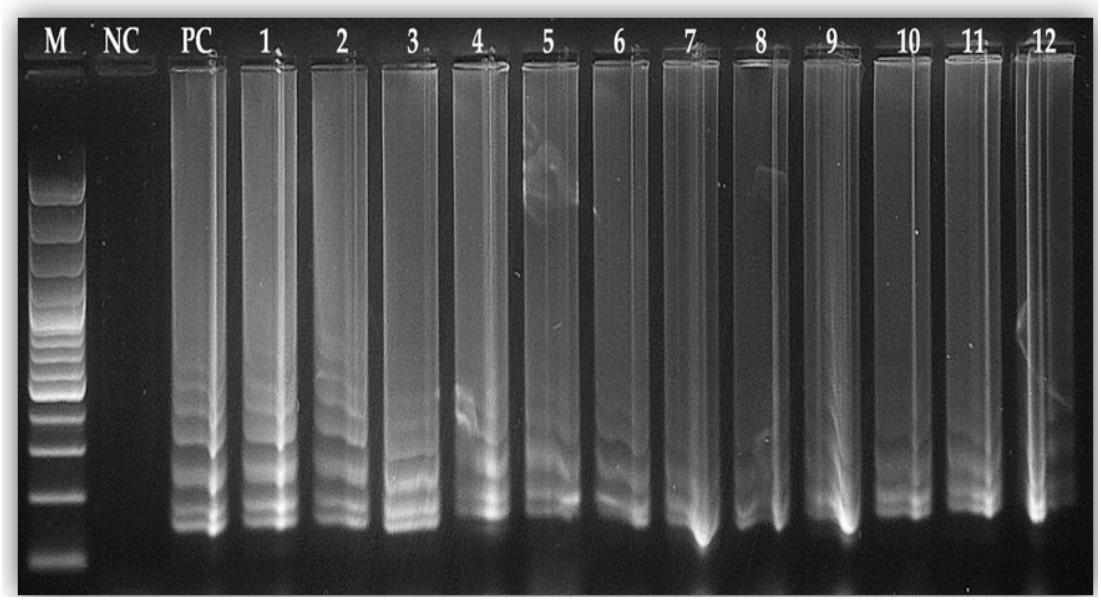

Figure 24: Agarose gel electrophoresis of serotype 'O' FMDV RT-LAMP assay products on a 1.5% agarose gel. Legend: lane M, 100 bp DNA ladder (New England BioLabs); lane NC, negative control; lane PC, positive control; lane 1-12, test samples.

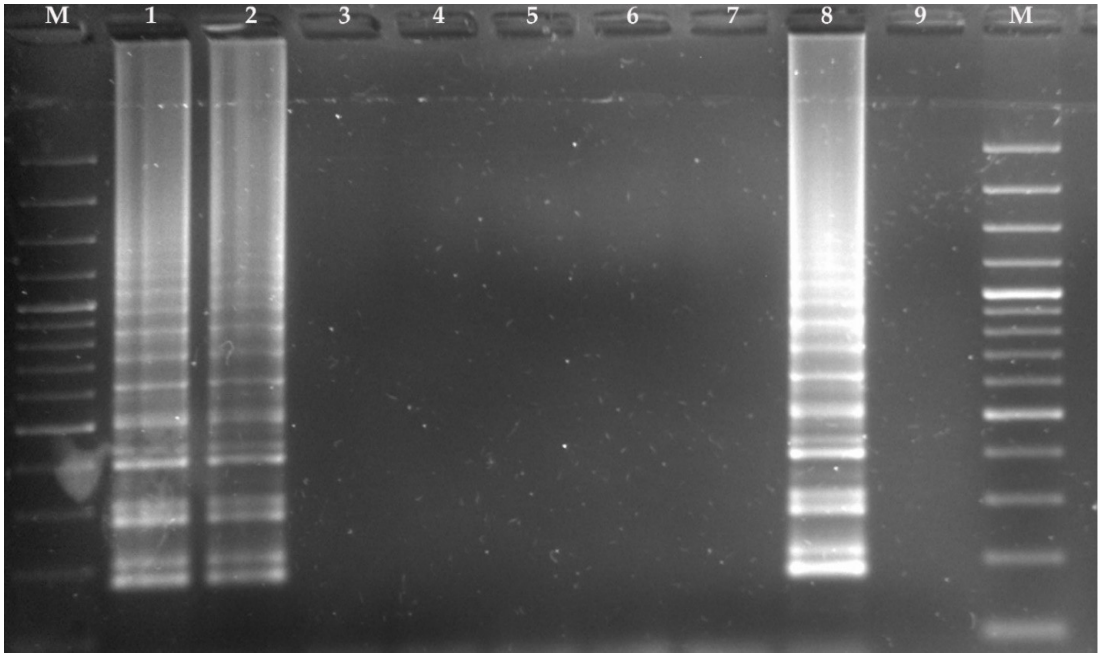

Figure 25: Agarose gel electrophoresis of limit of detection RT-LAMP assay products on a 1.5% agarose gel; Legend: lane M, 100 bp DNA ladder (New England BioLabs); lane 1-7 (10^{-1} to 10^{-7}); lane 8, positive control; lane 9, negative control

Evidenced by formation of ladder-like bands up to lane 2 (10^{-2} dilution) from an initial RNA template concentration of 450.278 ng/µl (Fig. 29), the detection limit of RT-LAMP assay was about 3.78 $\times 10^{-2}$ ng/µl. The sensitivity of the assay was 3.78 $\times 10^{-2}$ µg/µl.

CHAPTER FIVE

5.0 DISCUSSION

Foot-and-mouth disease (FMD) is endemic in Tanzania, with outbreaks occurring throughout the year in different regions of the country. Serotype ‘O’ FMDV is known to have the widest distribution in Tanzania (Kasanga *et al.,* 2014). Owing to its infectious nature, rapid diagnosis and discrimination of serotype ‘O’ FMDV strains is vital in initiating the effective control strategy. Detection, serotyping of FMDV, surveillance and control measures have been based on the laboratory findings. The generation of such relevant information is achieved via assays such as PCR-based methods that require sophisticated equipment and complex procedures for the detection and discrimination of serotypes and/or variants. This makes them unsuitable for resource-limited settings in developing countries such as Tanzania henceforth warranting the search for more simpler, sensitive, specific and user-friendly genomic-based diagnostic assays (Mori and Notomi, 2009). RT-LAMP is recognized as an alternative diagnostic tool that aims to solve this constrain.

The aim of this study was to develop RT-LAMP primers suitable for detecting VP1 gene of serotype ‘O’ FMDV strains circulating in Tanzania as well as to check the suitability of Pakistan RT-LAMP primers in detection of serotype ‘O’ FMDV strains circulating in Tanzania.

The findings of this study indicated that all samples (n=14) tested positive for FMDV genome, as indicated by both the presence of time to positivity and annealing temperature (Table 9). This observation was consistent with the results of agarose gel electrophoresis, where ladder-like bands were observed, indicating amplified positive product (Fig. 23). RT-LAMP assay was able to detect FMDV genome between 10-17

minutes, results which agree with previous finding by Dukes *et al.,* (2006) and Kasanga *et al*., (2014) which demonstrated the ability of RT-LAMP assay to detect FMDV within 60 minutes.

The RT-LAMP results using primers developed in this study were positive as indicated by the presence of amplification and annealing derivatives of VP1 gene of serotype 'O' FMDV (Fig. 18, 19) contrary to those developed by (Maryam *et al.,* 2017) which failed to amplify FMDV serotype 'O' isolates recovered from cattle in Tanzania (Fig. 20, 21). The failure of these primers to amplify the VP1 polyprotein gene from the Tanzanian serotype 'O' FMDV could be ascribed to genetic diversity which classify the Pakistan serotype 'O' FMDV isolates under different pool and genotype (Cathay and Middle East-South Asia -ME-SA) as compared to those from Tanzania (EA-2).

The assay was able to detect the VP1 gene of serotype 'O' FMDV at 13-26 minutes in the presence of AMV reverse transcriptase but for optimum reaction, the assay reaction times was set for 45 minutes (Table 9). These results agree with previous RT-LAMP assay findings by Madhanmohan *et al.,* (2013), Farooq *et al.,* (2015), Lim et *al.,* (2018) and Maryam *et al.,* (2017) that demonstrated RT-LAMP to detect VP1 gene of serotype 'O' FMDV within 60 minutes. The RT-LAMP annealing temperature (Derivative) ranged between 87.0°C-89.0°C (Table 9).

The study employed four designed primers that targeted six regions of the VP1 gene. The inclusion of loop primers increased the RT-LAMP assay product yield, amplification speed, efficiency and improved both specificity and sensitivity of the RT-LAMP assay. These results agree with previous studies conducted by Mori and Notomi, 2009; Madhanmohan *et al.,* 2013 and Maryam *et al.,* 2017. It was observed

that loop primers developed by Maryam *et al.*, (2017) amplified the VP1 gene of serotype 'O' FMDV isolates circulating in Tanzania. This led to a deduction that these loop primers targeted a conserved region in VP1 gene as they were able to successfully amplify both serotype 'O' FMDV isolates from Pakistan and Tanzania. It was also noted that addition of AMV reverse transcriptase had significant impact on amplification rate where it was observed to half the reaction time (Fig. 22).

Diagnosis of FMDV that employ molecular assay rely heavily on assay specificity and sensitivity. The efficiency of RT-LAMP assay relies on the specificity of the primer sets designed. The specificity of the assay was 100%. These results agree with previous studies conducted by Madhanmohan *et al.*, 2013 and Maryam *et al.*, 2017. Specificity was evaluated by conducting cross-reactivity studies where RT-LAMP primers were used to amplify VP1 genes of serotypes; A, SAT1 (Tanzania) and SAT3 (Namibia and Zambia). At the time there were no characterized data on SAT 2 from Tanzania. The RT-LAMP primers did not show any cross-reactivity with other serotypes used in the study, suggesting that the primers were specific to serotype 'O' FMDV. This was indicated by lack of both amplification and annealing derivatives for the samples used in the assay (Figs. 11 to 14).

In this study, the sensitivity of RT-LAMP assay for serotype 'O' FMDV was inferred from the detection limit findings. The RNA template concentration before serial dilution was 450.278 ng/µl. Using varying concentrations of RNA, the detection limit of the assay was about 3.78×10^{-2} ng/µl as shown in Fig. 15 and consistent gel results (Fig. 25). This finding was similar to that made by Madhanmohan *et al.*, (2013) whose detection limit was at 10^{-3} dilution. From this finding, it was inferred that the assay could detect VP1 gene of serotype 'O' FMDV up to a concentration 3.78×10^{-2} ng/µl.

CHAPTER SIX

6.0 CONCLUSION

This study has developed the RT-LAMP assay for detection and discrimination of serotype 'O' FMDV recovered in Tanzania whose optimum conditions were 65˚C, 45 minutes in the presence of both AMV reverse transcriptase and loop primers. The developed RT-LAMP assay was demonstrated to be specific to serotype 'O' FMD viruses with a sensitivity of 3.78×10^{-2}ng/µl. The study provides baseline information for control of outbreaks and development of serotype-specific vaccines.

6.1 RECOMMENDATIONS

From this study, it is recommended that;

1. Further evaluation studies are needed to determine whether the assay would be useful for detecting serotype 'O' FMDV strains circulating in other regions in Tanzania
2. Field evaluation of the RT-LAMP assay should be carried out using FMDV-infected epithelial tissue samples
3. Further studies are needed to evaluate the comparative sensitivity with RT-PCR, *q*RT-PCR and/or any other molecular tools should be carried out
4. Due to the high mutation rate of FMDV, the oligonucleotide primers need to be re-designed and improved after a certain period of time
5. Further development and evaluation of RT-LAMP assays using serotypes A, SAT1 and SAT 2 FMD viruses need to be carried out at both laboratory and field settings to infer its application for rapid diagnosis and discrimination of the field virus strains following FMD outbreaks.

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