APPLICATION OF REAL-TIME RT-PCR ASSAY FOR DETECTION AND TYPING OF FOOT-AND-MOUTH DISEASE VIRUS IN TANZANIA

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ABSTRACT

Speed is paramount in the diagnosis of highly infectious Foot-and-mouth disease (FMD). Recent developments in molecular biology have enabled specific detection of FMD virus by real-time RT-qPCR and sequencing in endemic settings in Africa. In this study, a laboratory-based experimental design was used to standardize real-time RT-qPCR assay for detection and typing of FMDV in selected regions of Tanzania. The optimized conditions for both pan-serotypic and serotype-specific real-time RT-qPCR assays were: reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10minutes followed by 95°C for 15 seconds), annealing and elongation temperature (at 60°C for 1 minute) for 52 cycles. FMD virus were detected in 100% (n = 14) archived samples that were collected between 2010 and 2013 from confirmed FMD-cases. The standardized real-time RT-qPCR assays revealed 100% sensitivity and 100% specificity for detection and typing of FMDV, respectively.The frequency of FMDV detection among FMD-suspected cases of cattle collected from 2008 to 2014 in selected regions of Tanzania was 92% (n = 23). Of the 23 positive samples, 56.5% (n = 13), 8.7% (n = 2), 21.7% (n = 5), and 8.7% (n = 2) were typed into serotypes O, A, SAT 1 and SAT 2 respectively. One sample (4.3%) was positive for both serotype A and SAT 1. These findings indicate that the standardized pan-serotypic and serotype-specific real-time RT-qPCR assays have a potential use in detection and typing of FMDV field strains in endemic settings of Tanzania and Africa at large. Application of standardized real-time RT-qPCR assays could hasten diagnosis of FMDV that guide selection of effective FMD-control measures in the region. In-depth studies, including sequencing of one sample that revealed positive for two serotypes, are required to elucidate the possibility of mixed infection among FMD cases.

DECLARATION

I, Herieth Rhodes Mero, hereby do 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 being concurrently submitted in any other institution.

_____ _____

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The above declaration is confirmed by;

_____ _____

Dr. Christopher J. Kasanga Date
(Supervisor)

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DEDICATION

With devotion, I dedicate this study to the government of United Republic of Tanzania especially all stakeholders of the Ministry of Livestock and Fisheries, both national and international researchers (molecular biologists, virologists and molecular epidemiologists) who are doing research on the long-known foot-and-mouth disease virus (FMDV) causing catastrophic FMD. I salute and celebrate the remarkable, magnificent research work you have done on FMD. Indeed your great achievements is an encouragement to young researcher such as myself, that FMD can successfully be controlled effectively only if, we work collaboratively within Tanzania and globally through extensive progressive research. Please tighten your belts because the battle has just begun and more is to be done on FMD ‘aluta continua’.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	Adenine
ABI	Applied Biosystem Inc
AG	Agarose Gel
AGE	Agarose Gel Electrophoresis
AMV	Avian Myeloblastosis Virus
Aº	Angstrom
AVE	Elution buffer
AVL	Virus Lysis Buffer
AW1	Wash Buffer 1
AW2	Wash Buffer 2
BEBO	4 – [(3-methyl-6- (benzothiazol-2-yl)-2, 3-dihydro- (benzo-1, 3-thiazole)-2-Methylidene)]-1-methyl-pyridinium Iodide
BHK-21	Secondary cell line from Baby Hamster Kidney
BHQ1	Black Hole Quencher 1
BOXTO	(4-[6-(benzoxazole-2-yl-(3-methyl-)-2, 3-dihydro-(benzo-1, 3-thiazole)-2-methylidene)]-1-methyl-quinolinium chloride)
bp	base pair
Bst	*Bacillus Stearothermophilus*
Bus	B-urylation site
C	Cytosine
Cap	7-Methyl guanosine
CCD	Charge Coupled Device
cDNA	Complementary DNA
CF	Complement Fixation

CFT	Complement Fixation Test
CIDB	Centre for Infectious Disease and Biotechnology
CO_2	Carbon dioxide
Ct	Cycle threshold
$Cy5^{TM}$, $Cy3^{TM}$	Cyanide dyes
D	Aspartic Acid
Da	Dalton
DABCYL	(4-Dimethylaminoazo) benzene-4-carboxylic acid
DABSYL	(4-(Dimethylaminoazo) benzene-4-Sulfonyl Chloride)
ddNTPs	2', 3'-dideoxynucleotides
DNA	Deoxyribonucleic Acid
dNTPs	2'-deoxyribonucleoside Triphosphates
dsDNA	double stranded Deoxyribonucleic acid
EA	East Africa
eIF4G	Eukaryotic transcription initiation factor
ELISA	Enzyme-Linked Immunosorbent Assays
ER	Endoplasmic Reticulum
e*t al*	and others
EtBr	Ethidium Bromide
etc.	Excetra
eTIFs	Eukaryotic Translation Initiation Factors
FMD	Foot-and-Mouth Disease
FMDV	Foot-and-Mouth Disease Virus
FP	Forward primer
FRET	Fluorescence Resonance Energy Transfer
Gly	Glycine

g	gravitational force
G-H loop	G and H amino acid loops of viral protein 1
G	Guanine
GDP	Gross Domestic Product
GNP	Gross National Product
HEX	Hexachloro-6- carboxyfluoroscein
HT-NGS	High throughput Next Generation Sequencing
i.e.	that is
IBRS-2	Pig Kidney cells
ID	Infectious Dose
IN	Identification number
IPC	Internal positive control
IRES	Internal Ribosomal Entry Site
TID	Tissue infectious Dose
KDa	KiloDalton
Km	Kilometer
LAMP	Loop-Mediated Isothermal Amplification
LED	Light Emitting Diode
LFD	Lateral Flow Devices
LPBE	Liquid Phase Blocking ELISA
L^{PR0}	Leader proteases
M	ambiguous nucleotide (A + C)
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium Sulfate
MMLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA

Nanometre	nM
NSPs	Non-Structural Proteins
nt	Nucleotide
OD	Optical Density
OIE	Office International des Epizooties
OP	Oesophageal Pharyngeal
ORF	Open Reading Frame
P	Probe
P1	Polyprotein 1
P2	Polyprotein 2
P3	Polyprotein 3
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCP	Progressive Control Pathway
PCP-FMD	Progressive Control Pathway for Foot-and-Mouth Disease
PCR	Polymerase Chain Reaction
PEC	Positive Extraction Control
Pfu	*Pyrococcus furiosus*
PH	Degree of acidity or alkalinity
Pi	Post infection
PI-2A	Capsid precursor
PK	Pseudoknot
Poly A tract	Polyadenylation tract
Poly C tract	Polyribocytidylic tract
PPi	Inorganic pyrophosphates

R	ambiguous nucleotide (A + G)
RFU	Relative Fluorescence Units
RGD motif	Arginine-Glycine-Aspartic Acid motif
ΔRn	Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline
RNA	Ribonucleic Acid
RP	Reverse primer
Rpm	Revolution per minutes
rRT-PCR	real-time Reverse Transcription Polymerase Chain Reaction
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
RT-LAMP-LFD	Reverse Transcription-Loop-Mediated Isothermal Amplication-Lateral Flow Device
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SAT	Southern African Territories
SPs	Structural Proteins
ssDNA	Single stranded Deoxyribonucleic Acid
ssRNA (-)	Negative sense, single stranded Ribonucleic Acid
ssRNA (+)	Positive sense, single stranded Ribonucleic Acid
SVD	Swine Vesicular Disease
SYBR Green 1	(2-[N-(3-dimethylaminopropyl)-N-propylamino]-4- [2,3-dihydro-3-Methyl-(benzo-1,3-thiazol-2yl)-methylidene]-1-phenyl-quinolinium)
T	Thymine
TAD	Transboundary Animal Disease
TAMRA	Non-fluorescent quencher
Taq	*Thermus aquaticus*

TET	Tetrachloro-6-carboxy-fluoroscein
TIF	Translation Initiation Factors
TM	Trade Mark
Tm°	Melting temperature
Tth	*Thermus Thermophilus*
TVLA	Tanzania Veterinary Laboratory Agency
U	Uracil
UK	United Kingdoms
US	United States
VI	Virus Isolation
VIAA	Virus Infection Associated Antigen
Viz	Example
VNT	Virus Neutralization Test
VP	Viral Protein
VP1	Viral Protein 1
VP2	Viral Protein 2
VP3	Viral Protein 3
VPg	Virus protein genome linked
VPO	1AB subunit
VTM	Virus Transport Media
Y	ambiguous nucleotide (C + T)
YOYO-1	(1, 1'-4, 4, 8, 8-tetramethyl-4, 8-diazaundecamethylene) bis [4-[(3-methylbenzo-1,3-oxazol-2-yl)methylidene]-1,4-dihydroquinolinium]tetraiodide)
α	Alpha
$A_v\beta_1$, $A_v\beta_3$, $A_v\beta_5$, $A_v\beta_6$, $A_v\beta_8$	Intergrin receptors

β	beta
%	Percentage
≤	Less than or equal to
≥	Greater than or equal to
®	registered trade mark
°C	Degree Celsius
µl	Microlitre
µM	Micromolar
3’ UTR	Three prime Untranslated Region
$3C^{PRO}$	3C protease
$3D^{POL}$	RNA-dependent RNA polymerase
5’ UTR	Five prime Untranslated Region
6-FAM	6-carboxyFluorescein

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Long-term persistence and catastrophic economic repercussion, has made Foot-and-mouth Disease (FMD) a popular but unusual animal disease which draws attention of the world since 1890s because its eradication is still a dream to come true. FMD is a highly contagious, viral disease affecting both domestic and wildlife cloven-hooved animal species (Reid *et al.,* 2014) of the order *Artiodactylae* (Carrillo *et al.,* 2012; Picado *et al.,* 2010). Foot-and-Mouth Disease Virus (FMDV) of family *Picornaviridae* and genus *Aphthovirus,* is a causative agent of FMD (Kasanga *et al.,* 2014). There are no other new FMDV serotypes apart from the co-existing seven immunologically distinct serotypes (Sangula *et al.,* 2011; Reid *et al.,* 2014) that are differentially distributed around the globe (Rweyemamu *et al.,* 2008). These are serotypes A, O, C, Southern African Territories (SAT) 1, 2, 3 and Asia 1 (Alexandersen *et al*., 2003; Chang *et al.,* 2013; Reid *et al.,* 2014). Being a global Transboundary Animal Disease (TAD) of economic importance, permanent eradication and/control of FMD by vaccination have not been successful because high mutation rate of the virus attributes to existence of genetically and antigenically distinct serotypes/ topotypes with no cross-protection by a single vaccine (Kasanga *et al.,* 2012).

Furthermore, extreme contagiousness, broad host range and a wide though unequal geographical distribution of FMDV are other challenges to FMD-control contributed by inevitable economic activities that facilitate disease transmission through movement of human, animals and/ or their products from FMD-endemic areas (Africa, Asia and Southern America (Reid *et al.,* 2014) to FMD-free areas (some European countries,

Northern America, Australia, Iceland, Greenland and small island of Oceania) resulting into seasonal emerging and re-emerging outbreaks of FMD (Carrillo, 2012; Kivaria, 2003; Rweyemamu *et al.,* 2008). Re-emergence of devastating FMD-outbreaks in previously FMD-free countries like; Bulgaria (2010-2011), Japan and Korea (2000 and 2010), France and Netherlands (2001) and United Kingdom (UK) (2001 and 2007) is an evidence of periodic spillover of FMD virus from FMD-endemic settings (Reid *et al.,* 2014) as contributed by globalization and international trade of unscreened animals and /or their products. When it comes to economic losses that FMD outbreak impacts, a case study revealed that FMD incursion in United Kingdom (UK) in 2001 caused a loss of over 8 billion approximately with Gross Domestic Product (GDP) drop by 0.2% (Waters *et al.,* 2014). No nation is considered FMD-safe because of the highly contagious nature of FMD virus (Ding *et al.,* 2013) even though a lot of efforts are put to regain the FMD-free status in previously FMD-naive countries. Lack of skilled labour force, sound diagnostic capacity as well as poor law-enforcement against uncontrolled animal movement are among obstacles to the understanding of complexity of epidemiology and dynamics of FMD in Tanzania and Africa at large (Namatovu *et al.,* 2013). Scientific knowledge and comprehensive understanding of spatio-temporal distribution, genetic and antigenic characteristics of the FMDV, is a pre-requisite for effective control and eradication of FMD in endemic settings (Kasanga *et al.,* 2012) particularly in Tanzania.

Previous investigation on spatio-temporal distribution of FMD using both serological assays like Enzyme-linked Immunosorbent assays (ELISA) and molecular diagnostic tools, (gel-based reverse transcriptase polymerase chain reaction (RT-PCR), real-time reverse transcriptase polymerase chain reaction (RT-qPCR) and reverse transcriptase loop mediated isothermal amplification (RT-LAMP) have confirmed the presence of co-circulating A, O, SAT 1 and SAT 2 FMDV-serotypes in Tanzania (Kasanga *et al.,* 2012;

Mkama *et al.,* 2014). Despite such preliminary achievement, Tanzania is below stage 3 of Progressive Control Pathway for FMD (PCP-FMD), a strategic plan established by Office International des Epizooties (OIE) for successful control of FMD in endemic settings (Namatovu *et al.,* 2013). For that case, a rapid highly sensitive and specific molecular diagnostic assay is required for rapid detection and characterization of FMDV during outbreaks and continuous surveillance programs for effective FMD-control. In response to poor diagnostic capacity in Tanzania, this study focused on optimization and application of a highly sensitive and specific "one-step real-time RT-qPCR assay" for rapid detection and typing of the circulating FMDV field strains.

1.2 Problem Statement

FMD is a constraint to international trade of animals and/ or animal products (Ma *et al.,* 2011; Waters *et al.,* 2014) and thus reduces Gross National Product (GNP) (Goodwin *et al*., 2009). Moreover, FMD outbreaks contributes to decline of livestock industry, causing economic loss (Perry *et al.,* 2007; Ding *et al.,* 2013) among livestock keepers (Tanzanians inclusively) because it is characterized by high morbidity in adult animals, high mortality rate among young domesticated animals (Zhu *et al.,* 2013) and abortion for pregnant animals (Kivaria, 2003). Decline of livestock industry in turn contributes to deterioration of public health because livestock are the common sources of food (meat and milk) among Tanzanians.

Lack of rapid, highly sensitive and specific molecular diagnostic assay that can simultaneously diagnose and differentiate FMDV into specific serotypes and/ or topotypes is a major constraint to effective "on-time" control of FMD-sporadic outbreaks in Tanzania and other FMD endemic countries. Despite the fact that several molecular diagnostic assays ranging from conventional gel-based RT-PCR, real-time RT-PCR to

RT-LAMP are currently being used for both pan-serotypic and serotype-specific detection of FMDV, their application in endemic settings has not been largely instituted.

1.3 Justification

Application of one-step real-time RT-qPCR assay that can simultaneously detect and type four FMDV-serotypes (A, O, SAT 1 and SAT 2) that are co-circulating in different areas of Tanzania, will enable rapid detection and typing of FMDV in sporadic outbreaks as well as passive surveillance of FMD. This could therefore enhance immediate application of control-measures (quarantine, vaccination, eradication) against the disease. Appropriate and efficient control of FMD could improve productivity of livestock industry, public health and as well as economy of livestock keepers and the whole nation at large.

1.4 Expected Output

Application of serotype-specific real-time RT-qPCR will not only enable rapid 'on time' detection and typing of serotypes and/ or topotypes of FMDV that are responsible for sporadic outbreaks in different geographical areas of Tanzania, but also help in selection of candidate FMD vaccine strains. In addition, information generated from this study could guide the development of vaccines that match with circulating FMDV in Tanzania.

1.5 Research Questions

1. What were the optimum thermal cycling conditions of real-time RT-qPCR assay for detection and typing foot-and-mouth disease virus?
2. What was the specificity and sensitivity of real-time RT-qPCR assay for detection and typing foot-and-mouth disease virus?
3. What was the prevalence of Foot-and-mouth disease in selected areas of Tanzania from 2008 to 2014?

I.6 Hypotheses

i. The optimum thermal cycling condition of real-time RT-qPCR assay for detection and typing of FMD were; reverse transcription (at 55°C for 30 minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 90 °C for 10 minutes followed by 90 °C for 15 seconds), annealing temperature and elongation (55°C for 1 minute) for a total of 40 cycles

ii. Sensitivity and specificity of the real-time RT-PCR assay for diagnosing FMD was below 50% and 50% respectively

iii. Prevalence of foot-and-mouth disease was 40% in selected regions of Tanzania from 2008 to 2014.

1.7 Research Objectives

1.7.1 Overall objective

Overall objective of this study was to validate and deploy a one-step real-time RT-PCR assay for rapid diagnosis and simultaneous typing of FMD virus circulating in selected parts of Tanzania.

1.7.2 Specific objective

i. To establish the optimum conditions for one-step real-time RT-qPCR assay for detection and typing of FMDV,
ii. To determine the sensitivity and specificity of one-step real-time RT-qPCR assay and
iii. To determine the prevalence of FMDV in selected areas of Tanzania using optimized real-time RT-qPCR assay.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Foot-and-Mouth Disease Virus (FMDV)

2.1.1 Classification of FMDV

Foot-and-mouth disease virus (FMDV) is a member of (family; *Picornaviridae* and genus; *Aphthovirus*) known to cause Foot-and-mouth disease (FMD) (Goodwin *et al.,* 2009; Habiela *et al.,* 2010; Ding *et al.,* 2013; Zhu *et al.,* 2013). This virus is classified in the order *Nidovirales* (Longjam and Tayo, 2011).

2.1.2 Nomenclature of FMDV

The seven existing serotypes of FMD virus (O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1) (Genchwere and Kasanga, 2014; Kasanga *et al.,* 2014) were named after the geographical area where they caused FMD outbreak for the first time. O and A serotypes were discovered first in 1922 by Valle and Carre, who demonstrated that cattle from France that recovered from FMD infection, were re-infected with FMD after being mixed with another herd of cattle from German, infected with another serotype of FMD. This phenomenon explained the fact that, two distinct serotypes with no antibody cross-protection were responsible for FMD outbreaks in France and Germany (Jamal and Belsham, 2013). For that reason, the FMDV which caused disease outbreak in France was named Serotype O (‘O’ for department of Oise in France) and the FMDV which caused an outbreak in Germany was named Serotype A (‘A’ for Allemagne, which literally means ‘Germany’ in French language) (Jamal and Belsham, 2013). The outmost effort of Waldman and Trautwein, lead to the discovery of Serotype C in 1926 Waldman and Trautwein (1926), cited by Sangula, *et al*. (2011). However, FMD virus serotype C is considered the least prevalent one because it was reported last in 2004 when it caused

FMD outbreak in Brazil and Kenya, Roeder and Knowles (2009), cited by Sangula *et al.* (2011).

Intensive research done by Brooksby lead to the discovery of FMDV-serotypes which were responsible for FMD outbreak in South Africa and thus three distinct serotypes identified were named South African Territories (SAT) 1, 2 and 3 (Brooksby, 1958).

The seventh FMDV serotype was first isolated from Water buffalo in India in 1951 Dhanda *et al.* (1957), cited by Valarcher *et al*. (2009) and Pakistan in 1954 Brooksby and Rogers (1957), cited by Jamal and Belsham (2013) hence, it was named Asia 1 after the continent from which it was originally found. Up to this moment, Asia 1 serotype is primarily endemic in Afghanistan, India, Pakistan, Bhutan and Nepal countries which are collectively grouped in the Indian subcontinent (Valarcher *et al.,* 2009).

Despite the fact that, different comprehensive research have been conducted since the initial discovery of FMDV, yet no another new FMDV-serotype have been discovered (isolated) apart from seven existing serotypes. The existing FMDV serotypes (O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1) (Ding *et al.,* 2013) have been more than enough for human beings to handle since the first FMD outbreak, because each of FMDV serotype exist in different variants or sub-types (strains, topotypes) with no vaccine cross-protection (Kasanga *et al.,* 2012).

2.1.3 Host range of FMDV

Primarily, FMDV infects both domestic animals mainly cattle goat, sheep, pigs (Alexandersen and Mowat, 2005; Reid *et al.,* 2014) and over 70 wildlife species of order *Arteodactylae* (even-toed ungulates) (Porta *et al.,* 2013) in the family Bovidae, Cervidae,

Juidae, Tayasuidae, Camelidae, Giraffidae and Antilocapridae (Arzt *et al.,* 2011a,b; Picado *et al.,* 2010). Apart from domesticated and wildlife cloven hoofed animals that are susceptible to FMD (Zhu *et al.,* 2013), experimental studies have proved that, some non-cloven hoofed animals like Mice and Hamsters can also be infected with FMDV (Office International des Epizooties, 2012).

2.1.4 Geographical distribution of FMD virus

There's inequity in the manner by which the seven co-existent FMDV serotypes (A, O, C, SAT 1 SAT 2, SAT 3 and Asia 1) (Ma *et al.,* 2011; Reid *et al.,* 2014) are disseminated around the globe Knowles and Samuel (2003), cited by Reid *et al.* (2014). Among the seven FMDV serotypes, C is the least prevalent since it was reported last in 2004 (Reid *et al.,* 2014) unlike serotypes O and A which are responsible for causing most of FMD-outbreaks in different parts of the world (Valarcher *et al.,* 2009) despite the fact that, O is the most prevalent of all (Jamal and Belsham, 2013). Despite its low prevalence, Sangula *et al*. (2011) reported that serotype C was responsible for causing FMD-outbreaks in Kenya. The fact that, FMD is known to be enzootic in developing countries of Africa, Asia and South America (Carrillo, 2012) not all FMD virus are confined in these regions. Speaking of geographical confinement, (SAT 1, SAT 2 and SAT 3) are not strictly confined in sub-saharan region of African continent because evidence have shown that, they have been responsible for a number of FMD outbreaks in other continents (Reid *et al.,* 2014). This is because globalization has permitted their spill over from Africa to other continents (*viz* Europe) through international trade of animals and/ or animal products (Carrillo, 2012). Asia 1 serotype is long known to be confined in Asian continent even though it was recently reported that the virus was responsible for recent FMD outbreak in Greece (Reid *et al.,* 2014).

2.1.5 Physical structure of FMDV

Lack of surface pit (canyon) and highest buoyant density are the salient characteristic features which distinguish the non-enveloped FMDV with an icosahedral capsid (25-30nm in diameter) from other picornaviruses (Monaghan *et al.,* 2004; Carrillo, 2012; Jamal and Belsham, 2013; Han *et al.,* 2015). The icosahedral capsid encases a linear, positive sense (+), single stranded genomic RNA of about 8500 nt long in size (Sobrino *et al*., 2001; Grubman and Baxt, 2004; Carrillo, 2012). Sixty copies of each of the four dissimilar (asymmetric), structural viral proteins (VP1, VP2, VP3, and VP4) are the building blocks of the icosahedral capsid of FMD virus (Monaghan *et al.,* 2004; Carrillo, 2012) as shown in (Fig.1) below. The structural proteins are more variable than the non-structural proteins, even though the structural variation is unequal between the four structural proteins (VP1, VP2, VP3, and VP4) (Ma *et al.,* 2011). The antigenic variation is most frequent in VP1 of capsid structure because VP1 has versatile roles such as giving specificity to viruses (Ma *et al.,* 2011).

Furthermore, VP1 confers protection to the virus against the host's immunity (Ma *et al.,* 2011). Because of its high antigenic diversity between and within subtypes of FMDV, the gene encoding VP1, is normally targeted during serotyping of FMD virus (Sobrino *et al.,* 2001; Grubman and Baxt, 2004; Carrillo, 2012). Among the four structural proteins (VP1, VP2, VP3 and VP4), VP4 is the smallest protein with a molecular weight of 8.5KDa and each of the rest proteins (VP1,VP2 and VP3) which make the smooth, densely packed icosahedral capsid of FMDV have molecular weight of about 24KDa (Longjam and Tayo, 2011). Since VP4 protein is buried inside the icosahedral capsid rather than being exposed on the surface, it is believed to be in contact with the viral genomic RNA (Frank, 2002; Longjam and Tayo, 2011). In contrast to surface structural proteins of a viral capsid, the unique VP4 is covalently linked to a myristyl group at its N-terminal

(Longjam and Tayo, 2011). Among the three surface structural proteins of icosahedral capsid of FMD virus, VP3 and VP2 alternate around two-fold and three-fold axes unlike the VP1 which is located around the five-fold axes (Longjam and Tayo, 2011).

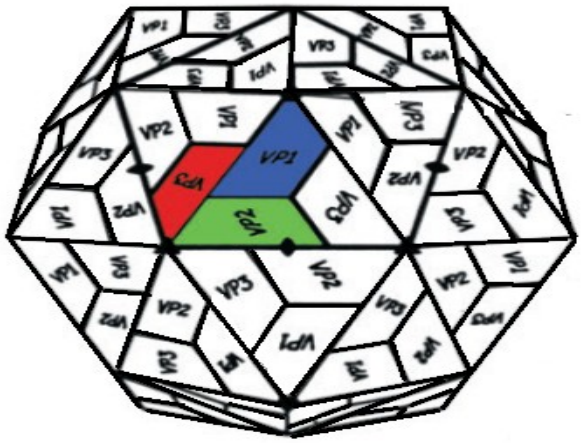


Figure 1: **Icosahedral capsid structure of FMDV made up of sixty copies of each of viral proteins (VP1, VP2, VP3 and VP4).**
Source: Fry *et al.,* 2005.

2.1.6 Genome structure of FMDV

The linear genomic RNA of FMDV, which is linked to a small genome-linked virus protein (VPg) at its 5'end (Longjam and Tayo, 2011) consist of three different regions namely 5' Untranslated region (5'UTR), 3' Untranslated region (3'UTR) and coding sequence (Jamal and Belsham, 2013) as shown in (Fig. 2) below. The 5' Untranslated region (5'UTR) and 3' Untranslated region (3'UTR) of about 1300 and 90 nucleotides sequence long respectively, are interspaced by a protein coding region of about 700 nucleotides sequence (Jamal and Belsham, 2013).

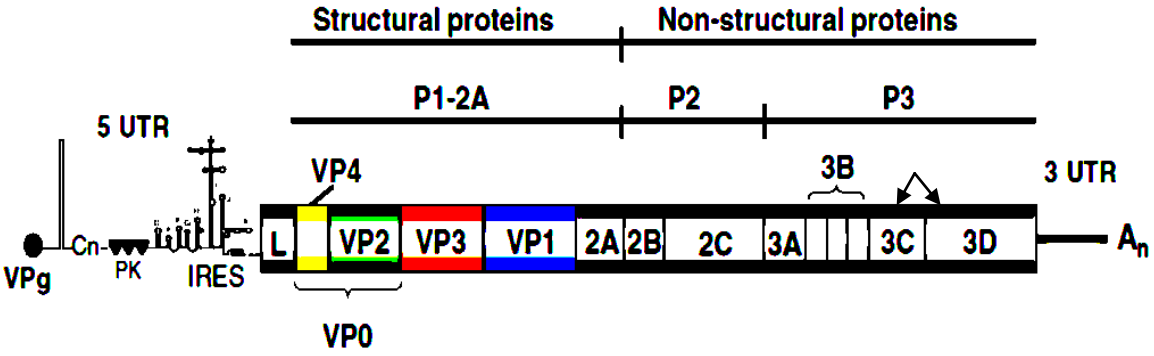


Figure 2: Genomic orientation of Foot-and-mouth disease virus. Source: Jamal and Belsham, 2013.

2.1.6.1 5' Untranslated region (5'UTR)

S-fragment, Poly C tract, a series of three to four RNA Pseudo knots structures, a Cis-acting replication element (cre) and the Internal Ribosomal Entry Site (IRES) constitute the 5'UTR which is covalently-linked to a small protein (VPg) (Grubman and Baxt, 2004; Carrillo, 2012; Jamal and Belsham, 2013). To date, the function(s) of a variety of components constituting the 5'UTR are still unknown (Carrillo, 2012).

More intensive research are ought to be conducted so as to unravel the unknown functions of Pseudo knot structures (Carrillo, 2012; Jamal and Belsham, 2013). The essence of knowing the functions of different genomic structure comes in during development of molecular diagnostic assays, production of serotype-specific vaccines and/ or anti-viral drugs against FMDV. Moreover, according to description by Carrillo (2012), Poly C tract is said to be related to virulence of FMD virus, even though this arguments was opposed by other scientists. The cis-acting replication element (cre) with a conserved AAACA motif and characteristic stem loop structure, is a template for viral RNA polymerase-dependent uridylylation of the VPg encoded by (3B $_{1\text{-}3}$) gene (Jamal and Belsham, 2013) and this is essential for circularization of viral RNA during transcription (Carrillo, 2012).

2.1.6.2 Coding region

Expression of the coding region of the genomic RNA encodes structural proteins (VPI (1D), VP2 (1B), VP3 (1C) and VP4 (1A) that constitute the viral icosahedral capsid (Carrillo, 2012) whilst the rest of genes codes for non-structural proteins (NSPs) which are; L, 2A, 2B, 2C, 3A, $3B_{1-3}$, 3C, 3D as well as 3AB and 3ABC protein complexes (Ma *et al.,* 2011). Immediate translation of the viral coding region (as one ORF) results into one primary polyprotein precursor which is cleaved to produce three polyproteins namely P1-2A, P2 and P3. Further cleavage of the named polyproteins yield a number of proteins that play a major role in expression of viral proteins. (2B, 2C) and (3A, three different VPg proteins ($3B_{1-3}$), $3C^{PRO}$, $3D^{POL}$) are proteins that are produced following definitive cleavage of the P2 and P3, respectively. $3D^{POL}$ is RNA-dependent RNA polymerase which catalyzes replication of viral genomic RNA (Jamal and Belsham, 2013). Being covalently linked to the viral genomic RNA molecule, each of three different forms of VPg proteins that are encoded by $3B_{1-3}$ genes play role as primers during RNA replication (Grubman and Baxt, 2004). The role of 2B and 2C proteins is not yet known, but the fact that these proteins are always found in Endoplasmic Reticulum (ER)-derived vesicles, they are believed to play certain role in replication of viral genomic RNA (Carrillo, 2012). 3A is an integral membrane protein with different functions and among those functions one is to exhibit markers related to the host as well as to boost viral RNA synthesis (Carrillo, 2012). $3C^{PRO}$ is a protease responsible for processing viral protein produced immediately after translation. P1-2A is cleaved by $3C^{PRO}$ to yield VPO, VP3 and VP1 proteins that constitute the viral capsid (Jamal and Belsham, 2013).

2.1.6.3 3’ Untranslated region (3’UTR)

3’UTR of FMDV which spans about 90 nucleotide downstream the coding region, is made up of a specific three stem-loop structures and a Polyadenylation (Poly A) tract,

which are presumed to play a major role in translation of the linear single-stranded genomic RNA (+) of FMDV (Grubman and Baxt, 2004; Jamal and Belsham, 2013). To prove that, a non-infectious viral RNA with diminished translation efficiency under experimental conditions was produced as a sequel of excision of the named two structures of 3'UTR (Grubman and Baxt, 2004).

2.2 Molecular Pathogenesis of Foot-and-Mouth Disease

Development of molecular diagnostic tools requires comprehensive knowledge on important virulent genes playing vital role in pathogenesis of FMDV, prior to designing of primers which target such genes (Arzt *et al.,* 2010).Furthermore, knowledge on molecular pathogenesis is the key to understanding the complex molecular epidemiology of FMDV and this makes development of specific molecular diagnostic assays easier as well as application of rational FMD-control measure such as serotype-specific vaccines(Arzt *et al.,* 2010),

Pathogenesis of FMD which is characterized by pre-viraemic, viraemic and post-viraemic phases (Grubman and Baxt, 2004), begins when there's specific interaction between viral virulence factors and host's cell receptor after an animal has received enough viral tissue infective dose (TID) mainly through respiratory route.

In pre-viraemic phase, FMDV infect the cell and multiply at the primary replication site predominantly the Oesophageal Pharyngeal (nasopharynx) region of an animal (Alexandersen *et al.,* 2003; Arzt *et al*., 2010; Rodriguez-Calvo *et al.,* 2011). The FMDV which is known to have high tropism to stratified epithelial tissues recognizes these cells either by RGD-dependent mechanism or RGD-independent mechanism (Jamal and Belsham, 2013). Speaking of RGD-independent recognition mechanism, virus infect

susceptible hosts' cells by recognizing Heparan Sulfate proteoglycan cellular receptors (Jamal and Belsham, 2013) rather than cellular intergrin receptors in tissue culture (in vitro) even though internalization of viruses is via caveola-mediated endocytosis (O'Donnell *et al.,* 2008; Ruiz-Saenz *et al.,* 2009). In contrast to RGD-independent recognition mechanism, FMDV infects the host naturally (i.e RGD-dependent recognition mechanism) by using a conserved tripeptide Arg-Gly-Asp (RGD) sequence of highly variable G-H loop of viral structural protein (VP1) (Fox *et al.,* 1989) which binds specifically and complementarily to a family of trans membrane glycoprotein receptors (intergrins) of stratified epithelium (Grubman and Baxt, 2004; Sobrino *et al.,* 2001; Burman *et al.,* 2006). So far, FMDV is known to infect an animal by recognizing a number of intergrin receptors such as $\alpha_v\beta_1$ (Jackson *et al.,* 2002),αvβ6 (Jackson *et al.,* 2000),and $\alpha_v\beta_3$, αvβ5, αvβ8 (Ruiz-Saenz *et al.,* 2009). Speaking of RGD-dependent recognition mechanism, the virus is internalized by clathrin-mediated endocytosis (O'Donnell *et al.,* 2008) and release of viral genomic RNA in the cytosol (Carrillo, 2012) is preceded by capsid dissociation into 12 Pentamers under mildly acidic PH condition inside early endosomes (Grubman and Baxt, 2004; Ruiz-Saenz *et al*., 2009). Being positive sense (+), the single stranded genomic RNA of FMDV acts as a messenger RNA (mRNA) molecule (Carrillo, 2012) and it is translated directly into a 'polyprotein precursor' by cellular protein synthesis machinery (80s Ribosomes).

Initiation of cap-independent translation of positive-sense, single-stranded genomic RNA, begins once Ribosomes binds to Internal Ribosomal Entry Site (IRES) to produce a single polypeptide precursor (Sobrino *et al*., 2001; Monaghan *et al.,* 2004; Jamal and Belsham, 2013). Following translation of viral genomic RNA, four primary protein products are formed as a result of proteolytic cleavage of the precursor polyprotein. These proteins are Leader protease (L^{pro}), Capsid precursor (P1-2A), Polyproteins (P2 and P3)

which are the precursor for viral Non-Structural Proteins (NSPs) (Jamal and Belsham, 2013). In primary cleavage, the L^{pro} cleave itself autocatalitically from the polypeptide precursor and inhibits cellular gene expression by chopping the host's eukaryotic transcription initiation factor (eIF4G) to halt transcription initiation of host's genes (Jamal and Belsham, 2013). The case is the same for 2A (18 amino acid), which removes itself from P2 and become associated with P1 to form a capsid precursor (P1-2A protein) which will later undergo cleavage and maturation to produce four structural proteins (VIP, VP2, VP3 and VP4) (Grubman and Baxt, 2004; Jamal and Belsham, 2013). The remaining part of the polypeptide precursor is cleaved by protease ($3C^{pro}$) to yield P2 (2B, 2C) and P3 (3A, 3B, $3C^{pro}$, $3D^{pol}$) non-structural proteins (Fig. 4) (Grubman and Baxt, 2004; Sobrino *et al.,* 2001; Jamal and Belsham 2013). The products of $3C^{PRO}$-dependent proteolytic cleavage of capsid precursor (P1-2A), which are protein monomers namely (1AB (VPO), 1C (VP3), 1D (VP4)), constitute the viral capsid structure. N-terminal myristoylation of the 1D (VP4) protein monomer triggers the self-assembly of icosahedral viral capsid via pentameric protein subunits (Goodwin *et al.,* 2009).

Even though its mechanism is not yet understood, translation of viral RNA is ought to stop before the onset of synthesis of new ssRNA (+) for the progeny virus (Grubman and Baxt, 2004). After the halting of viral translation, few copies of viral genomic RNA (+) in cytoplasm, serve as templates and associates with vesicles generated from Smooth Endoplasmic Reticulum (SER) to form replication complexes where the viral genomic ssRNA (+) is amplified (Monaghan *et al.,* 2004). Within the replication complex, RNA-dependent RNA polymerase ($3D^{POL}$) catalyzes synthesis of new strands of ssRNA (+) via a negative strand intermediate ssRNA (-) for the progeny viruses. Replication of viral genomic ssRNA (+) in the vesicular structures (replication complexes) results into rearrangement of cell membrane causing the accumulation of both smooth and rough

endoplasmic reticulum, golgi apparatus and smooth membrane-bound vesicles on one side of the nucleus (Monaghan *et al.,* 2004) that accounts for preliminary vesicles formation in the oesophageal pharyngeal region. Despite the fact that, a negative sense RNA molecule also posses VPg at its 5' end, only one of the three forms of VPg protein products encoded by (3B $_{1\text{-}3}$) genes, is covalently-linked to the newly synthesized positive sense RNA molecule before assembly of a virion (Grubman and Baxt, 2004). The newly synthesized genomic ssRNA (+) together with a total of 12 pentamers forms an immature Provirion which undergoes maturation cleavage specifically on VPO (1AB) subunit to produce VP2 and VP4 protein monomers.

A fully matured and infectious virion with unique antigenic specificity will be formed after assembly of the monomeric protein subunits (VP1, VP2, VP3 and VP4) even though the VP4 remains buried inside the capsid, leaving the rest of proteins (VP1, VP2 and VP3) exposed on viral surface. The infection will spread very fast from Oesophageal Pharyngeal (OP) region to the surrounding tissues due to extracellular release of massive progeny viruses by rupturing of cell membrane (lytic cycle).

After viruses have colonized the OP, these small creatures are disseminated to all parts of animal's body through blood (i.e viraemic phase) (Grubman and Baxt, 2004), as they are being transported by macrophages from pharynx to secondary sites of infections predominantly mouth and feet, where they also cause vesiculation (Arzt *et al*., 2011b). Infected animals develop a chronic FMD condition after the viraemia phase and all clinical signs disappear even though FMDV may become persistent in OP region and other body tissues rather than blood (Arzt *et al.,* 2011b).

2.3 Transmission of Foot-and-Mouth Disease (FMD)

The multiplicity of routes of infection is one of the reasons for a highly contagious nature of FMD and this has hindered control efforts. Among a variety of routes by which FMD is transmitted directly from FMD-infected to FMD-free animals, is through physical contact (Chase-Topping *et al.,* 2013) with bodily fluids such as (semen, milk, urine, feces, vesicular fluids, saliva, nasal and lachrymal fluids etc.) secreted and excreted from infected animals (Alexandersen *et al.,* 2003). FMD-naive animals with pre-existed abrasions, wounds or any sort of tissue damage; are more likely to succumb FMD if contacted with bodily fluids from infected animals and the time that lapse until the disease has clinically developed is dependent on type of bodily fluid that an animal contacted because these bodily fluids differ significantly in terms of virus titer, faeces and urine being the least (Grubman and Baxt, 2004). With exception of other FMDV serotypes, only SAT 1-3 are sexually transmitted among African buffalos (*Syncerus caffer*) (Van Vuuren *et al.,* 1999). Apart from physical contact, indirect transmission of FMD occurs through vehicles (trucks and shoes of people from FMD-infested area to FMD-free area), fomites or environment contaminated with FMDV. Example, oral transmission of FMD occurs when animal consumes animal feeds (hays, milk, meat products, offal etc.) and water or drinks that has been contaminated with infectious dose, (ID) of FMDV (Alexandersen *et al.,* 2003). Young animals (calves, piglets) normally get FMD infection directly by suckling milk from infected lactating animals. In dairy centers FMDV is likely to be disseminated among animals if the same milking machine is used during milking process without being sterilized before being used by each animal (Chase-Topping *et al.,* 2013). Pigs are most likely to acquire FMD-infection through consumption of animal products contaminated with FMDV rather than aerosols. Being the most common and uncontrollable route of FMDV dissemination, airborne

transmission is dependent on a number of parameters and affected by a number of meteorological conditions (Sorensen *et al.,* 2000; Donaldson and Alexandersen, 2002).

The infectious droplets and/ or droplet nuclei (aerosols) exhaled by FMD-infected animals are normally transmitted to susceptible hosts by wind even though transmission efficiency is dependent on animal species, proportion of infected to non-infected animals in a defined area, strain of virus and its virulence level without forgetting environmental factors like weather and topography (Donaldson and Alexandersen, 2002). High dose of large-sized droplets and/ or droplet-nuclei under favour climatic conditions (55% – 60% relative humidity, turbulent air and lower temperature) are pre-requisite for successful long-distant airborne transmission of FMD (up to 20 Kilometers) (Alexandersen *et al.,* 2003). Different strains of FMD virus have different potential to cause FMD, since they differ in terms of virulence and produce disease with different severity. In addition, the amount of virus that an animal emits depends on the strain type of the virus (Donaldson and Alexandersen, 2002). Be it short-distant or long-distant aerogenous transmission of FMD, the proportion of FMD-infected animals to naive animals in a defined area matters significantly as far as disease development is concerned. Provided that, the area of animals' herd is constant, susceptible animals are more likely to develop FMD at high rate if the number of animals emitting the virus via their respiratory route by exhalation, is higher than the number of naive susceptible animals and vice versa is true. Different animal species that are susceptible to FMD succumb the disease differentially following aerogenous transmission of disease. Pigs are known to be the producers of aerosolized exhaled air with high viral load, even though they are not highly susceptible to aerosols produced by other animals (Alexandersen *et al.,* 2003).

2.4 Clinical Signs/ Manifestation of Foot-And-Mouth Disease

Of recent, symptomatic FMD manifests in a broad spectrum of clinical signs depending on the virulence level of a certain type of FMD virus and host species, (Carrillo, 2012) as opposed to asymptomatic FMD. Generally, FMD-infected animals experience short-lasting fever (Chase-Topping *et al.,* 2013), loss of appetite (Carrillo, 2012), depression (Alexandersen *et al.,* 2003) and epithelial lesions which are preceded by formation and rupturing of fluid-filled vesicles or bullae, which occur normally on tongue, feet, inside and around the mouth (Chase-Topping *et al.,* 2013) 24 to 72 hours post infection (pi) (Carrillo, 2012). Epithelial lesions are formed on feet specifically on interdigital cleft and coronary band of hooves, bulb of heel and knees of feet resulting into lameness (Chase-Topping *et al.,* 2013) as a result of pain (Grubman and Baxt, 2004).Apart from reluctance of animals to mating and hence reduced animal productivity due to formation of painful vesicles and ulceration on genital organs, formation of these vesicles or ulcerations on mammary glands predispose the lactating animal to secondary infections like mastitis whose consequence is reduction in milk production that diminishes dairy industry (Alexandersen *et al.,* 2003; Kivaria, 2003). Ulcerative lesions inside the mouth, nose and around muzzle cause excessive salivation, drooling (Chase-Topping *et al.,* 2013) and mucoid to mucopurulent nasal discharge (Alexandersen *et al.,* 2003). FMD is also characterized by abortion in pregnant animals and weight loss due to failure of animals to eat as a result of pain and ulceration in the mouth (Alexandersen *et al.,* 2003). Mortality rate is significantly low in FMD outbreaks among adult animals as compared to young and immunocompromised animals (Carrillo, 2012). In contrast to high morbidity in adult animals (Zhu *et al.,* 2013), sudden mortality of young animals (calves, lamb and piglets) with no evident vesicles is caused by myocarditis (Alexandersen *et al.,* 2003) and basing on postmortem examination the heart normally appears to have several whitish to grayish or yellowish bands i.e “tiger heart”

(Alexandersen *et al.,* 2003). So far we don't know whether the FMD virus will clinically manifest in a different way in the near future because of its high mutation rate.

2.5 Diagnosis of Foot and Mouth Disease

A highly sensitive and specific laboratory-based definitive diagnosis is essential for effective FMD-control in endemic settings, because this disease is insignificantly diagnosed solely on the basis of clinical signs since it is contradicted with other vesicular diseases like Swine vesicular diseases (SVD), Vesicular stomatitis, Vesicular exanthema, Encephalomyocarditis, Herpangina (Carrillo, 2012; Office International des Epizooties, 2012; Jamal and Belsham, 2013). Literally, FMD can be diagnosed directly by targeting the whole virus particle (FMDV) or its antigen (epitope) or its nucleic acid in sample. Alternatively FMD can be detected indirectly by targeting anti-FMDV antibodies secreted by animals in response to FMD infection. Varieties of assays that are used for diagnosis of FMD can be classified into two major groups namely; molecular diagnostic tools and non-molecular diagnostic tools. The molecular diagnostic tools are mainly the PCR-based diagnostic assays as well as DNA sequencing technologies. In this section, discussion of the non-molecular diagnostic tools will be preceded by molecular diagnostic tools.

2.5.1 Molecular diagnostic tools for FMD

"Molecular diagnosis" is literally a broad terminology that does not only encompass PCR-based disease detection tools, because DNA sequencing is also a part of it. Such molecular diagnostic tools which are globally used for detection and/ or serotyping of FMDV include conventional gel-based Reverse Transcription Polymerase Chain Reaction (gel-based RT-PCR), real-time reverse-transcription polymerase chain reaction (real-time RT-qPCR), Reverse-Transcription Loop Mediated Isothermal Amplification (RT-LAMP) as well as first-, second-, and third-generation High-Throughput Next generation Sequencing technologies (HT-NGS).

2.5.1.1 PCR-based molecular diagnostic tools for detection and typing of foot-and-mouth disease (FMD) virus

Before explaining about each of the PCR-based diagnostic tools for detection and/ or characterization of FMD (i.e. conventional gel-based RT-PCR, real-time RT-qPCR, RT-LAMP, RT-LAMP- LFD), understanding of the general principle of Polymerase Chain Reaction (PCR) which is core to all other PCR types is very important. Literally speaking, the chemistry of PCR is based on the fact that the double helix structure of DNA molecule relies on complementary binding of Purines (Adenine and Guanine) and Pyrimidines (Thymine and Cytosine) (Pestana *et al.,* 2010) by formation of non-covalent (Hydrogen) bonds between them. Another fact is that, high temperatures approximately (above 90ºC) can denature the double helical structure of DNA by breaking the Hydrogen bonds between Purines and Pyrimidine; hence the antiparallel strands become separated. In a living system, DNA polymerase catalyzes synthesis of new DNA strand which is complementary to the parental DNA template by incorporating 2'-deoxyribonuocleoside-5'-Triphosphates (dNTPs) to the RNA primers.

Knowing those facts about DNA structure, Kary Mullis innovated a Thermal cycler (PCR machine) that mimics the in vivo DNA replication process by using synthetic DNA oligonucleotide primers and a thermal stable *Taq* polymerase (naturally expressed by a thermophile bacteria, *Thermus aquaticus*) (Valasek and Repa, 2005). Because the *Taq* polymerase enzyme is heat-stable, it can amplify DNA exponentially through denaturation, annealing and elongation steps. Presently, *Taq* polymerases are not the only thermal stable enzymes which catalyze PCR reaction; others are *Tth*-and-*Pfu* polymerases which are naturally expressed by *Thermus thermophilus* and *Pyrococcus furious* bacteria. The named polymerase enzymes with salient thermal-stable feature are the heart of Polymerase Chain reaction (PCR) because without them, no DNA amplification will

occur even though other PCR ingredients (dNTPs, Mg^{2+} Buffers, DNase-free water, reverse-and-forward primers, fluorescent-probes etc.) are present at optimum concentration and they are of required quality. The polymerase enzymes which are the sole driving force of an energy-dependent DNA amplification differ significantly in terms of their proof-reading (5'-3'exonuclease) activity as well as polymerization rate which are the essential determinants of their efficiency.

Of the named three DNA-dependent DNA polymerase, *Taq* polymerases and *Tth* polymerases have extension rate of about 2Kb-4Kb/ min and 5'-3' exonuclease activity unlike *Pfu* polymerases which have 3'-5'exonuclease activity rather than 5'-3' exonuclease activity despite having a lower extension rate of about IKb-2Kb/ min (Dorak, 2006). As opposed to *Taq*- and *Tth*-polymerase, *Pfu* polymerases doesn't have tendency of adding extra Adenine (A) residue at the end of newly synthesized strands (Dorak, 2006). Even though DNA polymerases have potential to monitor DNA amplification in a controlled logarithmic manner despite temperature variation (thermal circularization) attributed by denaturation, annealing and polymerization steps, challenges associated with these enzymes are inevitable. Dorak (2006) pointed out that false priming is a challenge in most PCR reactions that are attributed to the use of wild type polymerase enzymes.

The case is true with wild type *Taq* polymerase enzyme because it catalyzes background reactions before PCR onset. After mixing all PCR master mix reagents, wild type *Taq* polymerase catalyzes annealing of primers to non-targeted gene sequences. To eliminate false priming, scientists are manufacturing more efficient, genetically engineered hot-start polymerases which commence to catalyze DNA amplification only at its optimum high temperatures (≥95ºC). With hot-start polymerases, background reactions (i.e false

priming) are strictly eliminated because the active site of these enzyme are tightly bound by either heat-labile proteins or specific antibodies which will be denatured and released from the enzyme at high temperatures (≥95ºC) optimum for catalytic activity of these enzymes. At high denaturation temperature, the active site of the hot-start DNA-dependent DNA polymerase is set free from heat-labile proteins or specific antibodies so that incoming dNTPs molecules can be incorporated into the growing new DNA strand during elongation step of PCR.

(a) Real- time (quantitative) polymerase chain reaction (real-time qPCR)

The ability to amplify nucleic acids and quantify them simultaneously, has given real-time PCR credit to be used in multitude of biological research that cuts across a number of fields such as Genetics, Physiology, Genetic engineering, Biotechnology, Microbiology, Clinical medicine and Biomedical sciences (Vasalek and Repa, 2005). By the outmost efforts of scientific researchers, the real-time PCR which can make a lot of copies of few nucleic acid templates was developed (Higuchi *et al*., 1992) so as to complement weaknesses of the gel-based PCR assay (Mullis *et al.,* 1987). Because of contamination of amplicons as a result of post-PCR manipulation in gel-based PCR technique, Higuchi and his colleagues developed a real-time PCR assay (Higuchi *et al.,* 1992) with capacity to detect, amplify and quantify target sequences at enormous specificity and the amplification procedure can be monitored at the same time i.e "in real time" (Valasek and Repa, 2005).

Even though the amplification phase of the real-time PCR resembles that of traditional, conventional gel-based PCR with denaturation, annealing and elongation steps, its mode of amplicons detection is quite different as compared to the conventional PCR.After Higuchi and his colleagues managed to use fluorescent dsDNA-binding dyes to detect the

newly synthesized strand during amplification of nucleic acids, quantification of the amplicons produced during real-time PCR became possible.

Regarding the first real-time PCR version which was made commercially available by Roche molecular system in 1996 (Valasek and Repa, 2005), Ethidium bromide was the first DNA-binding dye of choice to be used as a fluorescent reporter (Arya *et al.,* 2005; Valasek and Repa, 2005). The good reproducibility, sensitivity, rapidity and reduced risk of carry-over contamination of real-time PCR assay (Mackay *et al.,* 2002), is attributed to its automated detection methods of PCR-products (amplicons). The amplicon detection systems such as (DNA-binding fluorophores, Hydrolysis probes, Fluorescence resonance energy transfer (FRET)-based hybridization probes etc.) have reduced the risk of carrying over contamination (Mackay *et al.,* 2002) to next diagnostic steps (*viz* sequencing) as opposed to conventional PCR whose amplicon detection is based on Agarose Gel Electrophoresis (AGE) or Poly Acrylamide Gel Electrophoresis (PAGE).

(i) Principle underlying chemistry of real- time PCR

Generally, the real-time PCR make use of both probes and primers to targets specific nucleotide sequences within a target DNA-template for amplification and detection respectively, compared to the conventional gel-based PCR or LAMP which uses only primers for nucleic acids' amplification and PCR amplicons are analysed by different means. Likewise for other PCR types, one complete cycle of the real-time PCR is achieved following denaturation, annealing and elongation steps.

(ii) Denaturation of dsDNA molecules

Before making many copies of DNA, two supercoiled strands of a target DNA (double helix) gets separated completely after being heated to 94-96 ºC (Pestana *et al.,* 2010) .

Heating of the DNA double helix results into breakage of Hydrogen bonds between Purines and Pyrimidines (Pestana *et al.,* 2010). The essence of separating the two strands of DNA double helix is to give room to DNA polymerase to synthesize two daughter strands that are complementary to each of sense and non-sense parental strands of dsDNA molecule (Pestana *et al.,* 2010) by extending the gene-specific primers.

(iii) Annealing of primers and probes to DNA templates

Following denaturation of a double stranded DNA (dsDNA) by high temperature generated from heating block of real-time PCR machine, the specific primers anneal to its complementary sequence on the target DNA followed by binding of non-extendable sequence-specific oligonucleotide probes. Primers are made to initiate polymerase-catalyzed PCR amplification even though their nucleotide composition determines annealing temperature which is normally less than their melting temperature (Tmº) by 5ºC (Pestana *et al.,* 2010). Literally speaking, there are different varieties of fluorescently-labeled probes which are presently used in real-time PCR or real-time RT-PCR assays and they recognize and binds complementarily to the same target DNA template even though at distinct sequences from primer's recognition sequence. In reality, primers anneal to specific sequence on the target DNA template before probes hybridize to another sequence upstream primer's recognition site. Because there's a significant difference between each type of fluorescently labeled Probe in terms of their underlying chemistry, detailed discussion for dsDNA- intercalating agents, Hydrolysis probes, Hybridization probes, Molecular beacons and scorpions follows shortly in this dissertation.

(iv) PCR elongation step

Because real-time PCR amplify and quantify DNA simultaneously, detection of the PCR amplicons is normally done at the end of elongation step of every cycle followed by software-based quantification of amplicons. The core function of the polymerase enzyme during PCR elongation step is to synthesize new DNA strands which complement the parental strands of DNA duplex by extending both forward and reverse primers that annealed to specific sites on each strand of target DNA molecule. Because quantification of DNA amplicons in real-time PCR is preceded by amplicon detection, more details of different amplicon detection systems which are normally generated after real-time PCR are provided below.

(v) Amplicons detection systems used in real-time PCR

Fluorescent dyes that are bound (tagged) to sequence-specific, non-extendable oligonucleotide probes or free (unbound) fluorescent dyes are normally used for amplicon detection following real-time PCR (Vasalek and Repa, 2005; Dorak, 2006). Unlike the latter amplicon detector, most of the former amplicon detectors are based on Fluorescence Resonance Energy Transfer (FRET) between quencher and reporter fluorescent dyes and all of them are tagged on the same probe even though they are located in close proximity**.** There's an obvious difference between the two named amplicon detection systems of real- time PCR in terms of efficiency, versatility and purchase cost.

(vi) Free (unbound) fluorescent dyes as amplicon detectors of real-time PCR

A variety of intercalating, double-stranded DNA-binding fluorescent dyes are used as amplicon detectors following real-time PCR (Arya *et al.,* 2005). SYBR® Green 1, EvaGreen™ (Dorak, 2006), Ethidium bromide (EtBr), BOXTO, BEBO and YOYO-1

(Valasek and Repa, 2005) are examples of free fluorescent dyes with different potential to bind to dsDNA molecule and fluoresce.

The radioactive Ethidium bromide (EtBr) dye which was previously used in the preliminary version of real-time PCR developed in 1990s, was replaced by fluorescent colors such as SYBR® Green 1 because it pose carcinogenic effects to laboratory personnel (Arya *et al.,* 2005; Valasek and Repa, 2005). Presently, SYR® green 1 is the most popular intercalating dsDNA-binding dye which is currently used in real-time PCR assays (Arya *et al.,* 2005). Characteristically, the SYBR® green 1 binds only to any double standed DNA molecule (ssDNA exclusively) by intercalating its minor groove and fluoresce to give a green-coloured light signal (Fig. 3) with 1000 fold intensity as compared to when it is not excited nor unbound to dsDNA molecule in solution (Wittwer *et al.,* 1997; Pestana *et al.,* 2010). Because the amount of green fluorescence color of SYBR green is directly proportional to the number of dsDNA copies yielded after PCR-elongation step (Arya *et al.,* 2005; Vasalek and Repa, 2005), quantification of amplicons as well as monitoring of real-time PCR is possible. EvaGreen™ is a more potent fluorescent dye because it yield higher fluorescent signal and it doesn't inhibit the real-time PCR reaction as compared to SYBR® Green 1, even though it is quite expensive (Dorak, 2006). BOXTO is the most potent of all named fluorescent dyes because it emits fluorescent signal above EvaGreen™ and SYBR® Green 1 (Dorak, 2006). Even though their uses have not gained popularity, BEBO and YOYO-1 are among other dsDNA-binding dyes which are also used in different real-time PCR assays (Valasek and Repa, 2005).

Despite their wide application because of their low cost (Valasek and Repa, 2005), we cannot erase the fact that these dsDNA-binding dye has some weaknesses when it comes

to generation of quality and precise data in different real-time PCR or real-time RT-qPCR assays.

Specificity of is a major issue of concern when it comes to amplicon detection in real-time PCR or real-time RT-PCR because these agents are capable of binding non-specifically to any dsDNA molecule. The dsDNA-binding dyes can bind to both the targeted dsDNA, and non-targeted dsDNA molecules (primer-dimers) and this results into false positive results (Valasek and Repa, 2005; Pestana *et al.,* 2010). For that reason, there's an extra requirement for researchers to analyze melting curves of all PCR-products (amplicons) so as to rule out whether amplification of target gene was specific or artifacts like primer-dimers were among the PCR products (amplicons).

To overcome the issue of reduced specificity of real-time RT-PCR because of using dsDNA-binding dyes, scientists are ought to compare and contrast melting points of all amplicon in terms of homogeneity. If PCR-based amplification of DNA was efficient and specific to the target gene, millions of homogenous DNA copies that are complementary to target DNA with the same melting Temperature (Tm^o) will be obtained. The amplicons will be considered homogenous only if they are exact copies of target DNA, and must have the same length as well as the same nucleotide sequence (Pestana *et al.,* 2010). Amplicons of target DNA, primer-dimers and non-targeted dsDNA contaminants normally have different melting curves with distinct melting temperatures (Tm^o) (Pestana *et al.,* 2010). A melting curve for amplicons generated after real-time PCR is normally obtained by plotting the relative fluorescence units (RFU) against melting temperature (Tm^o) of each PCR product (Arya *et al.,* 2005).

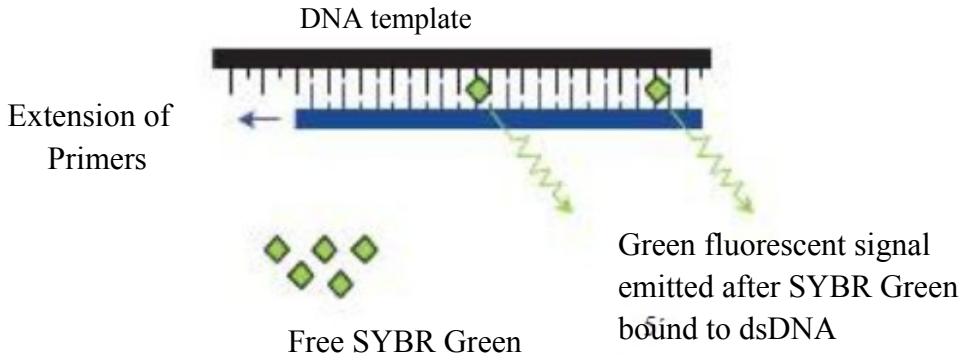


Figure 3: **Amplicon detection by free dsDNA-binding dyes (Intercalator dyes).** Source: Arya *et al.,* 2005.

(vii) Fluorescent resonance energy Transfer (FRET)-based amplicon detectors in real-time PCR assays

The non-extendable oligonucleotide probes are normally labeled (tagged) with two distinct dyes among which one serves as a fluorescent reporter and another one serves as quencher of the fluorescent light signal. Characteristically, the reporter fluorophore differs from quencher in terms of the light wavelength at which they can be excited (excitation wavelength even though both of them depend on each other for amplicon detection by Fluorescence Resonance Energy Transfer (FRET) mechanism.

During FRET, the reporter fluorophore absorbs light at specific excitation wavelength from a light source (*viz* Laser, LED, and Lamp) (Arya *et al.,* 2005). The energy acquired after excitation of reporter fluorophore is high but short lived (Dorak, 2006). After absorbing light from the source, the reporter fluorophore emit the absorbed light (transient energy) at higher wavelength than the excitation wavelength. The emitted light energy is inturn absorbed by a quencher (energy receiver) which is in close proximity (about 10 to 100 A^{o}) to the reporter fluorophore (energy donor) and consequently, complete quenching of the fluorescent light signal of fluorophore reporter (energy donor) occurs (Dorak, 2006).

All fluorophore reporters must strictly fluoresce with higher emission wavelength once excited by light at their optimum wavelength. Some of the commercially available fluorophore reporters includes; 6-Carboxyfluorescein (6-FAM) (Arya *et al.,* 2005; Valasek and Repa, 2005), Tetrachloro-6-carboxy-fluorescein (TET), Hexacholoro-6-carboxyfluoroscein (HEX) (Arya *et al.,* 2005), Cal Fluor Gold 540™, Cal Flour Orange 560™, Redmond Red™, Cy5™, Cy3™, Fluorescent™, Palsar 650®, Yakima Yellow™, Quasar® 670 etc.

Unlike the fluorophore reporters, quenchers are compounds that may or may not fluoresce once they absorb light energy (which must be at their maximum absorbance wavelength) from fluorophore reporters. Quencher compounds which have potential to fluoresce, they normally emit light signal at higher wavelength than its absorbance wavelength. Dark quenchers don't fluoresce after absorbing energy from fluorophore reporters and these includes; DABSYL (4-(Dimethylamino) azobenzene-4'-Sulfonyl chloride), a series Black Hole Quenchers, Eclipse™ and Lowa Black™ (Dorak, 2006), 4(Dimethylaminoazo) benzene-4-carboxylic acid (DABCYL) (Arya *et al.,* 2005; Valasek and Repa, 2005). These FRET-based amplicon detection systems which make use of fluorophore reporters and quenchers include; Hydrolysis probes and a group of Hybridization probes that covers (Dual-hybridization probes, Molecular beacons and Scorpions).

(viii) Hydrolysis probes (5' exonuclease probes)

The ability of hydrolysis probes to bind specifically to certain sequences on target DNA molecule just downstream the primer's binding sequence has made them more powerful amplicon detector in real-time PCR and/ or RT-qPCR as compared to dsDNA-binding (intercalating) dyes.

Literally, the principle underlying amplicon detection by hydrolysis probes is based on the fact that, DNA polymerase enzymes (*viz Taq* polymerase) have two distinct active sites among which, one is exonuclease activity and another is a polymerase activity (Arya *et al.,* 2005).

After primer has annealed to specific sequence on target ssDNA strand or cDNA and subsequent binding of fluorescently labeled probes to the same target ssDNA or cDNA but upstream to primer's binding sequence; the polymerase enzyme starts to move from 3' to 5' end of the template while scanning the template so as to incorporate new dNTPs that are complementary to the template's nucleotides. Once the polymerase meets the probe which is covalently labeled with fluorophore reporter and quencher at its 5' terminus and 3' terminus respectively (Arya *et al.,* 2005), this probe is digested from its 5' terminus by the 5' to 3' nuclease activity of the polymerase enzyme and a number of cascade reaction occurs before color emitted by a fluorophore reporter is detected (Fig. 4). Before hydrolysis of probe by 5' to 3' nuclease activity of DNA polymerase, no fluorescent color is emitted because the fluorophore reporter and fluorophore quencher are closely attached to the same oligonucleotide probe and thus quencher absorbs energy from reporter fluorophore which acts as energy donor (Valasek and Repa, 2005). Energy is transferred from the reporter fluorescent dye (donor) to a quencher dye (recipient) in form of Fluorescence Resonance Energy Transfer (FRET) i.e. Forster transfer (Valasek and Repa, 2005).

Following degradation or 5' to 3' nuclease-catalyzed hydrolysis of dually labeled fluorescently-labeled probes, the fluorophore reporter is set free from being in close proximity to a quencher and after being excited (ignited) by energy source (either Lamp, Light Emitting Diode (LED) or a Laser) of real-time PCR machine (Valasek and Repa,

2005), the former emits a light signal of specific wavelength that is detected by a photo detector after being filtered by filters with specific wavelength.

The hydrolysis (oligonucleotides) probes which are structurally sequence-specific, have completely outweighed the use of dsDNA binding dyes (*viz* EtBr, SYBR® green 1, BEBO, YOYO-1 etc.) in real-time PCR because unlike the latter amplicon detectors, the former ones makes the real-time PCR assay more sound by strengthening detection specificity and hence its precision and accuracy. This is because these oligonucleotide probes are sequence specific and eliminate the likelihood of detecting non-specific amplicons apart from the target (viz primer-dimers, non-targeted DNA contaminants) (Arya *et al.,* 2005; Valasek and Repa, 2005). Presently, these hydrolysis oligonucleotide probes are broadly used in disease diagnosis and even in detection of mutations that occurs in specific targeted genes (Arya *et al.,* 2005), because they are sequence -specific.

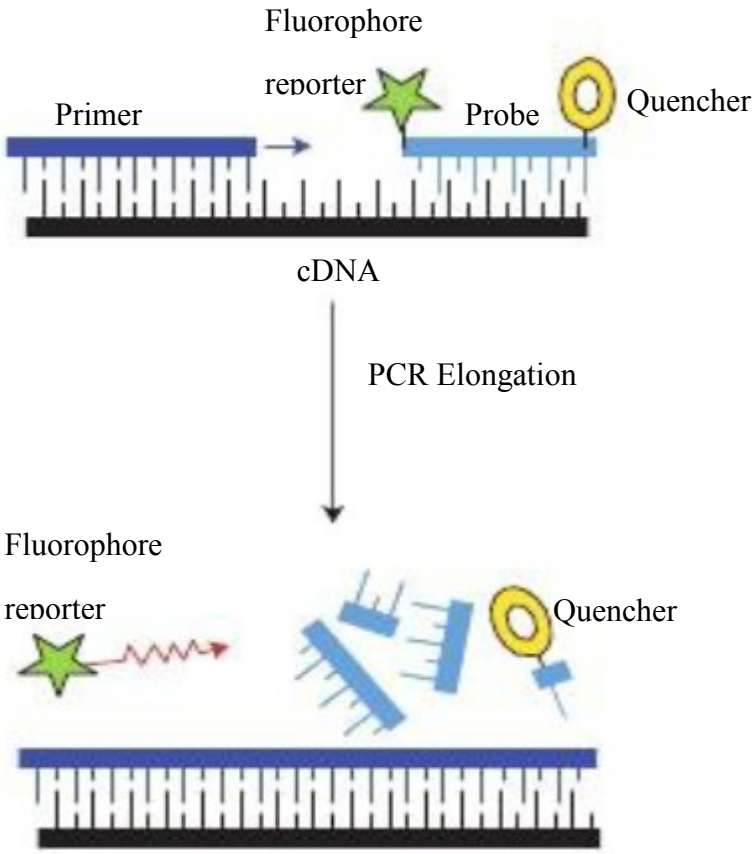


Figure 4: **Illustration of Hydrolysis probes (Taqman probes) as amplicon detectors of real-time PCR**

Source: Arya *et al.,* 2005.

(ix) Dual hybridization probes

Amplicon detection in a real-time RT-PCR assay by the use of hydrolysis probes is quite different from dual hybridization probes because in the latter detection system two different probes with different specificity are used. Among the two sequence-specific oligonucleotide probes, one is covalently labeled to an acceptor fluorophore at its 5' terminus and the other one is labeled with a donor fluorophore at its 3' terminus (Arya *et al.,* 2005).

The named oligonucleotide probes recognize and bind specifically to two distinct sequences on the same target DNA molecule during PCR-annealing step. Hybridization of the two probes one carrying fluorophore acceptor and another probe carrying fluorophore donor, brings the two fluorophores (donor and acceptor) into close contact and Fluorescence Resonance Energy Transfer (FRET) occurs from a fluorophore-donor to a fluorophore acceptor and allow the latter to emit fluorescence color at a certain wavelength (Arya *et al.,* 2005) as shown in (Fig. 5) below. The fluorescent color emitted is directly proportional to the number of amplicons at the end of real-time PCR reaction (Arya *et al.,* 2005; Valasek and Repa, 2005).

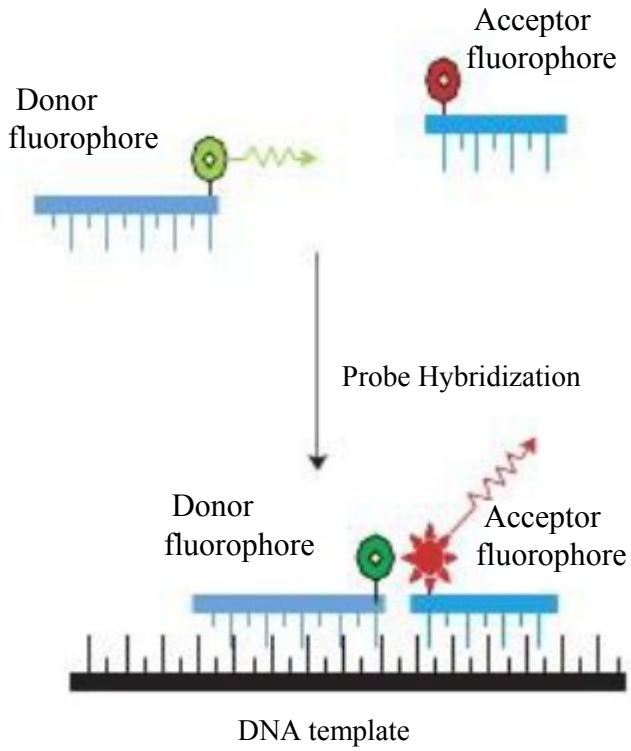


Figure 5: **Dual Hybridization probes**
Source: Arya *et al.,* 2005.

(x) Molecular beacons

In contrast to the above mentioned fluorescent-based amplicon detection systems, molecular beacons are one of the kinds because of its greater sensitivity regardless the fact that their design is a bit complex and needs optimization (Pestana *et al.,* 2010). Molecular beacons are designed to have a Stem-loop (hairpin) structure but the sequence-specific probe is part of the loop and the stem is built by two complementary arms (Arya *et al.,* 2005). The stem-loop structure of molecular beacon is normally created by addition of few (five to six nucleotide) bases at 5' terminus and 3' terminus of probe, but the added nucleotides flanking the probe sequence must be complementary to each other to form a stem arm structure (Pestana *et al.,* 2010).

Furthermore, both fluorophore reporter dye and quencher are covalently linked to 5'- and 3'-terminus of the stem-loop structure respectively (Arya *et al.,* 2005). The stem of a hairpin structure configuration, permits the fluorophore and quencher to be in close proximity such that, emission of fluorescent signal is impossible because the quencher absorbs energy from a fluorophore i.e. Fluorescent Resonance Energy Transfer (FRET) and emit it in form of heat rather than light signal (Pestana *et al.,* 2005). Since amplicon detection by molecular beacons doesn't depend on 5' to 3' exonuclease activity of DNA polymerase enzyme, sequence-specific probe located on loop structure hybridize to its complementary sequence on target DNA template and this results into conformational change of a stem loop structure (Fig. 6).

The stability of a probe-template hybrid causes breakage of hydrogen bonds that are holding two strands of a stem arm, and subsequently hairpin structure linearize and thereafter the quencher and fluorophore become distant. As the hairpin structure of molecular beacon linearize following hybridization of probe to target DNA, the fluorophore reporter emits fluorescent color after being separated from a quencher (Arya *et al.,* 2005; Pestana *et al.,* 2010). The emitted fluorescent color is of specific wavelength and is detected by a photo-detector after passing through light filters (Valasek and Repa, 2005). Following polymerase-catalyzed elongation of a new strand complementary to the DNA template, structure of molecular beacon will be reorganized in solution after polymerase has displace it due to its movement from 3'-5' direction of a template (Pestana *et al.,* 2010).

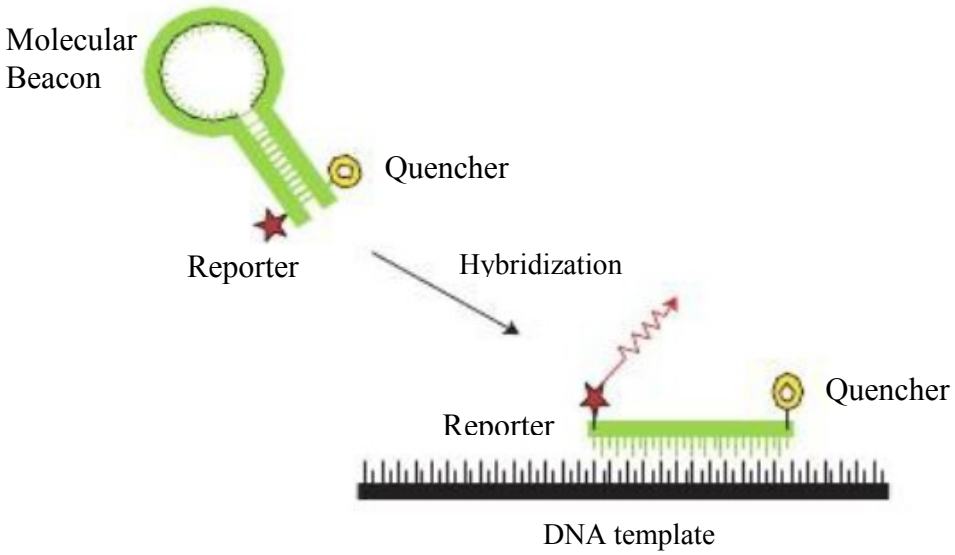


Figure 6: **Molecular Beacons.**

Source: Arya *et al.,* 2005.

(xi) Scorpions

By considering structural conformation, scorpions are obviously different from molecular beacons even though they all possess a stem-loop (hairpin) structure when they are not hybridized to a target DNA template (Arya *et al.,* 2005). A stem-loop (hairpin) structure labeled with both fluorophore reporter and quencher is just one component of a scorpion apart from sequence-specific primers, probes and non-amplifier monomer (PCR-stopper) element (Arya *et al.,* 2005; Pestana *et al.,* 2010) as shown in (Fig. 7) below.

The unique thing about scorpions is that probes and primers are not separated but they are all covalently joined together by an intermediate non-amplifier monomer (PCR- stopper) element (Pestana *et al.,* 2010). Function of the non-amplifier monomer is to stop PCR amplification (prevent read through) and subsequent unfolding of the hairpin structure before a sequence complementary to its loop element (probe) is synthesized. Unlike molecular beacons, scorpions hybridize to specific sequence on target DNA via a primer rather than a sequence-specific oligonucleotide probe. For that matter, as DNA polymerase enzyme extend the primer that has annealed to DNA template during PCR elongation step, other structures constituting the scorpion are left hanging and unbound to the targeted DNA template. After polymerase-directed elongation of the annealed primer, the loop of the Scorpions' hairpin structure (probe) hybridizes complementarily to the newly synthesized daughter strand which is complementary to the target DNA parental strand. Eventually, conformational change of hairpin occurs.

The fluorophore reporter as well as quencher (Arya *et al.,* 2005), gets separated and fluorescent color of specific wavelength will be detected as explained above in the case of molecular beacons. Increased specificity of Scorpions over and above molecular beacons and other probe-types is due to intermolecular interaction of loop sequence of the hairpin structure with PCR-product (Pestana *et al.,* 2010). Moreover, the fact that principle behind scorpion probes is not based on 5'-to-3' exonuclease activity of a DNA polymerase, this makes amplicon detection process rapid as it applies to molecular beacons (Pestana *et al.,* 2010). The fact that scorpion probes have lower background noises, it should not escape our mind that, these probes are difficult to design, expensive and require good optimization.

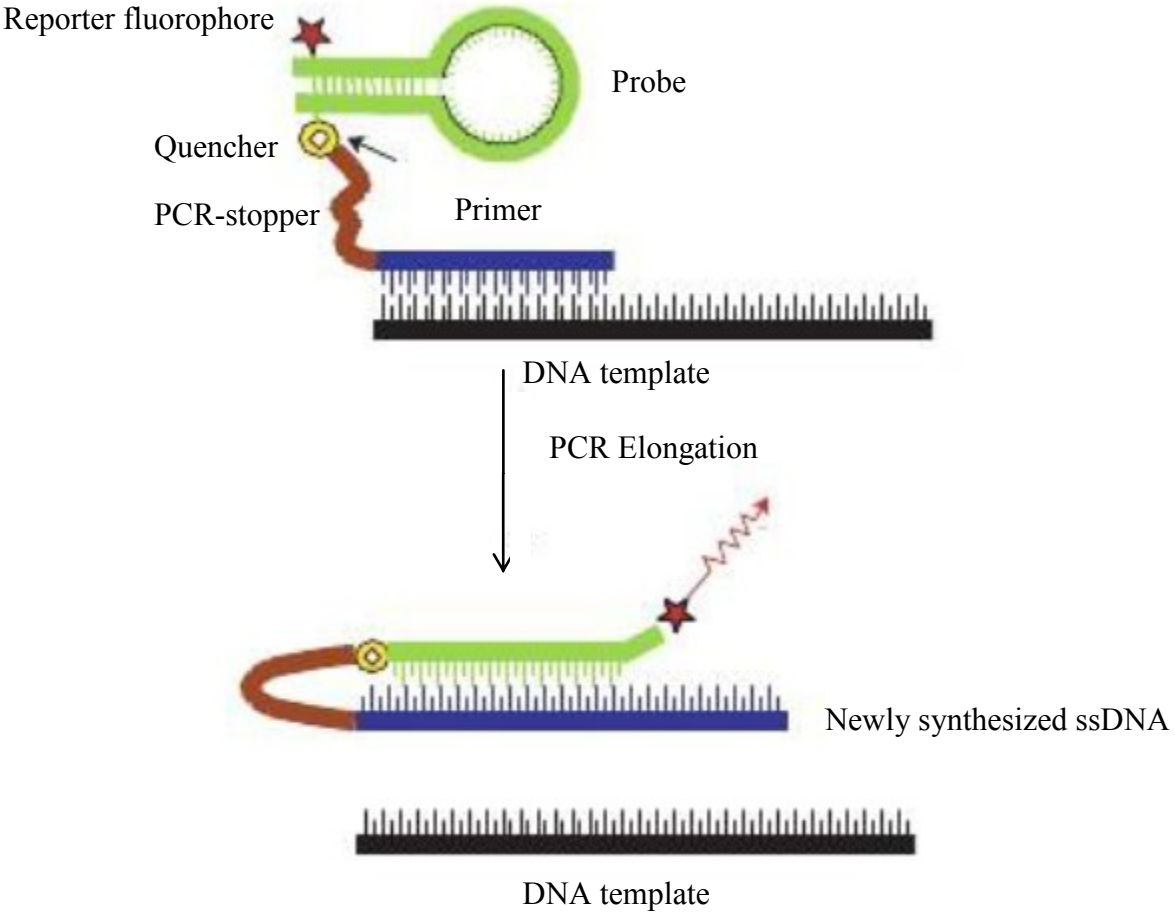


Figure 7: **Scorpions.**

Source: Arya *et al.,* 2005.

(xii) Real-time reverse transcription quantitative polymerase chain reaction (real-time RT- qPCR)

Real-time reverse transcription polymerase chain reaction (RT-qPCR) is a variant of the traditional real-time PCR and it is operating under the same principle (as discussed above)

except the fact that, real-time RT-qPCR is a special assay designed specifically for amplifying and quantifying Ribonucleic acids (RNA).

Unlike the traditional real-time PCR, real-time RT-qPCR has additional reverse transcription process for synthesizing Complementary DNA (cDNA) apart from denaturation, annealing and elongation steps. Apart from the fact that, DNA polymerase can use only DNA molecule as its template and differences between RNA and DNA molecules in terms of nucleotide composition (RNA is rich in Uracil (U) and not Thymine (T)), have made DNA polymerase-directed amplification of RNA molecule impossible (Vasalek and Repa, 2005). That being the fact, a reverse transcriptase catalyzes synthesis of single stranded DNA (cDNA) which is complementary to RNA molecule. The reverse transcriptase (also known as RNA-dependent DNA polymerase), is naturally expressed by Retroviruses like Moloney Murine Leukemia virus (MMLV) (Office International des Epizooties, 2012), Hepatitis C, and Avian Myeloblastosis Virus (AMV) (Vasalek and Repa, 2005) even though other genetically engineered reverse transcriptase enzymes (*viz* Omniscript, Powerscript, Stratascript, Superscript II) are commercially available (Vasalek and Repa, 2005). The synthesized cDNA will in turn serve as a template for DNA polymerase-directed amplification just like the traditional real-time RT-qPCR. Since cDNA is complementary to RNA, quantification of RNA molecule by real-time PCR is possible because the copy number of the synthesized cDNA is proportional to the RNA molecules in the sample (Vasalek and Repa, 2005).

Even though it is not user-friendly, the real-time RT-qPCR assay is still the sound molecular diagnostic assay over and above the conventional gel-based RT-qPCR assay,

because of simplicity of its automated detection method of PCR products, high sensitivity and specificity (Arya *et al.,* 2005; Vasalek and Repa, 2005). Pan-serotypic and serotype-specific real-time RT-PCR assays are the two variants of real-time RT-qPCR assay used in diagnosis of FMD, even though none of them lack some strengths and weaknesses.

(xiii) Pan-serotypic real-time RT-qPCR assay

It has been proved that, generic pan -serotypic real-time RT-qPCR assay (rRT-PCR) which targets the highly conserved $3D^{POL}$ gene encoding the RNA-dependent RNA polymerase or the highly conserved 5' Untranslated Region (5' UTR) is at least equal to virus isolation by cell culture which is regarded as the FMD gold standard test Ferris *et al.* (2006) cited by Reid *et al.* (2014). On the other side of a coin, this generic pan-serotypic real-time RT-PCR assay only confirms the presence of FMD infection generally and cannot distinguish or point out the candidate serotype of FMD virus responsible for disease outbreaks (Reid *et al.,* 2014).

(xiv) Serotype-specific real-time RT-qPCR assay

Molecular diagnosticians saw the need to differentiate FMDV into respective serotypes during field or laboratory diagnosis of FMD for accurate serotype-specific vaccine administration, during FMD control since there's no vaccine cross-protection between FMDV serotypes (Reid *et al.,* 2014). Serotype-specific real-time RT-qPCR assay make use of serotype- specific primers which target the most genetically divergent VP1 (1D) gene between FMDV-serotypes. In contrast to the pan-serotypic real-time RT-PCR assay, serotype-specific real-time RT-PCR assay is more specific and hence narrow the FMD-diagnosis. One obstacle of serotype-specific real-time RT-PCR assay is that; there's delayed diagnosis of massive samples from FMD-suspected cases samples during FMD-epidemics because this assay uses one serotype-specific primer at once.

(b) Conventional gel-based reverse transcription polymerase chain reaction (gel-based RT-PCR) assay

The first Thermal cycler (PCR-machine) which Kary Mullis innovated was a typical gel-based PCR. Principally, the conventional gel-based RT-PCR have reverse transcription step in addition to denaturation, annealing and elongation steps in order to convert genomic RNA molecules into a complementary DNA (cDNA) which will later serve as template for subsequent PCR amplification. However, detection of RT-PCR products is based on separation of the DNA amplicons through a semi-solid Agarose Gel (AG) or Polyacrylamide Gel (PAG) matrix under electric field (i.e. gel electrophoresis). During Electrophoresis DNA molecules with negative polarity are separated based on their sizes (base pairs) under electric field as they move from Cathode to Anode.

(i) Pan-serotypic gel-based RT-PCR assay for detection of foot-and-mouth disease virus (FMDV)

In the pan-serotypic gel-based RT-PCR assay, any of the existent seven serotypes of FMDV are detected despite existence of a broad spectrum of variants/ subtypes within each FMDV serotype as a result of genetic and antigenic diversity. This is achievable by targeting specific gene sequence encoding RNA-dependent RNA polymerase ($3D^{POL}$) and/ or 5'Untranslated Region (5'UTR) of viral genomic RNA molecule. Primers are designed to target specific ($3D^{POL}$) gene sequence and/ or 5'Untranslated Region (5'UTR) which are highly conserved in all FMD virus regardless the serotype or topotype group they belong to.

(ii) Serotype-specific gel-based RT-PCR assay for typing foot-and-mouth disease virus (FMDV)

The case is quite different when it comes to characterization of FMD virus into serotypes and their respective subtypes/variants, because there's a broad spectrum of structural variations among all FMD virus. Great antigenic variation between and within the seven co-existing FMD virus accounted by genetic differences as a result of high mutational rate of genome (Longjam and Tayo, 2011), helped scientists to design an assay that distinguish each of FMD virus serotypes and/ or topotypes. That being the case, primers is designed to target specific sequences encoding the Viral Protein 1 (VP1), which is among proteins which constitute the surface of viral capsid. Eventually all post RT-PCR products are normally analyzed by either Agarose or Polyacrylamide-gel Electrophoresis and hence the assay is termed "gel-based RT-PCR".

Regardless it is either "pan-serotypic-" or "serotype-specific-" variant of gel-based RT-PCR assay, generally this diagnostic assay is getting out of date because its constraints or shortcomings have yet been replaced by more sophisticated PCR-based diagnostic techniques (*viz* Real-time RT-qPCR and RT-LAMP).

According to Alexandersen *et al.* (2000), cited by Reid *et al.* (2014) gel-based RT-PCR assay is cumbersome, time consuming and thus making it unsuitable for quick FMD-diagnosis during epidemics. Moreover, since detection method of this assay is based either on Agarose Gel Electrophoresis (AGE) or Polyacrylamide Gel Electrophoresis (PAGE), distinction of variants of FMDV-serotypes with a wide spectrum of genetic diversity (*viz* strains, topotypes), is difficult and many have proved that, sensitivity and specificity of this assay is poor in this aspect Reid *et al.* (2000), cited by Reid *et al.* (2014).

Moreover, if the genomic RNA of FMDV that was amplified and detected by conventional gel -based RT-PCR is to be used for further analysis (*viz* sequencing), there's high risk of carrying over contamination to the next steps because of hectic task to obtaining the nucleic acids from the gel unless a multi-step DNA purification is done prior sequencing. Last but not least, the conventional gel-based RT-PCR is certainly not user-friendly and for that case it cannot be used for field diagnosis of FMD.

(c) Loop mediated isothermal amplification (LAMP)

Loop-mediated Isothermal Amplification (LAMP) was innovated about a decade ago by Tsugunari Notomi and his research colleagues as an alternative to a traditional PCR machine (Notomi *et al.,* 2000). The innovation of LAMP as an alternative molecular diagnostic tool apart from gel-based PCR and real-time PCR has significantly helped to solve a wide spectrum of queries across multiple science disciplines as it is applied in research fields, disease diagnosis, quality checks in many food industries etc. due to its high specificity, sensitivity and rapidity (Notomi *et al.,* 2000).

The fact that, DNA template is detected by four different primers which identify six different sequences on the target DNA under isothermal conditions, accounts for the high sensitivity and specificity of LAMP (Notomi *et al.,* 2000).With LAMP, 10^9 copies of a single DNA template are synthesized by strand displacement in less than one hour under catalysis of *Bst* Polymerase enzyme isolated from *Bacillus Stearothermophillus* under isothermal conditions normally 65ºC (Notomi *et al.,* 2000). Amplicons obtain after LAMP can be detected (analyzed) by gel electrophoresis (Waters *et al.,* 2014) or visualization. Visual detection is normally done either by using PH-sensitive dyes,

turbidity change as well as metal-sensitive indicators (Tanner *et al.,* 2015). Pyrophosphates (PPi) and Hydrogen ions (H+) are the by-products of nucleic acid amplification when 2'-deoxyribonucleoside-5'- triphosphates (dNTPs) is incorporated at 3' end of the growing DNA strand (Tanner *et al.,* 2015). The pyrophosphate (PPi) produced following DNA amplification form Magnesium pyrophosphates precipitates with Magnesium sulfate ($MgSO_4$) which ultimately makes the solution mixture cloudy. This enables visual detection of LAMP-amplicons by observing/ assessing percentage cloudiness of the resulted solution even though cloudiness of mixture (turbidity) is best detected by using the turbidity instrument (Turbid meter). The use of metal-sensitive indicators is another simple, rapid and cost-effective method for detection of post-LAMP-products. Such metal-indicators include Calcein, Hydroxynaphthol blue and Malachite green which change colors from dark yellow to yellow, dark blue to blue, and dark blue to light blue respectively.

Of recent there are two variants of LAMP that are widely used for diagnosis of FMD. These are reverse transcription loop-mediated isothermal amplification (RT-LAMP) and reverse transcription loop mediated isothermal amplification-lateral flow device (RT-LAMP-LFD).

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

Of all PCR-based molecular diagnostic assays, RT-LAMP has the highest sensitivity (Notomi *et al.,* 2000) because at least six pair of primers are used per reaction unlikely Real-time RT- PCR and conventional gel-based RT-PCR (Chen *et al.,* 2011) which uses only one pair of primer.

According to research work done by Chen *et al.* (2011), sensitivity of RT-LAMP was found to be higher than the conventional gel-based RT-PCR assays despite problems

associated with its amplicon detection systems. On another perspective RT-LAMP still stands as the best molecular diagnostic assay for detection and characterization of the candidate FMDV-serotypes responsible for epidemics in the field due to its rapidity, cost-effectiveness and simplicity (Ding *et al.,* 2013).

Pan-serotypic RT-LAMP is normally designed for detection of FMDV by targeting 3D gene which is highly conserved in all FMDV serotypes whilst the VP1 gene with high genetic diversity among different FMDV is targeted during characterization of the virus. The amplicon detection methods for both pan-serotypic and serotype-specific-RT-LAMP are more or less the same.

(ii) Reverse transcription loop-mediated isothermal amplification-lateral flow device (RT-LAMP-LFD)

Assay in order to complement constraints of each diagnostic tool, scientists designed Reverse Transcription Loop-mediated Isothermal Amplification-Lateral flow device (RT-LAMP-LFD) assay which is literally a combination of RT-LAMP and Lateral Flow Devices (LFD) for detection and characterization of FMD (Waters *et al.,* 2014). RT-LAMP-LFD assay have a higher analytical sensitivity about 10,000 fold than when Immunochromatographic LFD is used alone for FMD diagnosis (Waters *et al.,* 2014). However the RT-LAMP-LFD assay bypasses the RNA extraction step and thus makes detection of FMD less time consuming. The potential of this assay to detect and/ or characterize FMDV genome in both surrounding air exhaled by infected animals as well as in their biological samples, makes it a strong user-friendly tool for rapid evaluation of pre-clinical infection and carrier status of FMD (Waters *et al.,* 2014).

Since detection of RT-LAMP and RT-LAMP-LFD products is based on visualization (i.e. turbidity-or fluorescent color change) of the reaction mixture, their interpretation is difficult (Waters *et al.,* 2014). Difference in visual abilities of different diagnosticians greatly compromises the sensitivity and specificity of RT-LAMP-based assays for any disease (FMD inclusively).

2.5.1.2 DNA sequencing technologies

Up to this moment, there is an ongoing evolution of a variety of DNA sequencing technologies in the world since Allan Maxam and Walter Gilbert's first innovated the DNA sequencing technique which was based on chemical degradation of DNA (Maxam and Gilbert (1977), cited by Pareek *et al*. (2011). However, the technique was replaced by a manual Sanger enzymatic di-deoxy DNA sequencing method whose principle was based on chain termination (Sanger *et al.,* 1977). The need for a quick diagnosis stimulated the development of advanced and highly sophisticated High Throughput Next Generation Sequencers (HT-NGS) which can sequence multitude of genomes from lower to higher animals, generate massive data about 100 folds than the automated Sanger's sequencing technology at high speed and at cheap cost (Pareek *et al.,* 2011). So far sequencing technologies have been classified into First generation-HT-NGS, Second generation HT-NGS and Third generation HT-NGS). These sequencing technologies are used at different part of the world. However, these three generations of High-Throughput Next generation Sequencers (HT-NGS) differ in terms of speed, costs and quality of the sequence output.

(a) First generation high-throughput next generation sequencing technologies

The automated Sanger's sequencing technology is a typical example of first generation of HT-NGS. Frederick Sanger who innovated this sequencing technology took advantage of the ability of DNA polymerase to replicate DNA (invitro) by using fluorescently-labeled 2', 3'-dideoxynucleotides (ddNTPs) which are analogues of the normal 2'-deoxynucleotides (dNTPs) (Sanger *et al.,* 1977). Unlike the latter nucleotide, the former nucleotide lacks hydroxyl group at Carbon 3 of the ribose sugar important for phosphodiester bond formation (Sanger *et al.,* 1977). Literally speaking, the automated

Sanger's sequencing technology is based on chain-termination of growing chain once 2', 3'-dideoxynucleotides (ddNTPs) is incorporated in place of the normal 2'-deoxynucleotides (dNTPs). Because dNTPs ratio is 9: 1, amplicons of different length will be yielded at the end of PCR reaction. For the automated Sanger's sequencing, the amplicons/ extension products are separated by capillary electrophoresis using a denaturing flowable polymer. A chromatogram with multiple peaks that are colored Blue, Black/ Yellow, Green or Red which stands for C, G, A or T. However, before development of the automated Sanger's sequencer, amplicons or sequence-products of preliminary manual version were analyzed by gel-based electrophoresis. Development and commercialization of automated version of Frederick Sanger's sequencing method has helped so far to answer a lot of questions on Genomics of variety of organisms across multiple disciplines through sequence-based DNA analysis using Bioinformatics tools (*viz* human genome project). Innovation of Genetic sequencer 20 by 454 Life Sciences the in 2005 marked the beginning of new era of Next Generation Sequencing technologies.

(b) Second generation high-throughput next generation sequencing technologies

SOLiD, Illumina and Pyrosequencing (Fakruddin *et al.,* 2012) are the commercially available second generation HT-NGS platforms which are more efficient than the first generation HT-NGS due to their fast sequencing speed of about 500 million base pairs per run (Pareek *et al.,* 2011). Second generation HT-NGS were developed to complement constraints of the first generation HT-NGS which was based on 2', 3'di-deoxynucleotide chain-termination.

(c) Third generation high-throughput next generation sequencing technologies

Because of sequencing errors of the second generation HT-NGS due to mis-incorporation of nucleotide bases by polymerase during PCR- based amplification, a number of third

generation HT-NGS were developed. These less cost-prohibitive third generation HT-NGS which can sequence massive nucleotides within a short period of time includes; Heliscope™ Single Molecule Sequencer, Single molecular real-time sequencer, Single molecule real-time sequencer, Nanopore DNA sequencer, Real-time single molecule DNA sequencer platforms developed by VisiGen Biotechnologies, Multiplex Polony techologies without forgetting the Ion Torrent Sequencing Technology (Pareek *et al.,* 2011).

2.5.2 Non-molecular tools (assays) for diagnosing foot-and-mouth disease (FMD)

(a) Virus isolation (cell culture)

Even though cell culture is considered as the most ancient method for diagnosing FMD, this assay still holds as the gold standard test despite having some constraints (Office International des Epizooties, 2012). Diagnosis of FMD by cell culture has now become insensitive because this assay confirms the presence or absence of disease by virus isolation without further characterization of the virus into respective serotypes and or/ topotypes/ strains. The use of molecular diagnostic tools (PCR and sequencing technologies) together with bioinformatics tools, have greatly assisted the understanding of molecular epidemiology of FMD unlike the conventional cell culture. As opposed to cell culture (VI), analysis and interpretation of output data from molecular diagnostic tools by using bioinformatics tools have greatly assisted to understand evolutionary rate, phylogenetic relatedness of FMDV with high mutation rate. Furthermore, it is not possible to trace the possible source of infection when FMD outbreaks occur in both endemic and non-endemic settings by cell culture (virus isolation) only (Jeirani *et al.,* 2012). Isolation of FMDV is accomplished by incubating known amount (volume) of sample from FMD-suspected animals into susceptible cell culture (invitro). Alternatively the virus can be amplified in a living system (in vivo) *viz.* unweaned mouse of 2-7days

old (Office International des Epizooties, 2012). FMDV can be isolated in cell culture by using primary bovine (calf) thyroid cells or kidney cells of Pig, Lamb, or Calf (Office International des Epizooties, 2012). In addition to primary cells, a group of secondary cell lines such as BHK-21 from (Baby Hamster Kidney) cells and Pig kidney cells (IBRS-2 cells) have shown good results in virus isolation even though they are less sensitive for samples with low viral load as compared to primary cell lines (Office International des Epizooties, 2012). Jeirani *et al*. (2012) described that FMDV from epithelial scrapings were isolated after being cultured in Porcine Kidney cells at 37^{o}C within a humidified incubator with five percent carbon dioxide (5% CO_2) for 72 hours. Cell culture is time consuming and it takes few days to a week for a virus to be isolated (Oem*et al.,* 2009), and this results to delayed control response of FMD in the field.

(b) Immunological (serological) assays for diagnosing foot-and-mouth disease (FMD)

As opposed to virus isolation, serological assays are meant to confirm that an animal got an infection currently or previously regardless whether the clinical disease developed or not. Serological assays can diagnose FMD directly or indirectly detecting viral antigens (structural or non-structural proteins) produced in response to infection. Serological assays for detection of either antibodies against structural proteins (SPs) or non-structural proteins (NSPs) are normally used for serotyping as well as pan-serotypic detection of FMD virus respectively (Office International des Epizooties, 2012; Ding *et al.,* 2013). Such serological assays include; Enzyme-linked Immunosorbent Assay (ELISA), Virus Neutralization Test (VNT), Complement Fixation Test (CFT), Lateral Flow Devices (LFD) etc. Apart from confirming presence of FMD infection in suspected cases, the serological assays which detects the viral NSPs are globally used to serve a range of purposes such monitoring the efficacy of vaccine during the emergence or routine vaccination programs. In other instances, ELISA assays are used for screening FMD-susceptible animals prior to export or import in international trade, detection/ monitoring

of virus activity as well as to distinguish non-vaccinated from FMD-infected animals (Office International des Epizooties, 2012).

(i) Lateral flow devices (LFD)

Lateral Flow Devices (LFDs) is preferably used for field diagnosis of FMD in the field areas. Field diagnosis of FMD by using LFDs prevents the risk of transmitting the highly contagious FMD virus from the field area to laboratory. On another perspective, rapid diagnosis of FMD during outbreaks by the use of LFD, confirm presence of FMD and hence assist in immediate (on time) implementation of effective FMD-control measures against specific serotypes. Furthermore, with LFDs FMD can be diagnosed at cheap cost since there's no need of expensive instrument (*viz* real-time RT-PCR machine). Moreover, the LFD is easy to use and does not necessarily require technical expertise to operate it. Even though the LFD have not yet been validated to be used for FMD diagnosis (Office International des Epizooties, 2012), their field application in different endemic settings have proved that specificity of these assays have been out-ruled by Enzyme-Linked- Immunosorbent Assays (ELISAs) (Oem *et al.,* 2009).

(ii) Enzyme-linked immunosorbent assays (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is the most popular immunoassay used for either qualitative or quantitative diagnosis of FMD by detection of either an antigen or antibody in the sample (Office International des Epizooties, 2012; Ding *et al.*, 2013). A number of different ELISA formats have been developed in few past decades, mainly for detecting Structural proteins (SPs) and Non-structural Proteins (NSPs) of FMD virus (Office International des Epizooties, 2012). Antigen-capture ELISA, Liquid-phase blocking ELISA are currently used for viral antigen typing and antibodies detection against FMD virus serotypes O, A and Asia 1 respectively (Ding *et al.,* 2013). Moreover, varieties of ELISA kits for detection of antibodies against different Non-structural Proteins (NSPs) are used in different endemic and non-endemic settings for FMD

diagnosis as well as sero surveillance (Office International des Epizooties, 2012). 3D is not the only Non-structural Proteins (NSPs), which is targeted for FMD detection in different ELISA formats, other proteins includes 3A, 3B, 2B, 2C, 3AB and 3ABC (Office International des Epizooties, 2012; Ding *et al.,* 2013). Pan-serotypic ELISA test normally targets the so called Virus Infection Associated Antigen (VIAA) or 3D protein (Ma *et al.,* 2011). Different ELISA kits are available for differentiation of infected animals from FMD-vaccinated animals in the field areas specifically by targeting antibodies against the 3ABC polyprotein complexes which are produced during in vivo virus replication (Ding *et al.,* 2013). Moreover, ELISA is currently used in assessment of vaccine purity during production chain as well as antigen analysis (Ma *et al.,* 2011).

However, false-positives results are one of the constraints of ELISA assays due to cross-reactivity with other viruses causing other vesicular diseases apart from FMD. Currently, failure to differentiate FMD-infected from-vaccinated animals, compromise FMD-control by vaccination. However, a number of ELISA tests which targets the non-structural proteins (3D, 3B and 3ABC), have been developed to differentiate the vaccinated from infected animals (Ma *et al.,* 2013). Unlike animals vaccinated with live attenuated virus (inactivated/ dormant), the named NSPs are only found in infected animal because the virus is actively replicating.

(iii) Virus neutralization test (VNT)

Virus Neutralization Test (VNT) has additional cell culture technique apart from virus neutralization with antibodies specific to it. VNT is normally used to detect presence of FMD in sample by neutralizing the virus infection prior to culturing the antibody-antigen mixture into primary or secondary cell-lines (Office International des Epizooties, 2012). Viruses in serum are normally neutralized by mixing it with antibodies that are specific to it. If the plaques will form on cell culture this indicate that the animal didn't have the disease of interest (Office International des Epizooties, 2012).

(iv) Complement fixation test (CFT)

Complement fixation test is one of immunological diagnostic assays which is used for either typing and/or subtyping or detection of FMD virus. Generally, this assay makes use of complement proteins (components of humoral innate immunity system), antigens, standardized sheep Red Blood Cells together with anti-SRBC antibodies in order to detect antibodies in animal's serum (Office International des Epizooties, 2012).

2.6 Prevention and Control of FMD

A rapid, (Reid *et al.,* 2014; Waters *et al.,* 2014), highly specific and sensitive diagnostic tool, veterinary staff who are well-trained on FMD without forgetting powerful veterinary administration with financial support from government and/ or NGOs are pre-requisite for efficient 'on time' FMD control (Ding *et al.,* 2013; Namatovu *et al.,* 2013; Reid *et al.,* 2014; Paton *et al.,* 2009). To prevent spread of outbreaks, (Quarantine law) is normally applied to restrict transboundary animal movements or products within and across FMD-infested countries (Ding *et al.,* 2013). Eradication is an immediate control measure mostly applicable in developed countries to quickly stop transmission of FMDV from infested premises to FMD-free premises by burning all the stamped or slaughtered infected and susceptible animals **(**Alexandersen *et al.,* 2003; Sinkala *et al.,* 2014). Vaccination is both a control and preventive measure for FMD by triggering immune response against pathogens through administration of immunogenic avirulent (attenuated) live virus or their antigenic proteins (Alexandersen *et al.,* 2003). However, control of FMD by using live attenuated vaccines greatly assists in reducing the detrimental impacts of FMD, though there are many challenges/ or obstacles associated with this control measure. The fact that, FMD virus is highly contagious, has made the production of the live attenuated vaccines very complex and costly because these vaccines needs to be

produced in highly confined environment so as to ensure environmental safety. For that reason different parts of the world still experience vaccine shortage.

Furthermore, despite the probability of viral reversion to virulence in conventional attenuated live vaccine, there's also failure of one vaccine to provide cross-protection against all FMDV-serotypes strains and topotypes (Grubman and Baxt, 2004) because the FMD virus exists as seven antigenically distinct serotypes. Moreover, storage and transportation of the live attenuated vaccines is a bit hectic because literally these vaccines are highly unstable under ambient environmental conditions and must be stored in cold chain (4-6°C) all the way from the manufacturing industry to filed areas before application. Last but not the least challenge is that, the live attenuated vaccines can only provide short-term protection against FMD and the need for periodic vaccine re-administration makes the whole process very expensive especially for developing countries where the disease is endemic. Regarding all challenges of live attenuated vaccines, research advancement and knowledge integration in the fields of Vaccinology, Molecular biology and Biotechnology improved the production of more advanced vaccines namely DNA, peptide, empty viral capsid vaccines (Arzt *et al*., 2011b; Grubman and Baxt, 2004). Peptide vaccines are synthetic viral peptide mimicking the antigenic binding site (RGD) which can trigger host's immune response (Li *et al.,* 2014).

Recently, there are ongoing research at Pirbright Institute, Diamond Light Source and Synchrotron Radiation Source as funded by Science and Technology Facility Council and Welcome Trust to produce a new vaccine (synthetic virus from a live virus) that is expected to overcome weaknesses of the current live attenuated vaccine in use (http://www.stfc.ac.uk/files/3149/3149). The new capsid vaccine is synthetic in nature and hence ensure environmental safety because the virus in non-infectious. Moreover,

storage and transportation of the new (synthetic) capsid vaccine will be simple in field condition because of high stability nature of the virus which can tolerate up to 56°C. In addition, because the new capsid vaccine is man-made (synthetic), researchers are planning to develop a diagnostic assay that will uniquely distinguish vaccinated from non-vaccinated animals in the field (http://www. stfc.ac.uk/files/3149/3149). Almost the same case have been mentioned by Grubman and Baxt (2004) that empty capsid vaccines can be produced by invitro expression of P1-2A and $3C^{pro}$ coding regions of FMDV genome and recombinant DNA technology is currently applied in production of (transgenic FMDV) vaccine, whose RGD receptor binding site on VP1 has been deleted. For that case, still there's a lot to be done before scientists announce publically that a new immunodominant vaccine that can offer protection against all seven co-existent FMDV serotypes, including their topotypes and/ or strains with an increasingly wide spectrum of antigenic diversity is commercially available and affordable in all places around the world.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Areas

This study was conducted in Tanzania. Samples were collected from different regions in the country and laboratory analysis was performed at the Centre for Infectious Disease and Biotechnology (CIDB), Tanzania Veterinary Laboratory Agency (TVLA) located in Temeke, Dar-es-salaam.

3.2 ResearchDesign

Both case-control and *in-silico* laboratory-based experimental designs were used in this study.

3.3 Sample Collection

(a) Sample type

Sample from FMD-suspected cases of cattle (basing on clinical signs) were collected during different outbreaks of vesicular diseases in different geographical locations of Tanzania. The collected samples in question were epithelial tissue from ulcerations/lesions on foot (hooves, interdigital cleft), in and around mouth parts (tongue, gums, and muzzle) of cattle.

(b) Geographical location for sample collection

Samples from both clinically sick cattle (FMD-suspected cases) and the apparently healthy cattle (control group) were collected from sporadic FMD-outbreaks in Rukwa, Tabora, Morogoro, Mwanza, Dar-es-salaam, Tanga, Zanzibar, Kagera, Mbeya and Kigoma regions in Tanzania.

Archived samples were collected from different sporadic outbreaks of FMD from 2008 to 2013, and stored at the Centre of Infectious Disease and Biotechnology (CIDB) of the Tanzania Veterinary Laboratory Agency (TVLA) in Dar-es-Salaam prior to laboratory analysis for FMDV.

Negative control samples were collected from FMD-naive animals unlike positive controls which were attenuated FMD vaccines (*viz*Serotype O Kenya vaccine strain Serotype A Ethiopia vaccine strain). For SAT1 and SAT2 serotypes of FMD, Internal Positive Controls (IPC) were used at CIDB, TVLA.

(c) Sample packaging, transportation and storage

The Epithelial tissues collected from lesions/ulcerations on foot and mouth (tongue) of FMD-suspected cases of cattle were given different sample identification (IN) numbers and packed in sterile Falcon tube with enough Virus Transport Media (VTM) such that, the tissue was immersed in the media. The samples were transported in cold chain (cool box with icepacks) from field area to TVLA before they were stored in a freezer at -80 ºC for subsequent laboratory analysis

3.4 Sampling Technique

Non-randomized (purposive) sampling technique was used to collect epithelial tissue samples from both cattle with evident clinical signs of FMD (FMD cases) and clinically healthy cattle with no evident clinical signs for FMD as Negative Control (NC) group.

3.5 Sample Size

A total of 45 epithelial tissue samples were analyzed by one-step real-time RT-PCR assay in this study. Two Internal positive controls (IPC) (for serotype SAT1 and SAT 2), 2 FMD vaccines (Serotype A and O) and 2 samples collected from clinically healthy (FMD-free) cattle as negative controls, were used during optimization and application of

the one-step real-time RT-qPCR assay by utilization of 14 archived samples from confirmed cases of FMD (known FMD-positive samples) and 25 samples from the FMD-suspected cases of cattle with evident clinical signs obtained from previous FMD-outbreaks in different regions of Tanzania from 2008 to 2014 respectively.

3.6 Activities and Laboratory Analysis

3.6.1 Primer designing

Primer design express software was used to design both 3D gene-specific primers and VP 1 gene-specific primers. The latter and former primers were used for typing and detection of FMDV, respectively. 3D gene-specific primers (Table 1) were designed for Pan-serotypic detection of FMD virus regardless serotype group to which, the virus belong because the 3D gene is highly conserved in all FMD viruses.

Table 1: **3D gene-specific (forward and reverse) primers and probes for pan-serotypic detection of FMD**

Oligo name	Sequence (5'-3')
FMDV/3D/EA/FP	ACTGGGTTTTACAAACCTGTGA
FMDV/3D/EA/RP	GCGAGTCCTGCCACGGA
FMDV/3D/EA/P	[6FAM]TCCTTTGCACGCCGTGGGAC[TAM]

The designing of serotype-specific primers was accomplished after multiple sequence alignment of VP1 gene sequences of topotypes of each serotype (A, O, SAT-1 and SAT-2) by using *(Clustal W software*) to obtain the highly conserved sequence of VP1 gene which target the highly conserved sequence of VP1 gene for each FMDV serotypes (A, O, SAT 1, and SAT 2) (Table 2).

Table 2: **Forward primer (FP), Reverse primer (RP) and Probe (P) specific to VP1 gene of each of FMDV serotypes: A, O, SAT1 and SAT 2.**

Oligo name	Sequence (5'-3')
FMDV/A/EA/FP	GCCACRACCATCCACGA
FMDV/A/EA/RP	GAAGGGCCCAGGGTTGGACTC
FMDV/A/EA/P	[6FAM]CTCGTGCGMATGAARCGGGC[BHQ1]
FMDV/O/EA/FP	CCTCCTTCAAYTACGGTG
FMDV/O/EA/RP	GCCACAATCTTYTGTTTGTG
FMDV/O/EA/P	[6FAM]CCCTCTTCATGCGGTARAGCAG[BHQ1]
FMDV/SAT1/EA/FP	CTYGACCGGTTCACYCTG
FMDV/SAT1/EA/RP	CCGAGAAGTAGTACGTRGC
FMDV/SAT1/EA/P	[6FAM]CAGGAYTGCGCCCACCA[BHQ1]
FMDV/SAT2/EAiv/FP	CRATCCGCGGTGAYCG
FMDV/SAT2/EAiv/RP	CGCTTCATYCTGTAGTARACGTC
FMDV/SAT2/EAiv/P	[6FAM]TTCGGKTTYGTGACCGCCG[BHQ1]

A total of five pairs of primers and five different fluorescently-labeled probes were used for optimization and application/deployment of one-step real-time RT-PCR assay which can detect and characterize FMD virus simultaneously. Four pairs of primers for FMD Virus characterization were specific to FMDV-serotypes: O, A, SAT1 and SAT2 that are co-circulating in different geographical locations of Tanzania.

3.6.2 Preparation of epithelial samples

Epithelial tissues sample collected from FMD-suspected cases with different IN were thawed after long-term storage in -80ºC freezer before analysis. 1g of each of collected epithelial tissue samples was removed from Virus Transport Medium (VTM) under sterile conditions of Biological safety cabinet II and dried using a sterile tissue paper so as to remove residues of VTM from the tissue. The epithelial tissues were ground with sterile sand and 100ml of Phosphate Buffer Saline (PBS) by using mortar and pestle in the Biological safety cabinet II.

Supernatant of the tissue homogenate was separated from sand and other tissue debris after centrifugation of the mixture at 200 rpm for 10 minutes. Aliquots of the supernatants of tissue homogenate were placed in sterile eppendorf tubes of about 1.5ml by volume for long-term storage at -80ºC before subsequent total RNA extraction followed by real-time one-step RT-PCR procedures.

3.6.3 Extraction of total RNA form epithelial tissues

Total RNA was extracted from epithelial samples collected from both FMD-suspected cases and clinically healthy animals by using QIAamp® Viral RNA Mini Kit (QIAGEN, Valencia, CA,United States of America). Lysis of free cells to release total RNA was done after incubation of 140 µl of epithelial tissue homogenate in 560 µl of viral lysis buffer (AVL) at 15-25 ºC for 10minutes prior to brief centrifugation. 560 µl of Absolute alcohol (96-100% Ethanol) was mixed with the lysate followed by pulse vortexing for 15s and brief centrifugation. All of the resulted solution was dispensed onto QIAamp mini column and centrifuged at 6000xg (8000rpm) for I min. However, this procedure was repeated twice because only 630µl of the solution mixture was added onto silica-gel membrane of the mini column at once before centrifugation. Elution of total RNA from the silica-gel membrane of the QIAamp mini spin column to a sterile eppendorf by using 60µl of Elution buffer (AVE) was preceded by two-step washing of the RNA molecules. Total RNA molecules which were bound onto the silica-gel membrane were washed using two distinct washing buffers (Wash Buffer 1 (AW1) and Wash Buffer 2 (AW2) at different centrifugation speed. 500µl of Wash Buffer 1 (AW1) and 500µl of Wash Buffer 2 (AW2) were distinctly dispensed onto QIAamp mini spin column containing total RNA and centrifugation was done at 6000xg (8000rpm) and 12,000xg (14,000rpm) respectively, even though washing of total RNA with AW2 was preceded by AW1 and distinct sterile collection tubes were used to collect filtrates during each washing step

before they were discarded. Eventually, total extracted RNA which was eluted in 60µl of Elution buffer (AVE) was stored at -80 ºC before subsequent one-step RT-PCR step by using Fast 7500 Real-time PCR system machine.

3.6.4 Reconstitution of reagents

(a) Reconstitution of primers

Concentrated stock solution (100µM) of each of the four distinct primers specific to FMD virus: serotype A, O, SAT1, SAT 2 and 3D-gene specific primers for pan-serotypicdetection of FMD, were diluted to prepare a working solution (10 µM) of about 500µl after addition of 450 µl of nuclease-free water to 50 µl of 100µM stock solution of primers in sterile eppendorf tubes.

(b) Reconstitution of probes

Unlike primers, 500 µl of 5 µM working solution of each of four distinct probes was prepared after mixing 25 µl of 100 µM of Probe stock solution with 475 µl of nuclease-free water in a sterile eppendorf tube.

3.6.5 Preparation of master mix for one-step RT-qPCR assay

A total of five different RT-PCR master mix were prepared under septic conditions within a Biological Safety Cabinet level II for assay optimization, among which (serotype O, A, SAT1 and SAT2)-specific primers were used for typing FMD virus Serotype: O, A, SAT1 and SAT2) respectively in different samples collected from cases confirmed to have FMD infection. Apart from the named four serotype-specific primers which were used for characterization of FMD viruses into respective serotypes, primers specific to 3D-gene was also used for pan-serotypic detection of FMD.

(a) Preparation of master mix for pan-serotypic detection of FMD Virus

A master mix of 20µl by volume was prepared for each sample after mixing 12.5 µl of 2x reaction mixture, 1 µl of PCR water, 1.5µl of 5µM probe specific to 3D gene (3DP), 2 µl for each of 10 µM forward primer and 10 µM reverse primer specific to 3D gene, 0.5 µl of 1:10 Pre diluted ROX (standard dye) and 0.5 µl of superscript III RT/ Platinum Taqmix.

(b) Preparation of master mix for molecular typing of FMD virus by using one-step real-time serotype-O-specific RT-PCR assay

A master mix of 20µl by volume was prepared for each sample after mixing 12.5 µl of 2x reaction mixture, 1 µl of PCR water, 1.5µl of 5µM probe specific to VP1 gene specific to FMDV: Serotype O, 2 µl for each of 10 µM forward primer and 10 µM reverse primer specific to VP1 gene unique to FMDV: Serotype O, 0.5 µl of 1:10 Pre diluted ROX (standard dye) and 0.5 µl of superscript III RT/ Platinum Taqmix.

(c) Preparation of master mix for molecular typing of FMD virus by using one-step real- time serotype-A-specific RT-PCR assay

A master mix of 20 µl by volume was prepared for each sample after mixing 12.5 µl of 2x reaction mixture, 1 µl of PCR water, 1.5 µl of 5 µM probe specific to VP1 gene unique to FMDV: Serotype A, 2 µl for each of 10µM forward primer and 10µM reverse primer specific to VP1 gene of Serotype A, 0.5 µl of 1:10 Pre diluted ROX (standard dye) and 0.5 µl of superscript III RT/ Platinum Taqmix.

(d) Preparation of master mix for molecular typing of FMD virus by using one-step real- time serotype-SAT 1-specific RT-PCR assay

A master mix of 20 µl by volume was prepared for each sample after mixing 12.5 µl of 2x reaction mixture, 1 µl of PCR water, 1.5µl of 5µM probe specific to VP1 gene specific to FMDV: Serotype SAT 1, 2 µl for each of 10µM forward primer and 10 µM reverse primer specific to FMDV: Serotype SAT 1 gene, 0.5 µl of 1:10 Pre diluted ROX (standard dye) and 0.5 µl of superscript III RT/ Platinum Taqmix.

(e) Preparation of master mix for molecular typing of FMD virus by using one-step real- time serotype-SAT 2-specific RT-PCR assay

A master mix of 20µl by volume was prepared for each sample after mixing 12.5 µl of 2x reaction mixture, 1 µl of PCR water, 1.5µl of 5µM probe specific to FMDV: Serotype SAT 2, 2 µl for each of 10µM forward primer and 10µM reverse primer specific to FMDV: Serotype SAT 2, 0.5 µl of 1:10 Pre diluted ROX (standard dye) and 0.5 µl of superscript III RT/ Platinum Taqmix.

3.6.6 Loading of reagents and samples into 96 well plate before running one-step RT-PCR assay

Following preparation of master mix for Optimization of serotype-specific one-step RT-PCR, 20 µl of each of the four prepared master mix was loaded under septic conditions into each well of optic 96 well plate (MicroAmp®-PCR compatible DNA/RNA/RNase free) under septic conditions within the Biological Safety Cabinet level (Forma class II 2A; Thermal Electrical Corporation). 5 µl of total RNA suspension extracted from each FMD case was uniquely added in one well of 96 well plate containing 20 µl of other pre-loaded RT-PCR reagents depending on the primer used. FMD was detected in different samples containing total RNA by using 3D gene specific primers and different

VP1-specific primers were used for typing FMDV into serotypes: O, A, SAT1 and SAT2). The optic 96 well plate was covered with (MicroAmp®-PCR compatible DNA/RNA/RNase free) optic adhesive film before centrifugation of sample-reagents mixture at 3000 rpm by using Eppendorf centrifuge 5810 R machine for 3minutes, to ensure thorough mixing of the RNA template and reagents prior one-step RT-PCR.

3.6.7 Optimization of thermal cycling condition of real-time one-step RT-PCR assay for detection and typing FMD virus in archived samples from confirmed cases of FMD

The Applied Biosystem 7500 Fast Real-Time PCR system was pre-calibrated before running one-step RT-PCR assay. The assay was optimized by running one-step RT-PCR repeatedly to test same samples at different intervals of annealing and elongation temperatures (50°C, 55°C, 60°C, 65°C) for 1minute) at constant 52 numbers of cycles, reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10minutes followed by 95°C for 15 seconds). Each of five pairs of primers was used to test same samples at the same optimization conditions named above. Detection of amplicons was done at the end of elongation step by using fluorescent reporter (FAM) and Non-fluorescent quencher (Black Hole Quencher (BHQ1) in all probes specific to VP1 gene of FMDV serotypes O, A, SAT1 and SAT2). However, FAM and TAMRA were used as fluorescent reporter and non-fluorescent quencher for 3D gene-specific probe, respectively. By 7500 software (version 2.0.5) of Applied Biosystem 7500 Fast Real-Time PCR system, Threshold was set automatically before running the one-step RT-PCR assay. After the assay, results were interpreted. Sample with Cycle threshold (Ct) value less than 32 were considered FMD-positive, whereas samples with Ct value greater than 32 but less than 50 were retested for FMD confirmation because FMD status of such sample is considered ambiguous and those sample with no Ct value were considered FMD-negative.

3.6.8 Application of real-time one-step RT-PCR assay for detection and serotyping of FMD virus in field samples form FMD-suspected cases

After determining the optimum conditions (annealing temperature) at which the serotype-specific real-time one-step RT-PCR could detect and type FMD virus to serotype level in known selected FMD cases of cattle (assay optimization), the same primers were used to detect and type FMD virus in 24 cases of cattle whose FMD-status was unknown and 1 sample from clinically healthy cattle was used as a negative control. Four known positive controls for FMDV serotypes: O, A, SAT1, SAT2 and 1 positive control for pan-serotypic detection of FMD were used. Five pairs of primers were used concurrently to detect and serotype FMD virus in each of total 30 samples (positive and negative controls inclusively) collected from FMD-suspected cases under same optimum one-step RT-PCR conditions.

The thermal cycling conditions for one-step real-time RT-PCR were reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10minutes followed by 95°C for 15seconds), annealing and elongation temperatures (60°C for 1minute) at constant 52 numbers of cycles. Detection of FMD and serotyping of FMD-positive samples was achieved by running one-step RT-PCR by using one pair of (3D gene-specific primers) and four different kinds of VP1 gene-specific primers which can uniquely distinguish FMDV: serotypes O, A, and SAT2 respectively. The same 30 samples were tested for presence of FMD and they were repeatedly typed for presences of FMDV: serotypes O, A, SAT1 and SAT2 simultaneously.

3.7 Data Analysis

Microsoft office excel 2010 was used to calculate the sensitivity and specificity of the standardized/optimized real-time RT-PCR assay for detection and typing of FMDV.

(a) Sensitivity and specificity of a test for diseases diagnosis

Evaluation of how precise any diagnostic test (assay) can correctly /truly report diseased individuals and disease-free individuals among the tested population is a prerequisite after its optimization, just before its practical application for disease diagnosis. Evaluation of a diagnostic test which is independent of the tested population is normally done by determining its sensitivity and specificity by considering True Positives, False Positives, True Negatives and False Negatives (Table 3) among the total tested population. True Positives, False Positives, True Negatives and False Negatives are defined as subjects with disease and are tested positive by a diagnostic test, patients with no disease and are tested positive by a diagnostic test, patients with no disease and are tested negative by a diagnostic test, patients with disease and are tested negative by a diagnostic test respectively (Lalkhen and McCluskey, 2008). Literally sensitivity and specificity is the ability of a diagnostic test to correctly (truly) identify patients with disease and patients without disease respectively (Lalkhen and McCluskey, 2008). Sensitivity and Specificity of any diagnostic test can be determined by using the two formulas below.

Table 3: Two-by-two table showing disease status of animals and results of diagnostic tests (true positive, true negative, false positive and false negative).

Diagnostic test	**Disease status**		
	Present	**Absent**	**Total**
Positive	True positive	False positive	Total tested positive
Negative	False negative	True negative	Total tested negatives
Total	Total patients with disease	Total patients with no disease	Total tested population

Formula

Sensitivity = (True positive)/ (True positive + False negative) (Lalkhen and McCluskey, 2008).Specificity = (True negative)/ (True negative + False positive) (Lalkhen and McCluskey, 2008).

CHAPTER FOUR

4.0 RESULTS

4.1 Results of Optimization of One-Step Real-Time RT-qPCR Assay for Detection and Typing of FMDV

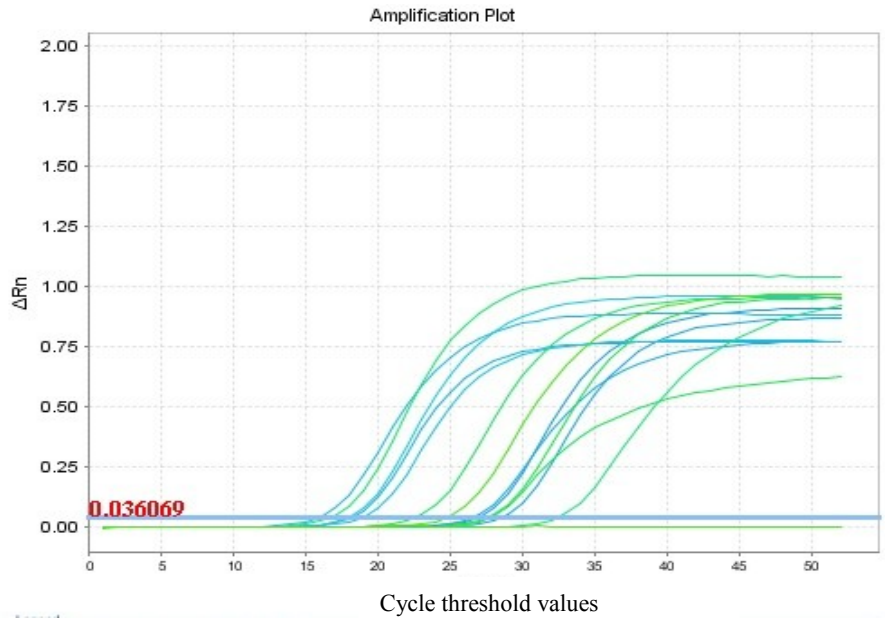
Following assay optimization it was evident that genome of FMDV was detected and typed simultaneously for serotypes O, A, SAT1 and SAT2 by using five different pairs of primers at the same thermal cycling conditions using one-step real-time RT-PCR. The standardized optimum conditions for the one-step real-time RT-PCR were reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10 minutes followed by 95°C for 15 seconds), annealing temperature and elongation (60°C for 1minute) at constant 52 numbers of cycles.

4.1.1 Results of optimization of one-step real-time RT-PCR assay for pan-serotypic detection of FMD virus

The genomic RNA of FMDV was detected in 14 confirmed cases of FMD by 3D-gene specific real-time RT-PCR at the standardized optimum thermal cycling conditions. With exception of a negative control, genomic RNA of FMDV was detected in 14 samples from FMD-cases of cattle by using 3D-gene specific real-time RT-PCR. Samples had Ct values ranging from 15.854 to 32.35 as shown in (Table 4). (Fig. 8 and 9) is the amplification plot of the 15 sample which were tested for presence of FMDV by using 3D-gene specific primers. FMD status was ambiguous for 1 sample (with 66 IN) because its Ct value was 32.35. However, Ct value of 32.35 is considered biologically significant because at this point, there's infection despite the fact that the sample has very few copies of genomic RNA of FMDV.

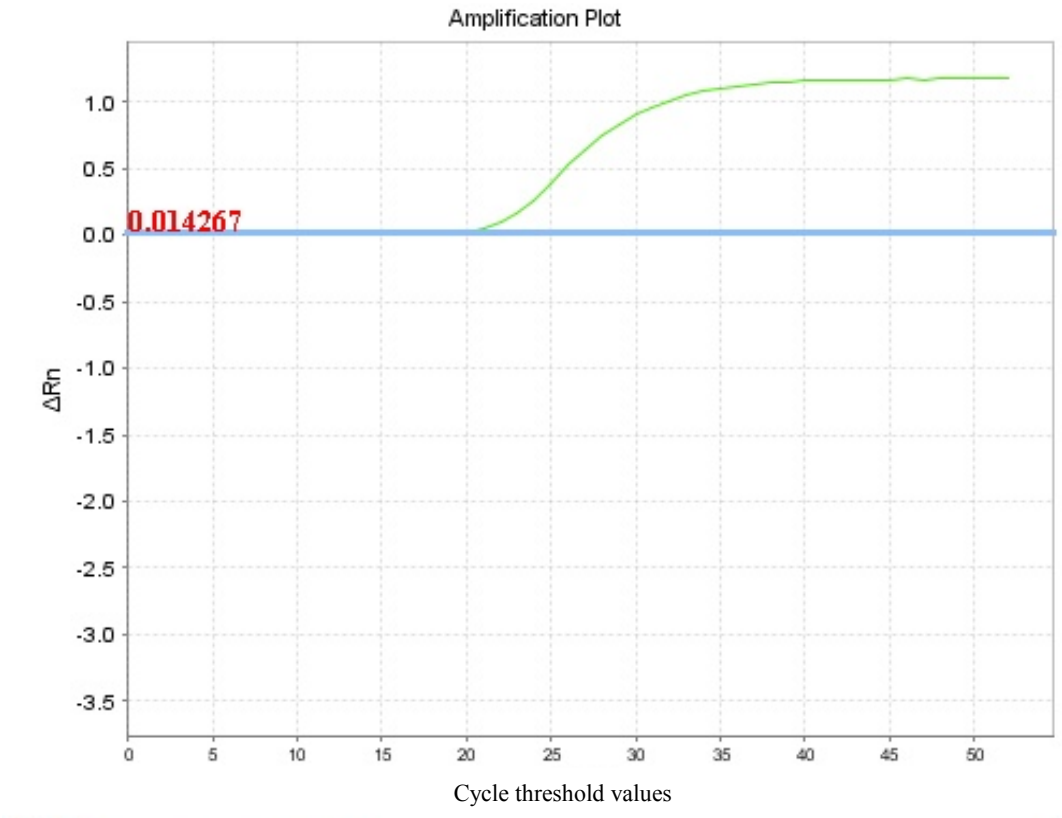
Table 4: Results of optimization of one-step 3D gene-specific real-time RT-PCR assay for pan-serotypic detection of FMDV in known FMD cases of cattle

Sample IN	Town/ Village	Region	Sample collection date	Ct values	FMD status
NC	Bigwa	Morogoro	3/11/2014	Undetermined	Negative
17	Sumbawanga	Rukwa	16/11/2010	24.716	Positive
46	Nzega	Tabora	2/9/2010	27.478	Positive
108	Masasi	Mtwara	20/7/2010	27.787	Positive
366	Mafinga	Iringa	25/4/2013	22.574	Positive
144	Mabuki	Mwanza	16/12/2010	16.681	Positive
66	Tabora Municipal	Tabora	21/09/2011	32.35	Ambiguous
370	Misenyi	Kagera	4/4/2013	19.477	Positive
3601	Kimara,Ilala	Dar-es-salaam	16/11/2010	17.854	Positive
3602	Kimara,Ilala	Dar-es-salaam	2/9/2010	18.876	Positive
360	Kimara,Ilala	Dar-es-salaam	15/12/2011	15.854	Positive
3612	Kimara,Ilala	Dar-es-salaam	21/9/2011	18.062	Positive
68	Masasi	Mtwara	19/12/2011	26.479	Positive
77		Morogoro	19/03/2012	28.479	Positive
126		Tanga	21/09/2011	26.842	Positive



Legend: ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

Figure 8: Amplification plots of FMD-confirmed cases (known positive samples) screened with FMDV: Serotype 3D-specific primers (sample 370 exclusively)



Legend: ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

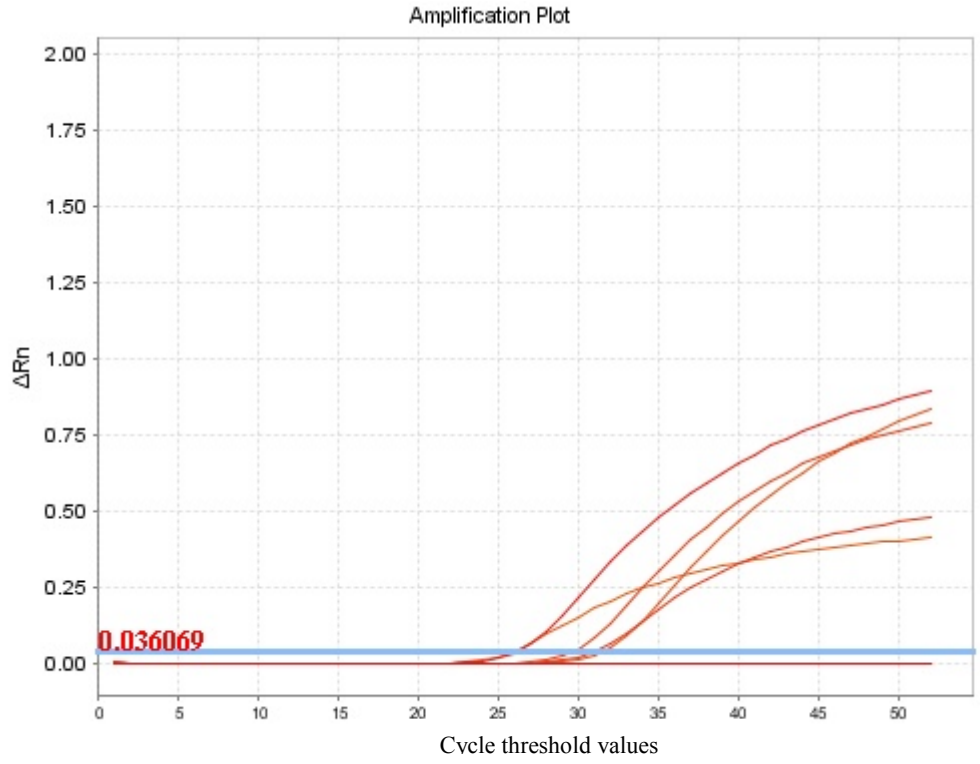
Figure 9: **Amplification plots of FMD-confirmed cases (known positive with samples IN NC, 370) screened with FMDV: Serotype 3D-specific primers.**

4.1.2 Results of optimization of one-step serotype O-specific real-time RT-PCR assay for typing FMD virus

Serotype-O-specific primers were used to type the candidate virus in five samples from clinical cases under standardized optimum thermal cycling conditions by real-time RT-PCR assay. The tested samples had Ct value which ranged from 25.807 to 30.983 as shown in (Table 5) and amplification plot (Fig.10) below.

Table 5: Results of optimization of one-step serotype O-specific real-time RT-PCR assay

Sample IN	Town/ Village	Region	Sample collection date	Ct values	FMD status
NC	Bigwa	Morogoro	3/11/2014	Undetermined	Negative
17	Sumbawanga	Rukwa	16/11/2010	26.052	Positive
46	Nzega	Tabora	2/9/2010	30.983	Positive
108	Masasi	Mtwara	20/7/2010	29.56	Positive
366	Mafinga	Iringa	25/4/2013	31.45	Positive
144	Mabuki	Mwanza	16/12/2010	25.807	Positive



Legend

ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

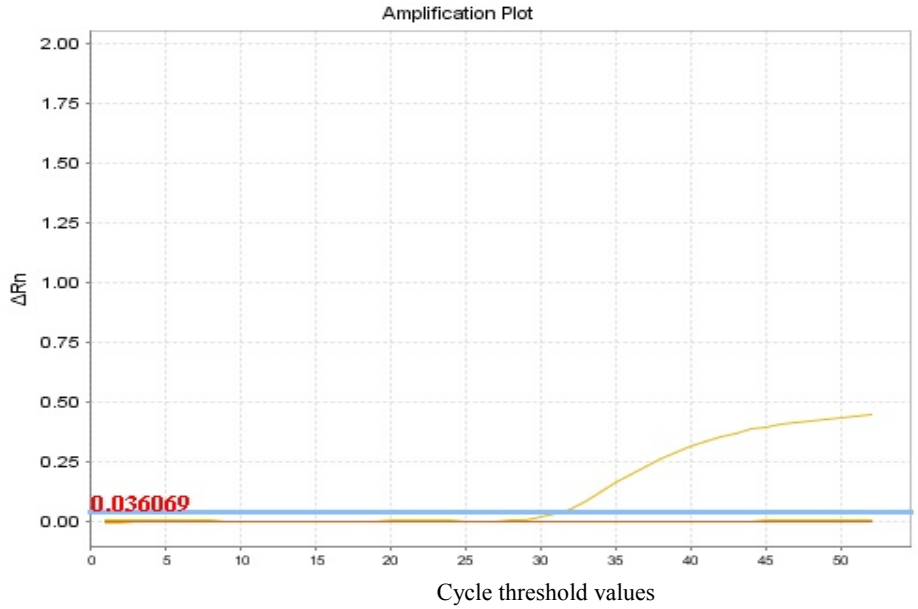
Figure 10: **Amplification plots of samples screened with FMDV: Serotype O-specific primers**

4.1.3 Results of optimization of one-step serotype A-specific real-time RT-PCR assay for typing FMDV

Only two samples confirmed to have FMD infection were typed by serotype-A-specific primers under standardized thermal cycling conditions of real-time RT-PCR assay. Samples with IN 66 and 370 had 20.385 and 31.259 Ct values respectively as shown in (Table 6) and amplification plots (Fig. 11and 12).

Table 6: Results of optimization of one-step serotype A-specific real-time RT-PCR assay

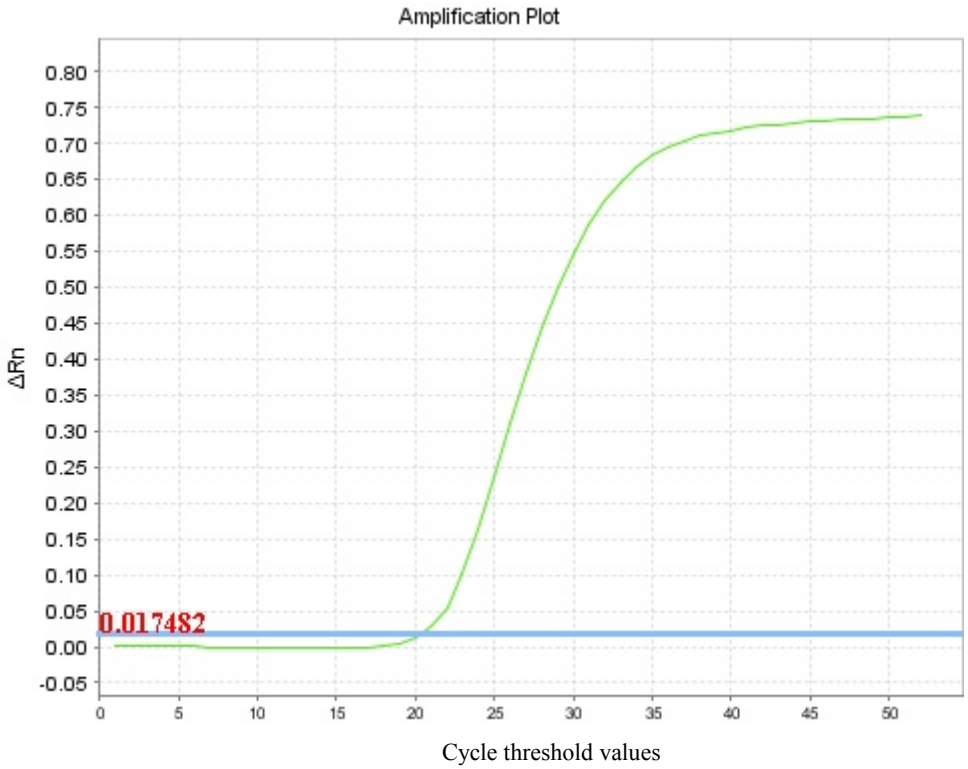
Sample IN	Town/ Village	Region	Sample collection date	Ct values	FMD status
NC	Bigwa	Morogoro	3/11/2014	Undetermined	Negative
66	Tabora Municipal	Tabora	21/9/2011	31.259	Positive
370	Misenyi	Kagera	4/4/2013	20.385	Positive



Legend

ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

Figure 11: Amplification plots of (NC, 66 samples) screened with FMDV: Serotype A-specific primers



Legend

ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

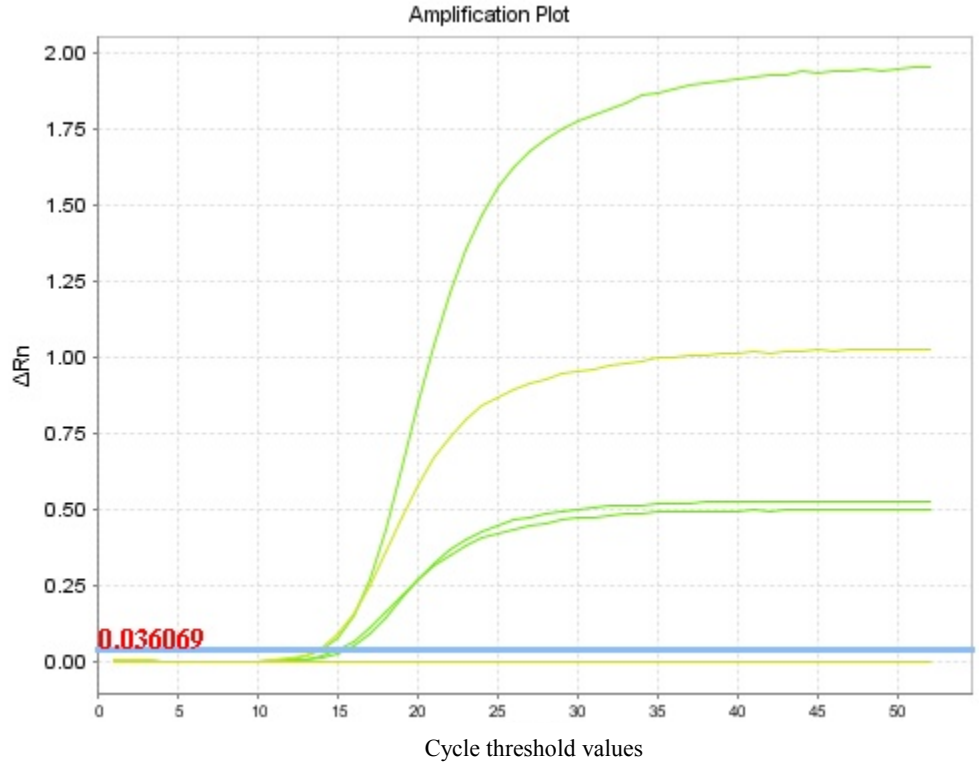
Figure 12: **Amplification plots of (NC and 370 samples) screened with FMDV: Serotype A-specific primers**

4.1.4 Results of optimization of one-step serotype SAT 1-specific real-time RT-PCR assay for typing FMD Virus

Under standardized/optimum thermal cycling conditions, samples from FMD-cases were confirmed to have serotype SAT 1 infection by one-step serotype SAT 1-specific real-time RT-PCR assay. These samples had Ct value ranging from 13.735 to 15.455 as shown in (Table 7 and amplification plot (Fig.13) below.

Table 7: Results of optimization of one-step serotype SAT 1- specific real-time RT-PCR assay

Sample IN	Town/ Village	Region	Sample collection date	Ct values	FMD status
NC	Bigwa	Morogoro	3/11/2014	Undetermined	Negative
3601	Kimara,Ilala	Dar-es-salaam	2/10/2012	13.735	Positive
3602	Kimara,Ilala	Dar-es-salaam	2/11/2012	14.032	Positive
360	Kimara,Ilala	Dar-es-salaam	2/12/2012	14.948	Positive
3612	Kimara,Ilala	Dar-es-salaam	2/13/2012	15.454	Positive



Legend

ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

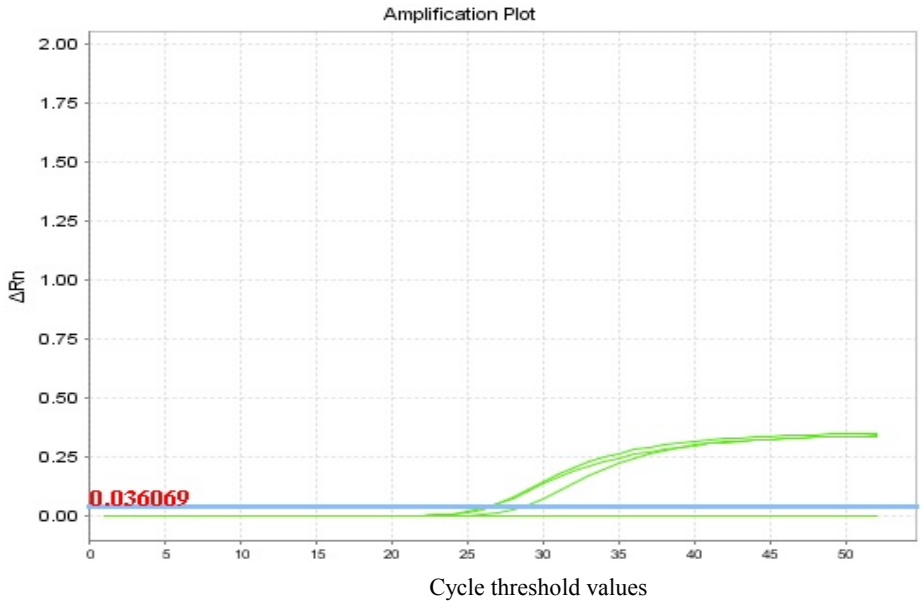
Figure 13: Amplification plots of samples screened with FMDV: Serotype SAT 1-specific primers

4.1.5 Results of optimization of one-step serotype SAT 2-specific real-time RT-PCR assay for typing FMDV

26.418, 28.467 and 26.285 were the Ct values of 68, 77 and 126 samples respectively which were confirmed to have FMD-infection by one-step serotype SAT 2-specific real-time RT-PCR assay as shown in (Table 8) and amplification plot (Fig. 14).

Table 8: Results of optimization of one-step serotype SAT 2-specific real-time RT-PCR assay

Sample IN	Town/ Village	Region	Sample collection date	Ct values	FMD status
NC	Bigwa	Morogoro	3/11/2014	Undetermined	Negative
68	Masasi	Mtwara	19/12/2011	26.418	Positive
77		Morogoro	19/03/2012	28.467	Positive
126		Tanga	21/09/2011	26.285	Positive



Legend

ΔRn = Fluorescence color emission of amplicon at each time - fluorescence color emission of the baseline

Figure 14: Amplification plots samples screened with FMDV: Serotype SAT 2-specific primers

4.2 Sensitivity and Specificity of One-Step Real-Time RT-PCR Assay for Pan-Serotypic Detection and Typing of FMDV

After optimization of serotype-specific real-time one-step RT-PCR assay, sensitivity and specificity of Serotype O-, A-, SAT1- , SAT2-, pan-serotypic (3D gene)-specific primers were (100%, 100%, 100%, 100% 100%) and (100%, 100%, 100%, 100%, 100%) respectively as computed below by using data in (Tables 9-13).

(i) Sensitivity and specificity of one-step 3D-specific real-time RT-PCR assay for pan-serotypic detection of FMD Virus

According to Table 9 below, True positive sample, True negative sample, False positive sample and False negative sample were 14, 1, 0 and 0, respectively.

Sensitivity = (14/14) × 100% = 100%

Specificity = (1/1) × 100% = 100%

Table 9: Two-by-two table showing FMD status and results when samples were tested by one-step 3D-specific real-time RT-PCR assay

	Foot-and -mouth disease		
Diagnostic test	**Present**	**Absent**	**Total**
Positive	14	0	14
Negative	0	1	1
Total	**14**	**1**	**15**

(ii) Sensitivity and specificity of one-step serotype O-specific real-time RT-PCR assay for typing FMD Virus

According to Table 10 below, True positive sample, True negative sample, False positive sample and False negative sample were 5, 1, 0 and 0, respectively.

Sensitivity = (5/5) × 100% = 100%

Specificity = (1/1) × 100% = 100%

Table 10: Two-by-two table showing FMD status and test results when FMD diagnosis was done by using serotype O-specific real-time RT-PCR assay

	Foot-and -mouth disease		
Diagnostic test	**Present**	**Absent**	**Total**
Positive	5	0	5
Negative	0	1	1
Total	**5**	**1**	**6**

(iii) Sensitivity and specificity of serotype A-specific one-step real-time RT-PCR assay for typing FMD Virus

According to Table 11 below, True positive sample, True negative sample, False positive sample and False negative sample were 2, 1, 0 and 0, respectively.

Sensitivity = (2/2) × 100% = 100%

Specificity = (1/1) × 100% = 100%

Table 11: Two-by-two table showing FMD status and test results when FMD diagnosis was done by using serotype A-specific real-time RT-PCR assay

	Foot-and -mouth disease		
Diagnostic test	**Present**	**Absent**	**Total**
Positive	2	0	2
Negative	0	1	1
Total	**2**	**1**	**3**

(iv) Sensitivity and specificity of serotype SAT 1-specific one-step real-time RT-PCR assay for typing FMD Virus

According to Table 12 below, True positive sample, True negative sample, False positive sample and False negative sample were 4, 1, 0 and 0 respectively.

Sensitivity = (4/4) × 100% = 100%

Specificity = (1/1) × 100% = 100%

Table 12: Two-by-two table showing FMD status and test results when FMD diagnosis was done by using serotype SAT 1-specific real-time RT-PCR assay

	Foot-and -mouth disease		
Diagnostic test	**Present**	**Absent**	**Total**
Positive	4	0	4
Negative	0	1	1
Total	**4**	**1**	**5**

(v) Sensitivity and specificity of serotype SAT 2-specific one-step real-time RT-PCR assay for typing FMD Virus

According to Table 13 below, True positive sample, True negative sample, False positive sample and False negative sample were 3, 1, 0 and 0, respectively.

Sensitivity = (3/3) × 100% = 100%

Specificity = (1/1) × 100% = 100%

Table 13: Two-by-two table showing FMD status and test results when FMD diagnosis was done by using serotype SAT 2-specific real-time RT-PCR assay

	Foot-and -mouth disease		
Diagnostic test	**Present**	**Absent**	**Total**
Positive	3	0	3
Negative	0	1	1
Total	**3**	**1**	**4**

4.3 Results of Application of Serotype-Specific Real-Time One-Step RT-PCR Assay for Detection and Typing Of FMD Virus

Each of 30 samples was screened for presence of FMDV by using five distinct primers and oligonucleotide probes by one-step real-time RT-PCR assay. 3D gene- specific primer was used for pan-serotypic detection of FMD and four distinct serotypes of FMDV

(O, A, SAT 1 and SAT 2) were typed by using four distinct pairs of serotype O-, A-, SAT 1- and SAT 2-specific primers respectively at same thermal cycling conditions at the same time.

Among 25 FMD-suspected cases 92% (n = 23) were confirmed to have FMD infection by one-step real-time RT-PCR assay. With exception of FMD positive controls (Vaccine strain serotype O, IPC-A, IPC-SAT 1 and IPC-SAT 2) 13, 2, 5, and 2 cases had infection from FMDV serotypes O, A, SAT 1 and SAT 2 respectively (Appendix 1).

Only 2 animals (with IN 29 and 179) were negative for FMD and 1 sample (with IN 374) was detected positive for both FMDV serotypes SAT 1 and A genomes.

CHAPTER FIVE

5.0 DISCUSSION

Different real-time RT-PCR assays are currently developed and optimized for diagnosis and discrimination of FMD viruses in different parts of the world. Because of enormous genetic and antigenic diversity among FMD viruses in different geographical regions, optimization of one-step real-time RT-PCR assay in this study was essential for diagnosis of FMD in selected regions of Tanzania.

In this study, the optimized one-step real-time RT-qPCR assay could detect and type FMD viruses into serotypes O, A, SAT 1, and SAT 2 with 100% sensitivity and 100% specificity at same thermal cycling conditions, which were: reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10minutes followed by 95°C for 15seconds), and annealing and elongation temperature (at 60°C for 1minute) with a total of 52 cycles. These conditions could be deployed for rapid detection and specific typing of FMDV into serotypes in endemic settings of Tanzania and neighboring countries.

Prevalence of FMD in cattle from 2008 to 2014 inKagera, Rukwa, Morogoro, Mbeya, Tanga, Dar-es-salaam, Tabora, Mwanza, Kigoma, Mtwara and Zanzibar was 92% (23/25) (Appendix 1).

The one-step serotype-specific real-time RT-qPCR was able to discriminate FMD viruses, which tested positive by the pan-serotypic one-step real-time RT-qPCR assay, into serotypes O, A, SAT 1 and SAT 2. The ability of this assay to discriminate viruses into four serotypes indicate that the assay is highly specific and could be used for rapid typing

of circulating field viruses which is necessary for appropriate recommendation of candidate vaccine strains that may be required for rational control of FMD in the region. Moreover, the application of this assay confirmed further that, two samples with IN 29 and 179 had no FMD-infection (Appendix 1).

The findings of serotype-specific RT-qPCR in the current study revealed that, one cattle had mixed infection of FMDV serotypes SAT 1 and A. Similar findings of mixed infection from distinct serotypes of FMDV were reported by Reid *et al.* (2014) who employed a tailored real-time RT-PCR assay for detection and typing of serotypes O, A and Asia 1 in Middle East. Sample with (O/PAK/4/2006) identification number had dual infections from (serotypes: O and A) with (28.33 and 31.55) Ct values respectively (Reid *et al.,* 2014).

Repeated cycles of infection from different FMDV serotypes at different duration in conjunction with the ability of such serotypes to persistently establish infection within same host for longer periods of time is one reason for multiple infections from different FMDV serotypes under natural field conditions. The current and previous findings demonstrate application of serotype-specific real-time RT-qPCR in discrimination of field FMDV strains in endemic settings of Africa, and reveal the potential use of the assay for identification of mixed FMDV infection, which is a rare scenario in cattle.

The possibility of detection of both serotypes in the same animal could also be ascribed to the design of oligonucleotide primers and/or probes in such a way that the degree of degeneracy is high leading to decreased specificity which increases chances for cross reactivity. This scenario was evident in this study where one sample collected from cattle in Kagera, tested positive for serotypes A and SAT 1 (Appendix 1). The elucidation of

this scenario requires further studies including VP1 sequencing and/ or experimental infection of susceptible animals.

Recent study which was done to develop a probe-based real-time RT-qPCR assays for detection and characterization of FMDV in West Eurasian region proved that, the assay detected and discriminated FMDV (Serotypes O, A and Asia 1) with (100%, 92% and 100%) sensitivity and (100%, 100% and 100%) specificity respectively, without primers' cross-reactivity between heterotypic viruses (Jamal and Belsham, 2015). Sensitivity of probe-based real-time RT-qPCR which was developed by Jamal and Belsham (2015) for typing serotype A was 92%, in contrast to 100% sensitivity of one-step serotype A-specific real-time RT-PCR assay which was optimized in this study.

The real-time RT-qPCR assay which typed serotype A of FMDV in West region of Eurasia was less sensitive (92%) as compared to the assay which typed the same serotype in different regions of Tanzania, which revealed 100% sensitivity. The possible reason for this difference in sensitivity of assay in the two countries could be due to less efficient designing of serotype-A specific primers used in West Eurasia and/ or genetic variation of the targeted viruses in the field.

Despite existence of genetic and antigenic heterogeneity between FMD viruses from distinct geographical regions, all serotype O-specific real-time RT-qPCR assays typed FMDV with 100% sensitivity and 100% specificity in distinct geographical regions in West Eurasia and Tanzania. These observations indicate that, all primers were properly designed such that they could detect and discriminate serotype O FMD virus with a maximum sensitivity of 100%.

From the findings of this study, it is evident that standardization of every molecular diagnostic assay is important before being used for FMD diagnosis in particular geographical location. This is because evolution of FMD virus could lead to the emergence of new variants (strains/ topotypes) that might be genetically and antigenically distinct from their ancestors. Hence, periodic development and optimization of molecular diagnostic assays will enable successful detection and typing of new variants of FMD viruses before implementation of rational control measures against FMD in specific geographical regions.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The findings of this study have shown that one-step real-time RT-PCR assay which was deployed for diagnosis of FMD, could detect and discriminate FMDV field samples into serotypes at the same thermal cycling conditions. The optimum thermal cycling conditions were: reverse transcription (at 60°C for 30minutes), denaturation of reverse transcriptase and activation of DNA polymerase (at 95°C for 10minutes followed by 95°C for 15seconds), and annealing temperature (at 60°C for 1minute) with a total of 52 cycles. The optimized/ standardized one-step real-time RT-PCR assay could detect and type FMDV with sensitivity and specificity of 100% and 100%, respectively.

6.2 Recommendations

(i) It is recommended that one-step real-time RT-PCR assay should be used for detection of FMDV in Tanzania and neighboring countries for rapid diagnosis and implementation of rational tailored FMD control methods in the region.

(ii) Further in-depth analysis including sequencing of VP1 coding region of FMDV genome from samples with suspected co-infection (sample with IN 374) should be performed to elucidate a possibility of co-infection.

(iii) Routine/periodic development of new real-time RT-PCR assays and their subsequent optimization should be done continuously in Tanzania and neighboring countries because of the possibility emergence of new variants and subtypes of FMDV that could not be detected by the established assays. This could ensure rapid and specific diagnosis of FMDV which is a pre-requisite for vaccine development and implementation of rational FMD control strategy in the region.

(iv) Inadequate number of highly qualified research scientists (molecular epidemiologists, virologists, molecular biologists and vaccinologists) is a challenge to effective-control of Foot-and-mouth disease in Tanzania. Furthermore, high cost of real-time RT-qPCR machine is one of limitations to diagnosis of FMD in different regions of Tanzania. Despite its high sensitivity and specificity (100% and 100% respectively), the real-time RT-qPCR assay is not user-friendly. The machine is not portable and cannot be carried easily to the field and used for surveillance and diagnosis of FMD during outbreaks in endemic settings of Tanzania. For that reason, researchers incur transportation cost of FMD-samples from filed areas to laboratory centers. Moreover, lack of reliable electric power supply in different endemic settings of Tanzania is another obstacle to the use of the real-time RT-qPCR machine for diagnosis of FMD. The mentioned facts calls for conduction of further applied research for innovation of new molecular diagnostic tools which are user-friendly, rapid, cost-effective, highly specific and sensitive for quick surveillance and diagnosis of FMD in the field areas for subsequent on-time application of rational FMD-control measures. In addition, the government and non-governmental organizations should work collaboratively to train more research scientists on FMD for successive control in the near future.

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APPENDIX

Appendix 1: Detection and typing of FMDV in FMD-suspected samples from different geographic locations in Tanzania

				Cycle threshold (ct) values						
FMDV serotypes				**All FMD serotypes**	**Serotype O**	**Serotype A**	**Serotype SAT1**	**Serotype SAT2**		
Targeted genes				**3D**	**VP1**	**VP1**	**VP1**	**VP1**		
Sample IN	**Village/ Town**	**Region**	**Date of sample collection**						**FMD status**	**FMDV candidate**
IPC-A	Misenyi	Kagera	4/4/2013	19.477	38.256	20.385	Undetermined	Undetermined	Positive	A
376	Meleba	Kagera	4/4/2013	22.318	24.286	Undetermined	Undetermined	Undetermined	Positive	O
19	Sumbawanga	Rukwa	16/10/2010	24.571	25.714	Undetermined	Undetermined	39.024	Positive	O
4	Ngara	Kagera	2/12/2010	29.477	29.451	Undetermined	Undetermined	Undetermined	Positive	O
77		Morogoro	19/03/2012	24.23	36.415	37.198	32.901	24.917	Positive	SAT 2
321		Mbeya	31/05/2012	20.139	21.873	Undetermined	Undetermined	38.098	Positive	O
IPC-SAT2		Tanga	21/09/2011	22.84	Undetermined	Undetermined	Undetermined	23.044	Positive	SAT 2
IPC-SAT1	Kimara Ilala	Dar-es-Salaam	2/10/2012	15.647	39.419	Undetermined	11.952	37.915	Positive	SAT 1
357		Morogoro	31/08/2012	22.365	34.207	34.814	20.768	Undetermined	Positive	SAT 1
319		Mbeya	31/05/2012	24.473	25.022	36.847	Undetermined	38.21	Positive	O
37	Municipal Tabora	Tabora	21/09/2010	19.986	22.12	Undetermined	Undetermined	Undetermined	Positive	O
Vaccine strain (Kenya serotype O)				10.191	10.295	Undetermined	Undetermined	Undetermined	Positive	O
142	Mabuki	Mwanza	16/12/2010	15.78	24.633	Undetermined	Undetermined	37.939	Positive	O
179		Mwanza	2011	34.32	Undetermined	Undetermined	Undetermined	Undetermined	Negative	NONE
355	Kingolowila	Morogoro	31/8/2012	20.848	35.871	Undetermined	16.611	38.038	Positive	SAT 1
360	Kimara Ilala	Dar-es-salaam	15/12/2011	23.566	35.759	Undetermined	20.467	37.502	Positive	SAT 1
361	Kimara Ilala	Dar-es-salaam	21/09/2011	14.472	37.651	Undetermined	9.718	Undetermined	Positive	SAT 1
368		Kagera	2013	27.862	Undetermined	31.9	24.67	Undetermined	Positive	SAT 1
374	Karagwe	Kagera	2013	21.874	38.332	22.89	25.968	Undetermined	Positive	A, SAT 1
379	Muleba	Kagera	2013	23.577	Undetermined	25.178	Undetermined	Undetermined	Positive	A
3	Wasela	Tanga	12/8/2008	23.967	22.735	Undetermined	Undetermined	Undetermined	Positive	O
8	Mabuki	Mwanza	16/12/2010	22.069	31.59	Undetermined	Undetermined	Undetermined	Positive	O
29	Kasulu	Kigoma	13/11/2010	37.173	Undetermined	Undetermined	Undetermined	Undetermined	Negative	NONE
NC		Mwanza	5/12/2011	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Negative	NONE
45		Tabora	13/05/2009	23.658	30.341	33.555	Undetermined	Undetermined	Positive	O
48	Nzega	Tabora	2/9/2010	27.652	31.893	Undetermined	Undetermined	Undetermined	Positive	O
61	Sumbawanga	Rukwa	2010	27.373	27.733	Undetermined	Undetermined	Undetermined	Positive	O
68	Masasi	Mtwara	19/12/11	21.372	Undetermined	Undetermined	Undetermined	23.241	Positive	SAT 2
116	Unguja	Zanzibar	23/10/2010	25.18	28.661	Undetermined	Undetermined	34.798	Positive	O
Vaccine strain (Ethiopian serotype A)				17.99	Undetermined	23.616	Undetermined	Undetermined	Positive	A