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MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUSES IN CATTLE FROM OUTBREAKS OCCURRED IN DIFFERENT PARTS OF ETHIOPIA FROM OCTOBER, 2017 TO MAY, 2018

MVSc Thesis

BY

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JUNE, 2018

BISHOFTU, ETHIOPIA

MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUSES IN CATTLE FROM OUTBREAKS OCCURRED IN DIFFERENT PARTS OF ETHIOPIA FROM OCTOBER, 2017 TO MAY, 2018

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By

Metages Yirgalem Tindashe

JUNE, 2018

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Signature and Approval Sheet

Addis Ababa University

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Title: **Molecular characterization of foot and mouth disease viruses in cattle from outbreaks occurred in different parts of Ethiopia from October, 2017 to May, 2018.**

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First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

BHK	Baby Hamster Kidney
CFT	Complement Fixation Test
CPE	Cytopathic Effect
CSA	Central Statics Authority
DMEM	Dulbecco's Modified Eagle Medium
EA	East Africa
ELISA	Enzyme-Linked Immunosorbent Assay
ETH	Ethiopia
EURO-SA	Europe South America
FAM	Carboxyfluorescein
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
G	Genotype
GDP	Gross Domestic Product
H2O2	Hydrogen per Oxide
Ig	Immunoglobulin
KOH	Potassium Hydroxide
MAB	Monoclonal Antibody
MEGA	Molecular Evolutionary Genetics Analysis
MEM	Minimal Essential Media
MoLF	Minster of Livestock and Fishery
Na2CO3	Sodium Bicarbonate
NAHDIC	National Animal Health Diagnostic and Investigation center
NaOH	Sodium hydroxide
NSP	Non Structural Protein
NVI	National Veterinary Institute
OD	Optical Density
OIE	Office International Des Epizootics

LIST OF ABBREVIATIONS (*Continued*)

ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
poly (C)	Polyribocytidylic
RdRP	Ribo Nucleic Acid Dependent Ribo Nucleic Acid Polymerase
RI	Replicative Intermidiate
rmp	Revolution Per Minute
RNA	Ribo Nucleic Acid
rRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
SAT	South Africa Territories
SNNPRS	Southern Nation Nationality People Regional State
TAMRA	Tetramethylrhodamine
UK	United Kingdom
UTR	Untranslated Region
VNT	Virus Neutralization Test
VP	Virus protein
WA	West Africa
WRLFMD	World Reference Laboratory for Foot and Mouth Disease

ABSTRACT

A cross sectional study with purposive sampling was conducted with the aim of molecular characterization of foot and mouth disease virus in cattle from outbreaks occurred in different parts of Ethiopia from October, 2017 to May, 2018. Outbreaks were investigated and individual animals were clinically examined. Samples were collected for virus isolation, serotyping and molecular characterization. A total of 125 animals from different outbreak sites were examined clinically for the presence of the disease. Of which, 56 (44.8%) animals were manifested clinical signs and lesions suggestive of FMD. From 37 clinical samples collected during the study period, only two serotypes (O and A) were identified using antigen detection ELISA and in 15/22 (68.18%) samples virus were isolated on BHK-21 cell. FMDV genome was also detected in 12/ 27 (44.44%) samples by real time RT-PCR. A total of 18 representative samples from both serotypes were submitted to world reference laboratory for FMD (WRLFMD), Pirbright, UK for confirmation and molecular characterization based on viral protein 1 (VP1) sequencing. The result of phylogenetic analysis revealed that, the current isolates of serotype O belonged to East Africa topotype -3 (EA-3) and the serotype A was clustered in African topotype of genotype -I (G-I). In addition, serotype O isolate shared 96.6% nucleotide similarity with Sudan's isolates while serotype A shared >97% nucleotide similarity with Kenyan and Tanzanian isolates which are grouped previously under African topotype of genotype I which indicates the presence of unrestricted animal movement. G-I is reported for the first time in Ethiopia. A total of 10.8% amino acid variations were recorded when VP1 sequence of the vaccine strain (O/ETH/38/2005) was compared with serotype O isolate in this study. Most of the variations were observed at amino acid position 133-158 and 194-213, which is known to be the immunodominant region. Thus, to enhance control of FMD in Ethiopia, detailed molecular analysis of outbreaks coupled with *in-vitro* vaccine matching to determine protecting potential of the vaccine strain currently in use is recommended.

Key words: *Ethiopia, FMD, Serotype A and O FMD viruses, Molecular characterization, Outbreak investigation.*

1. INTRODUCTION

Ethiopia is one of the countries that possess a huge number of livestock populations in Africa continent estimated to be 56.5 million cattle, 30.7 million sheep and 30.2 million goats were found in the country (CSA, 2017). The livestock sector contributes about 16.5% of the national Gross Domestic Product (GDP) and 35.6% of the agricultural GDP. It also contributes 15% of export earnings and 30% of agricultural employment (Leta and Mesele, 2014). However, the productivity is still remains marginal mainly due to malnutrition, prevalent disease, poor genetic potential of local breed, management problems, and inefficiency of livestock development services with respect to credit, extension, marketing, and infrastructure (Leta and Mesele, 2014). The direct consequence of prevalent diseases are loss of production and productivity, hindrance to access the international animal and animal products' market, reduction in the quality of skin and hide, death of the animals, loss of weight and poor fertility performance. Among prevalent livestock diseases, Foot and Mouth Disease (FMD) is constantly deteriorating the health and productivity of cloven hoofed domestic and wild animal (Chibssa, 2006).

FMD is an important contagious viral disease caused by foot-and-mouth disease virus (FMDV). The FMDV is non-enveloped icosahedral virus grouped under the genus *Aphthovirus* in the family *Picornaviridae* and possesses positive-sense single strand RNA genome. The viral genome is about 8.3kb long and enclosed in capsid protein. The capsid comprises 60 copies each of the four structural viral proteins (VP1-VP4); the VP1-3 proteins are directly involved in antigenicity and exposed on viron surface, while VP4 is internal (Bari *et al*., 2014; Gao *et al*., 2016). Three of these proteins, VP1, VP2 and VP3, contribute to the formation of five antigenic sites in serotype O (Aggarwal and Barnett, 2002) and four known antigenic site in serotype A (Bari *et al*., 2014; Kitching, 2005). Generally, the virus has got seven immunogenicaly distinct serotypes, namely, O, A, C, SAT1, SAT2, SAT3 and Asia1 (Balemual, 2018; Knowles and Samuel, 2003; Maree *et al*., 2011; Sahle *et al*., 2004). Within each serotypes, many subtypes can be identified by molecular and immunological tools (Knowles and Samuel, 2003; OIE, 2009). This

genetic heterogeneity of the virus mainly occurred due error-prone replication which improves viral fitness by changing antigenically important sites of the virus that enabled them to escape from protection by the developed vaccine (Grazioli *et al*., 2013). In Ethiopia five of the seven serotypes (O, A, C, SAT1, SAT2) were reported in bovine, swine, ovine, and caprine samples collected from different outbreaks occurred from 1981-2007. From the report, serotype O was the most predominant strain circulating in the country (73.3%) followed by serotype A (19.5%), SAT 2 (4.1%) and SAT1 (1.8%) (Ayelet *et al*., 2009). Currently, East Africa topotype (EA-3) (Negussie *et al*., 2013; WRLFMD, 2016) and EA-4 (Urge, 2017; WRLFMD, 2016) of FMDV serotype O and Africa topotype (G-VII) (Negussie *et al*., 2013; WRLFMD, 2016) and Africa topotype (G-IV) (Sulayeman *et al*., 2018) of FMDV serotype A were circulating in the country.

FMD is widely distributed and has occurred in most parts of the world with the exception of North America, Western Europe and Australia. It's common in most of poorer and developing countries of South America, Asia, Middle East and it is highly endemic in most sub-Saharan African countries where six of the seven serotype occur that aggravates the existing farming socio-economic problems (Baluka *et al*., 2014; Nsamba, 2015). Studies undertaken in Ethiopia revealed that the disease is still endemic and occur in different parts of the country mainly due to lack of effective vaccine, absence of livestock movement control and absence of systematic disease surveillance and reliable epidemiological data (Chibssa, 2006). The overall sero-prevalence ranges from 5.6% to 42.7% in cattle and from 4% to 11% in small ruminant (Abdela, 2017) and it pose a major threat to cattle in many parts of the country due to the endemic strains as well as the antigenic variant prevailing from neighboring countries. This disease is the most important livestock disease in the country due to the considerable economic losses through morbidity and mortality of affected animals (Baluka *et al*., 2014; James and Rushton, 2002; Mattion *et al*., 2004). Additionally, it is also the major constraint hampering export of livestock and livestock product to the Middle East and African countries; the Egyptian trade ban of 2005, in which Ethiopia lost more than US$14 million, being a recent memory (Leforban, 2005).

Clinically the disease is characterized by ropy salivation, lameness, inappetance, loss of condition, body temperature over 40^0c, and the appearance of vesicles and erosion on their tongue, gum, dental pad, lips, interdigital space of feet and mammary gland due to infection of epithelial cells. Generally, it is mild in adult animals although severe in young animals due to myocarditis (Hailu *et al*., 2017). Unrestricted animal movements are an important mechanism by which FMD is spread from one place to the other place (Habiela *et al*., 2010).

Effective control and eradication of FMD mainly depends on accurately and timely diagnosis of the disease in endemic areas and in backing of stamping out policies in FMD free regions (Seoke, 2016). Reverse transcriptase polymerase chain reaction (RT-PCR) (Xu *et al*., 2013) was the most widely used techniques for an efficient and rapid diagnosis of FMD in parallel with conventional assays such as virus isolation, serology and virus neutralization test (Callens *et al*., 1998). This technique coupled with automated nucleotide sequencing and phylogenetic analysis, is used to achieve a detailed virus identification to trace the origin of FMDVs associated with new outbreaks (Domingo *et al*., 2003). Phylogenetic analysis of the VP1 region of FMD viruses has been used to study the genetic relationships between different FMD virus isolates, geographical distribution of lineages and genotypes, the establishment of geographically and genetically linked topotypes and tracing the source of virus during outbreaks (Sahle *et al*., 2004). Topotypes are geographically clustered viruses in a single genetic lineage and sharing >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3) nucleotide similarity in the VP1-coding region (Samuel and Knowles, 2001). VP1 nucleotide sequences are used for re-constructing the evolutionary history of organisms and molecular evolutionary studies (Sahle *et al*., 2004).

The current measures to control FMD include vaccination using matching vaccine, movement control and slaughter of infected or susceptible animals. However, antibody generated by infection or vaccination against one serotype fails to cross-protect against the other serotypes (Domingo *et al*., 2003). Consequently, vaccine strain requirements

differ according to the types and subtypes of virus and vaccines have to be selected with great care (Mumford, 2007).

Currently, in Ethiopia there is no government strategy in FMD control through vaccination and movement control except some activities to start FMD control in selected parts of the country. Lack of vaccination strategies (quality, coverage and timing) and presence of free animal movement without certification are thus the main factors that could increase the spread of FMD along the cattle market chain. Moreover, the presence of lack of veterinary infrastructure to handle outbreaks on large scale greatly contributes to the increasing occurrence of the disease (Ayelet *et al*., 2009). According to MoLF (2018) report, in 2015 and 2016 a total of 60 and 114 disease outbreaks were reported. The absence of molecular study on the virus causing the disease especially in the current study area also aggravates the problem. Hence, determining the circulating serotype, establishing the geographic relatedness between isolates and molecular evolution of the virus is needed for planning a proper control and preventive measure in the country.

Therefore, the objectives of this study were:

1.1. General objective

- To make a molecular characterization on foot and mouth disease virus circulating in different parts of Ethiopia.

1.2. Specific objectives

- To isolate the virus from clinical sample collected from different outbreaks.
- To determine the serotype of the virus that circulates in the study areas.
- To detect and characterize the virus at molecular level.
- To compare the genetic relationship of serotype O isolates in this study with the vaccine strain (O/ETH/38/2005) currently used for vaccine production.

2. LITERATURE REVIEW

2.1. Definition

FMD is one of highly contagious and economically important viral disease affecting *artiodactylae*, mostly cattle, swine, sheep, goats, and many species of wild ungulates (Longjam *et al*., 2011). It is one of the list A of infectious diseases of animals of the Office International des Epizooties (OIE, 2011) and has been recognized as the most important constraint to international trade in animals and animal products worldwide (Grubman and Baxt, 2004). The disease is characterized by fever, loss of appetite, profuse salivation, lamness and vesicular eruptions on the feet, mouth and mammary gland (Yoon *et al*., 2012).

2.2. Etiology

2.2.1. Taxonomy and classification of FMDV

FMDV is first demonstrated by Loeffler and Frosch in 1897 as a filterable agent causing FMD (Grubman and Baxt, 2004). This is grouped under *Picornavirus* family, derived from the prefix 'pico' which means small, plus 'RNA', which refers to the genome type, of the virus. Now a day, picornaviruses are divided into twelve genera, including aphthovirus which have two type species, Equine rhinitis A virus and FMDV. (Figure1). Picornaviruses also includes poliovirus (PV, an enterovirus), human rhinoviruses and encephalomyocarditis virus (EMCV, a cardiovirus) (Sallu, 2016). Generally, picornavirus particles are characterized by the presence of single-stranded positive-sense RNA with icosahedral symmetry, which is important pathogens in animals and human and are classified into twelve genera (Longjam *et al*., 2011; Nsamba, 2015).

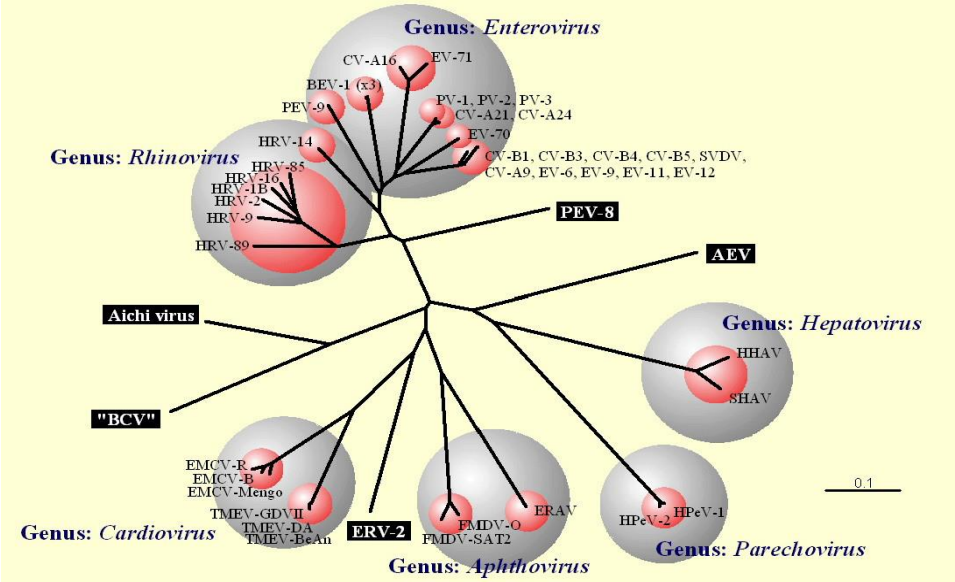

Figure 1: Taxonomy of Picornaviridae. Adapted from (Knowles *et al.*, 2008)

2.2.2. Physicochemical properties

FMDV genomes are enclosed by a non-enveloped protein shell (capsid) and that consists of polypeptides, which are devoid of lipoprotein and hence is stable to lipid solvents like ether and chloroform, alcohol, phenolic and quaternary ammonium disinfectants (Sahle *et al.*, 2004). However it is sensitive to pH and is inactivated when exposed to pH below 6.5 or above 11. Two percent NaOH or KOH and 4% Na_2CO_3 are also effective disinfectants for FMD contaminated objects; conversely, in milk and milk products, the virion is protected, and can survive at 70°C for 15 seconds and pH 4.6. The virus also can survive for long periods in meat. At temperatures below freezing, the virus is stable for a long period while exposure to 56°C for 30 minutes is sufficient to destroy most strains. On the contrary, sunlight has little or no direct effect on infectivity. The survival of the virus is also influenced by relative humidity with good survival above 60% relative humidity and speedy inactivation below 60% relative humidity (Geering and Lubroth, 2002).

2.2.3. Virus structure

The FMD virion appears to be a round particle with a smooth surface and a diameter of approximately 30 nm under electron microscope. The capsid is composed of 60 copies each of the four structural proteins, 1A (VP4), 1B (VP2), 1C (VP3), and 1D assembled in groups of increasing complexity (Carrillo *et al*., 2005; Knowles *et al*., 2016; Wang *et al*., 2015). The cores proteins, VP1 to VP3, consist of a highly conserved eight-stranded wedge-shaped β-barrel structures which fit together to form the majority of the capsid structure. However, the VP4 protein is buried within the capsid and has a myristyl group covalently attached to its N terminus. The strands of the β-barrels of VP1 to VP3 are connected by loops which form the outer surface of the virion (Belsham *et al*., 1991; Jackson *et al*., 2003). Unlike other picornaviruses, the FMDV capsid is easily dissociated at low pH (below 6.5). The reason for this instability is has been suggested that a His residues at the interface between VP2 and VP3 which become protonated at low pH could produce repulsive forces opening the capsid. This low pH induced instability of FMDV leads to differences in the mechanism of its uncoating upon infection of cells compared to that for other picornaviruses (Grubman and Baxt, 2004; Mason *et al*., 2003). The three dimensional arrangements of the structural proteins within the virion provide the antigenic sites that elicit responses to vaccination or infection. In addition, these structures mediate binding to cell receptors, entry of the genome into cells, and determine the stability of the capsid to environmental factors (Mason *et al*., 2003).

2.2.4. Genome organization

FMDV has a single-stranded positive sense RNA genome of approximately 8.5 kb in length and having sedimentation coefficient of 146S in sucrose gradient. The virus have a large single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (5′ UTR and 3′ UTR, respectively) (Du *et al*., 2007; Lau *et al*., 2008). The 5' UTR is usually long and contains of a short (S) fragment, a poly (C) tract, and a long fragment (5'LF-UTR), which contains three or four tandemly repeated pseudoknots (PKs), cis-Replicating Element (cre) and an internal ribosome entry site (IRES). The ORF encodes a large single

polyprotein that can be cleaved in to four functional structural proteins (VP4, VP2, VP3 and VP1) and 10 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C and 3D) usually by virally encoded proteases (Belsham *et al*., 1991; Yang *et al*., 2011). The VP1 is the most frequently studied protein due to its important role in virus attachment, protective immunity and serotype specificity, and nucleotide sequencing of this region has been extensively used for molecular epidemiology studies on FMD (Malirat *et al*., 2007). The 3A region has been implicated in virus virulence and host range, similar to the 3A proteins of other picornaviruses. The 3'UTR of about 90 residues with a poly (A) tail (35-100nt) at 3'-end is likely to be a site of interaction with viral and host proteins for RNA replication (Carrillo *et al*., 2005; Domingo *et al*., 2003).

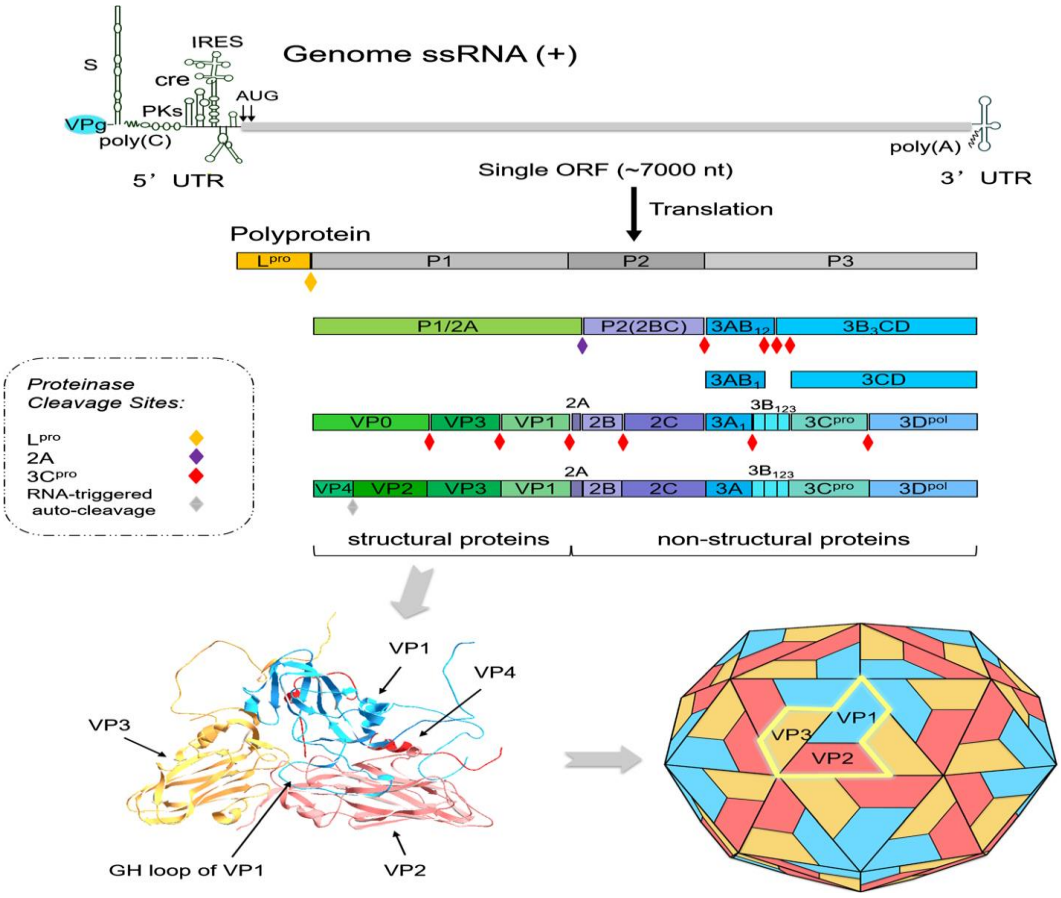

Figure 2: Schematic diagram of FMDV genome organization, processing of viral polypeptide and conformations of the structural proteins. Adapted from Gao et al. (2016).

2.2.5. Virus replication

FMDV initiates infection by binding to integrin receptors via an Arg-Gly-Asp (RGD) sequence found in the G-H loop of the structural protein VP1. Virions are then trafficked to endosomal compartments and the acidic P^H of endosomal membrane causes particle of the virus dissociates into pentamers and RNA is liberated into the cystol of the infected cell (Mateu *et al*., 1996). The incoming RNA uses the host cell protein-synthesizing machinery causing shut down of host cell replication. Thus, induction of viral RNA translation and cessation of cellular RNA translation occurs concurrently. The synthesis of cellular proteins is prevented by viral proteases that cleave cellular elongation factors, inhibiting CAP dependent translation. The viral proteins required for replication are immediately obtained from translation of the positive-sense viral RNA. These proteins also synthesize negative sense transcripts based on the positive-sense RNA template of viral genome. This reaction is catalyzed by the virally encoded RNA-dependent RNA polymerase enzyme (Carrillo, 2012). The negative-sense RNA then becomes the template used to synthesize *di novo* positive-sense viral genomes. Further synthesis of (+) RNA strands leads to the formation of multi-stranded replicative intermediates (RI) with a 3' poly [A] which are transcribed from the poly [U] tract in the RI. The RI generates a poo1 of (+) RNA for translation and some for synthesis of additional (-) RNA. As the protein level increases, some (+) RNA is packaged into virions. Proteolytic cleavages occur during shell assembly in a poly protein precursor of structural and non-structural proteins. Finally, complete virus particles are released by cell lysis (Racaniello, 2007).

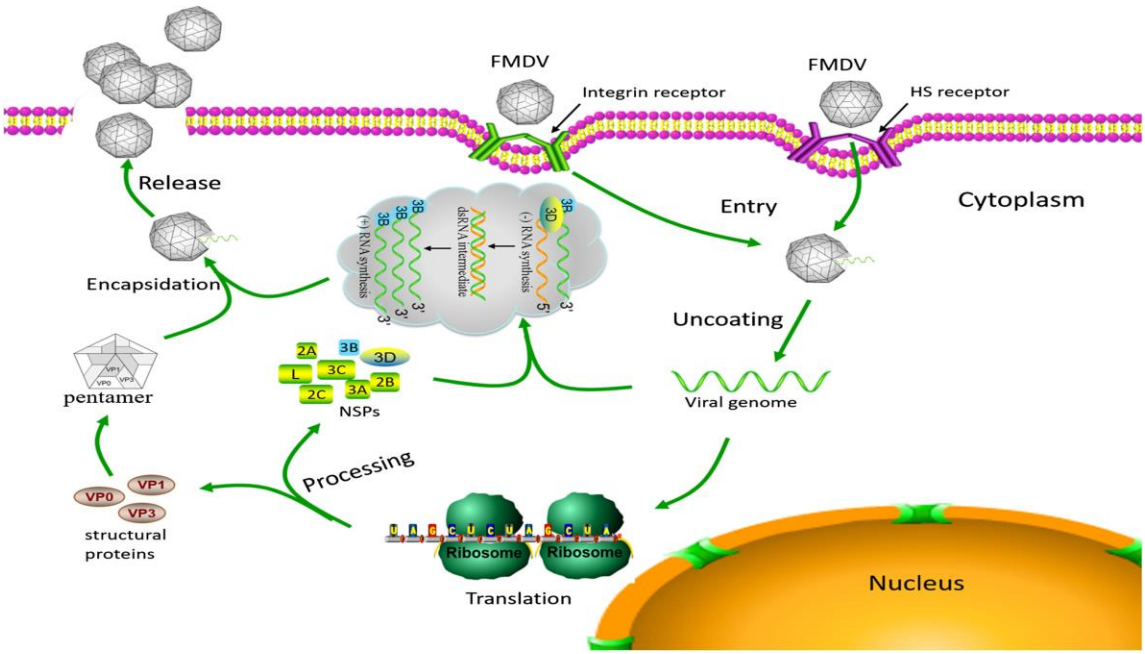

Figure 3: Lifecycle of FMDV in host cell. HS, heparin sulphate. Adapted from Gao et al. (2016).

2.2.6. Antigenic variation

Antigenic variation in FMDV leading to emergence of new variants is of great importance from epidemiological point of view and for formulating suitable control strategy. Evolvement of new subtypes and variation in FMDV occurs as consequence of mutation on the gene encoding the capsid protein which may give rise to immunological distinct variants that can re-infect individuals that have been previously infected by related viruses (Haydon *et al*., 2001). There is also increase in emergence of field variant due to antigenic variation over the time. Either the infected or vaccinated species of host may undergo immunologic pressure to generate antigenic variants. In addition, antigenic variation in FMDV has also been observed in tissue culture in the absence of immunologic pressure (Fares *et al*., 2001; Rudreshappa *et al*., 2012).

2.2.7. Genetic variation

The structure of FMDV and the organization and expression of its genome results on genetic variability and antigenic diversity, reflected in several serotypes and subtypes of the virus. Genetic variation in FMDV is of interest for two reasons. First, changes to the genes encoding capsid proteins results in antigenic variation, and affects vaccine efficiency and effectiveness of vaccination programs; second, genetic changes can lead to important insights into the transport of virus between countries, regions, herds, and individuals (Upadhayay and Ewam, 2012). The rise of new variants is inevitably caused by continued circulation of the virus in the field and the quasispecies nature of the RNA genome. It is a result of a two-step process. Firstly, the replication of viral RNA is error-prone due to the absence of proofreading in the 3D-encoded RNA dependent RNA polymerase. Secondly, competitive selection is continuously acting on the genome. Thus, those mutants with a selective advantage in the prevailing environment will be better represented than those with a selective disadvantage (Sahle *et al*., 2004). Genetic variation arise may be due to the following factors.

2.2.7.1. Mutation

Lack of proofreading and error prone activity of polymerase enzyme during viral replication, predispose RNA virus to higher rate of mutation such as FMDV (Ferrer-Orta *et al*., 2007). This virus have very high mutation rates, in the range of 10^{-3} to 10^{-5} per nucleotide site per genome replication, due to the lack of proofreading-repair activity of RNA polymerase during RNA replication. This high error rate leads to differences of FMDV replicated genomes from the original parental genome of 0.1 to 10 base positions, and populations that consist of genetically related but non-identical viruses known as *quasispecies*, i.e. pools of variant genomes statistically defined but individually indeterminate (Grubman and Baxt, 2004; Haydon *et al*., 2001). It is also estimated that a mutation rate of up to 10^{-8} to 10^{-9} nucleotide substitution per year during an epizootiological cycle of FMD viruses can occur. Therefore, new variants of FMD viruses are continuously arising after each replication cycle, which constitute an intratypic population of FMD viruses with different degrees of genetic relationships The

changes in the nucleotide compositions of the capsid genes are responsible for the antigenic variability of the virus (Sangare, 2005).

2.2.7.2. Recombination

Genetic recombination in RNA viruses involves the exchange of genetic material between two non-segmented RNA genomes resulting from polymerase 'jumping' during RNA synthesis. It has been shown that genetic recombination occurs between viruses of the same serotype as well as between serotypes (Chibssa, 2006). However, intratypic recombination occurs more frequently than intertypic recombination and the events in FMDV occur more readily in the 3' half of the genome, than in the capsid region of the virus. Evidence of recombination has been found in FMDV, although its direct impact on the diversity of a viral swarm has not been investigated (Heath *et al*., 2006). Mutations through recombination could result in the exchange of genetic material that could lead to the generation of new antigenic variants that may escape immune pressure. Therefore, recombination is an important factor in the creation of genetic diversity (Mwanandota, 2013).

2.2.7.3. Selective pressure

Selection is one of the other evolutionary mechanisms employed by RNA viruses. The immune system of an infected animal, which presumably provides a powerful selective force, is another driving force in viral evolution (Lewis-Rogers *et al*., 2008). Generally, the RdRP introduces mutations; selective pressures determine which mutations out-compete others and thus remain in the population in higher number. Therefore, it has an effect on genetic diversity from an environmental to cellular level. Viral success is dependent upon a virus’s ability to leave a host, its transmission capability, environmental stability, entry into a host, evasion of host immune system, entry into a cell and within cell replication efficiency. All of these selective pressures contribute towards the genetic diversity that persists within a viral population (Logan, 2017).

2.2.7.4. Population size

Genetic diversity also affected by Population size and replication speed. A greater population size, higher replication rate and a shorter generation times are responsible for higher mutation rate and greater overall genetic diversity in RNA viral population (Hague and Routman, 2016). Large population size allows for large numbers of variants to be present however a bottleneck transmission event can result in the fixation of a mutation and its propagation in the virus population regardless of its prevalence in the original large population. A negative correlation has been shown between the rate of nucleotide substitution and the size of genome regardless of population size (Holmes, 2003). The population size of FMDVs is large which is responsible for high antigenic variability together with continuous circulation of the field virus and plasticity of the major neutralizing sites on surface of the virion. They give rise to serious problems in spite of availability of good inactivated vaccine (Verma *et al*., 2010).

2.2.8. Antigenic site

The three structural proteins namely, VP1, VP2 and VP3 are responsible for the formation of five known antigenic sites of FMDV. However, each serotype may not contain all this sites. Interestingly, three of the sites are located within the flexible loops which connect the β-sheets of the viral proteins on VP1. The other sites are located on VP2 and VP3. All of the sites are appear to be necessary for a complete immunologic response to infection or vaccination. The major, immune-dominant antigenic site (Thomas *et al*., 1988), to which most of the immune response is directed and which is common to all of the serotypes, is located within the G-H loop of VP1. A structure for this loop was obtained by crystallographic analysis of chemically modified FMDV, and it consists of a short β-strand which precedes an Arg-Gly-Asp (RGD) which adopts an open-turn conformation, followed by a short helical region at the carboxy side of the RGD (Grubman and Baxt, 2004). The critical residues of site 1 is located at the position of 144, 148,154 and 208 (Oem *et al*., 2005). This site is composed of multiple, overlapping and continuous epitopes located at an exposed and mobile loop, the G-H loop of capsid protein VP1. Peptides representing this site were promising candidates for

a synthetic vaccine against FMD (Domingo *et al*., 2003). Amino acids at positions 31, 70-73, 75 and 77 of VP2 contribute to site 2. Site 3 is formed in part by residues at positions 43, 44 and 45 of the B-C loop of VP1. Only one critical residue, at position 58 of VP3, has so far been identified for site 4. The fifth site, formed at an amino acid position 149 of VP1, is probably formed by interaction of the VP1 loop region with other surface amino acids. Site 1 is linear and trypsin sensitive, whereas all other identified sites are conformational and trypsin-resistant (Oem *et al*., 2005). Antigenic site 2 of serotype O and site 3 of A10 are contained in the βB–βC, βE–βF and βH–βI loops of 1B while βB–βC corresponds to D2 of serotype C. The βB–βC loop of 1C compares to site 3 of A10 while βB–βC, βG–βH, βH–βI loops and C-terminal of 1D match epitopes identified for serotypes A, C and O. Antigenic site 1, which is found within the loop connecting G and H beta sheets is the immunodominant region of the virus (Seoke, 2016).

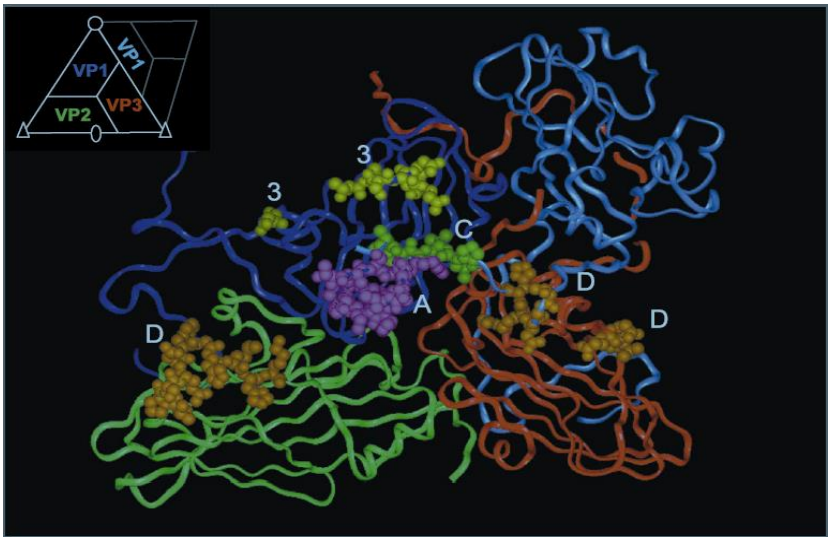

Figure 4: Crystallographic protomer of FMDV and location of antigenic sites. VP1, VP2, and VP3 location is shown schematically in the upper left. Site A corresponds to the mobile G-H loop of VP1 and it includes the RGD integrin-recognition triplet. Site C maps at the C-terminus of VP1. Site D involves the BC loop of VP2, the BB knobs of VP3 and the C-terminus of VP1. Adapted from Domingo *et al*. (2003).

2.2.9. Receptor specificity

Initiation of any infection requires the attachment and entry of potent infectious agent and these are governed by certain factors present at the site of predilection in target organ and most important among them are the cell surface receptors which specifically bind to the pathogen. FMDV enters to the cell by receptor-mediated endocytosis, in a process that begins with the initial attachment of the virus to cell-surface receptors (Ruiz-Saenz *et al*., 2009; Tang *et al*., 2012). Integrins and heparan sulphate proteoglycan are two classes of receptors known to which FMDV binds. By attaching VP1 RGD loop (on the viral capsid) to host integrins, FMDV initiates infection. Six types of integrins viz., αvβ1, αvβ2, αvβ3, αvβ4, αvβ5, and αvβ6 are found on the VP1 capsid protein of FMDV due to their ability of binding to the RGD amino acid found on the VP1 capsid protein which is highly conserved in nature. However, integrin αvβ6 is expected to be the principal receptor binding molecule for FMDV. Receptor(s) are considered as important host range determinants though with little evidence in support of it. The sequence of the capsid protein is also responsible for determination of infectivity of the virus in cells that are cultured (Chakraborty *et al*., 2014; Jackson *et al*., 2002).

2.2.10. Serotype and subtype

Currently FMDV were grouped in to seven immunologically distinct serotypes namely, O, A, C, Asia 1 and SAT (Southern African Territories) 1-3 based on the antigenicity of the capsid coating proteins (Ding *et al*., 2013). By using biochemical and immunological test over 60 subtypes have also been described within that serotype; and new subtypes occasionally arise spontaneously. All the FMDV serotypes are clustered into genetic lineages distinctly with about 30–50% differences in the VP1 coding gene (capsid region genes) (Xu *et al*., 2013). Infection with one serotype is fully susceptible with another due to lack of cross-protection. Antigenic diversity due to change in capsid protein led to variation in cross-productivity particularly evident within the serotype A. Vaccines prepared from a single strain of serotype A virus may not provide immunity against other strains (Chakraborty *et al*., 2014).

2.2.10.1. Specific characteristics of FMDV serotype

FMDV type O

FMDV serotype O is the most common serotype worldwide and has been studied most extensively (Klein, 2009). Recently, it has eight topotype (Samuel and Knowles, 2001) namely Cathay, Middle East-South Asia (ME-SA), South-East Asia (SEA), Europe-South America (Euro- SA), Indonesia-1 (ISA-1), Indonesia-2 (ISA-2), East Africa (EA) and West Africa (WA). Two of these topotypes are thought to be extinct (ISA-1 and ISA-2) and have not, so far, been detected outside Indonesia (Samuel and Knowles, 2001). From 1990 to 2013, EA-1, EA-2, EA-3, EA-4, ME-SA and WA topotype were circulating in Africa. From these topotype EA-3 and EA-4 were identified in Ethiopia in different time (Tekleghiorghis *et al*., 2016). This serotype also has five neutralizing antigenic sites on the surface. The most prominent surface projection is formed by surface exposed the G–H loop of VP1 and the C terminus of VP1 that contributes to antigenic site 1 with critical residues at positions 144, 148, 154 and 208 (Aktas and Samuel, 2000).

FMDV type A

Foot-and-mouth disease type A viruses have always been considered to be antigenically the most diverse of the Eurasian serotypes and has been a constant and frequent appearance of antigenically novel strains, particularly in western Asia with lack of cross-protection between them (Klein, 2009). There is also evidence that recombination in serotype A occurs much more than in the other serotypes (Jackson *et al*., 2007; Simmonds, 2006). In serotype A, four antigenic sites have been reported which are found to be in similar positions to that of serotype O except antigenic site 3. Two major antigenic sites were reported on VP1 (residues 140-160) coupled with two minor antigenic sites on VP1 (residue 169) and C terminus of VP1. Antigenic site 2 is found in VP2 at residue positions 72 and 79 (Kitching, 2005).

FMDV type SATs

The SAT (Southern African Territories) serotypes are usually found in Africa, but there have also been sporadic outbreaks in Saudi Arabia and Kuwait in 2000 due to movement of animal from Africa to Asia (Aidaros, 2002). There is a higher sequence variation within each of the three SAT types than in serotype O (Bastos *et al*., 2003).

For SAT-1 eight topotypes were identified throughout the region of which most had localized geographic distribution. However, SAT-2 showed higher genetic diversity with a total of 14 topotypes with five of these possibly extinct (Vosloo *et al*., 2004). Although SAT-3 has the most restricted distribution and is the type least frequently recovered from African buffalo, 6 topotypes were found with 25 genotypes of which four occurred in southern Africa and two were unique to East Africa (Bastos *et al*., 2003). In SAT2 there are two antigenic sites located in the G-H loop of VP1, downstream of the RGD motif, at residues 147, 148, 156, and 158 and residue 154 (Grazioli *et al*., 2013).

2.3. Epidemiology of the disease

2.3.1. Global distribution

FMD is one of extensively distributed disease worldwide. The disease is common in most developing nations of South America, Asia, Middle East and sub-Saharan African countries. Among 178 member states of World Organization for Animal Health, only 66 countries are FMD free (65 without vaccination, 1 with vaccination), 10 countries have FMD free zones. North America, Majority of South America, Western Europe, Australia, New Zealand and most Island countries in pacific are free of the disease (Depa *et al*., 2012). The seven serotypes of the disease are not equally distributed around the world. Types O and A have the broadest distribution and are continuously circulating in many parts of FMD endemic countries like Africa, southern Asia, the Far East (Gorna *et al*., 2014) and South America. Type C appears greatly in the Indian sub-continent and Asia 1 normally only occurs in southern Asia. In Africa all the different serotypes of the virus

are present except Asia1. The SAT serotypes are normally confined to sub-Saharan Africa (Saeed *et al*., 2015).

The global FMDV population can be roughly divided into seven regional pools. Pool 1 covers south-east Asia with spillover into eastern Asia. Pool 2 represents southern Asia. Pool 3 covers Euro-Asia (including the Middle East). In these three pool serotype O, A and Asia 1 are circulating virus. Pools 4, 5 and 6 cover eastern, western and southern Africa respectively. In pool 4 serotype O, A and SAT 1, 2 and 3 are circulating. In pool 5 serotype O, A, SAT 1 and 2 and in pool 6, only the SATs are tends to circulate. Pool 7 covers South America and has only type A and type O circulating (Logan, 2017). Ethiopia is grouped under pool 4 and currently serotype O, A, SAT1 and SAT2 are circulating in the country (Sulayeman *et al*., 2018)

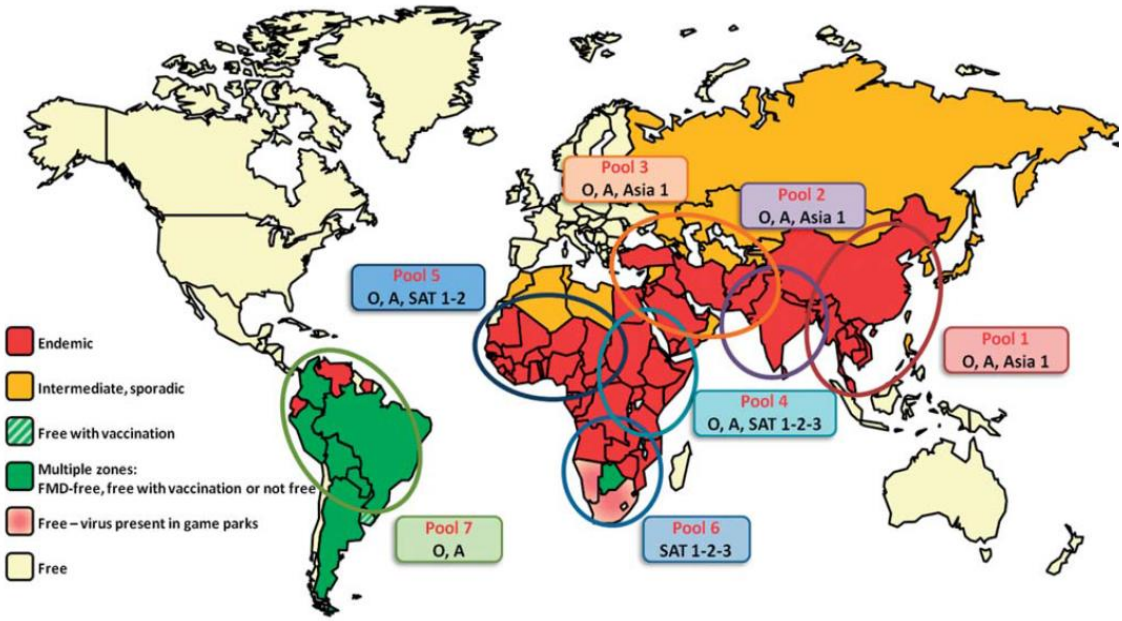

Figure 5: Global status foot and mouth disease and its regional virus pools and predominant virus serotypes. Adapted from Di Nardo *et al*. (2011).

2.3.2. Long distance trans-pool viral movements

The OIE/FAO FMD Laboratory Network has recently detected a number of viral lineages that have emerged from their established endemic pools to cause field outbreaks in

geographically distant locations. There is probably no single factor that underpins these changes, but these dynamic transboundary patterns are probably influenced by the migration of people in North Africa and the Middle East due to the escalation of regional political crises, as well as new trading patterns and demand for animal protein that arise due to increased prosperity in East Asian countries. These unexpected outbreaks caused by emerging viral lineages reinforce the importance of surveillance activities undertaken by the Network (WRLFMD, 2016).

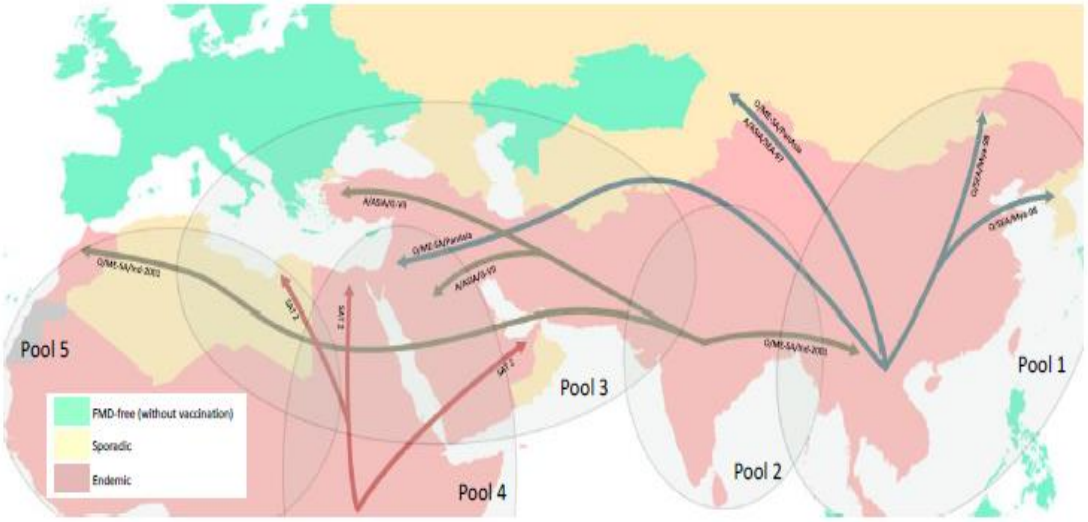

Figure 6: Long distance FMD virus movements within Asia and Africa (2009-2016). The different coloured arrows represent viruses from sub-Saharan Africa (red), Indian sub-continent (brown), and Southeast/East Asia (blue) that have moved into new geographical locations outside of the endemic pools (represented by shaded ovals). Adapted from WRLFMD (2016).

2.3.3. Distribution of FMD in sub-Saharan Africa

FMD is an endemic disease in most parts of sub-Saharan Africa countries. Its epidemiology is complicated by many factors. Not only six of the seven serotypes (i.e. all but Asia 1) have been recorded in the African continent but also marked regional differences in the distribution and prevalence of serotypes and intra-typic variants. In addition, uncontrolled movement of domestic and wild animal, and the presence of large numbers of persistently infected African buffaloes and other wild animal plays a great

role in epidemiology of the disease in the region (Tekleghiorghis *et al*., 2016). Trans-boundary live animal movements are part of the main characteristics of husbandry systems in many African regions; drought conditions, refugee movements and frequent cross-border trade have probably increased the risk of the entry and spread of FMD. In most pastoral areas of the continent livestock trade is active across border areas, dynamic and predominantly driven by price differential, and hence, livestock movement (both formal and informal) plays an important role in the spread and epidemiology of FMD (Sahle *et al*., 2004; Vosloo *et al*., 2002).

Regardless of the disease endemicity in nearly all countries of sub-Saharan Africa, the majority of outbreaks remains unrecorded and is not notified in a timely fashion due to trade restrictions and pastoral systems where inadequate in disease surveillance and sometimes absent; the transport of sampling material is difficult and expensive, few African laboratories are able to confirm the diagnosis of FMD (Rweyemamu *et al*., 2008). Due to underreporting of FMD outbreaks in sub-Saharan Africa there are major gaps in knowledge of currently circulating strains (Brito *et al*., 2015).

Serotypes O, A, and the South African Territories (SAT) FMDVs are the circulating serotype in the continent and serotype O is the most widely distributed in eastern and western Africa followed by A, whereas SAT viruses are mostly found in sub-Saharan Africa (Brito *et al*., 2015). Even though SAT viruses are restricted to sub-saharan Africa, some incursions of SAT1 have been reported in Greece while both SAT1 and SAT2 have occurred in the Middle East (Grubman and Baxt, 2004; Knowles and Samuel, 2003; Rweyemamu *et al*., 2008). FMDV serotype C isolate was lastly reported from Kenya in 2004 from cattle the isolate being suggested as re-introduction of the vaccine strain into the field (Roeder and Knowles, 2008).

Generally, Africa has been divided into 3 FMDV pools: East Africa (pool 4) with serotypes O, A, SAT-1, SAT-2 and SAT-3; West Africa (pool 5) with serotypes O, A, SAT-1 and SAT-2; and southern Africa (pool 6) with serotypes SAT-1, SAT-2 and SAT-3 (Paton *et al*., 2009). Six topotypes have been identified for serotype O, two for serotype

A, three for C and nine, fourteen and five topotypes for SAT-1, SAT-2 and SAT-3 respectively (Di Nardo *et al*., 2011). Co-occurrence of virus serotypes and topotypes occurs in Africa especially in the two pools (pool 4 and pool 5), for example, isolates of serotype O (topotype EA-3) from Niger (2007) and from Nigeria (Ayelet *et al*., 2009) in West Africa (pool 5) were genetically related to the serotype and topotype virus found in Eritrea (2004 and 2011), in Ethiopia (2005, 2006, 2008 and 2010-2012) and in Sudan (2005, and 2008-2011) in East Africa. In addition, for serotype SAT-2 topotype VII, similar isolates were found in the two pools in the East and West African countries (Tekleghiorghis *et al*., 2016). Majority of the disease-free areas are in Southern Africa (like parts of Botswana, Namibia and South Africa), where FMD control strategies such as cordon fencing and vaccination are implemented (Tekleghiorghis *et al*., 2016; Vosloo *et al*., 2006). But in other Africa countries, there are lack animal movement control measures and only a few practice preventative vaccinations required for international trade of animals and animal products (Brito *et al*., 2015) and this exacerbates the situation. As a result, it is practically impossible to eradicate FMD in sub-Saharan Africa unlike in North America and Western Europe because of the ever-present threat of the reservoir host, the African buffalo which transmits the disease to domestic animals, lack of animal movement control and few practices of vaccination program (Seoke, 2016).

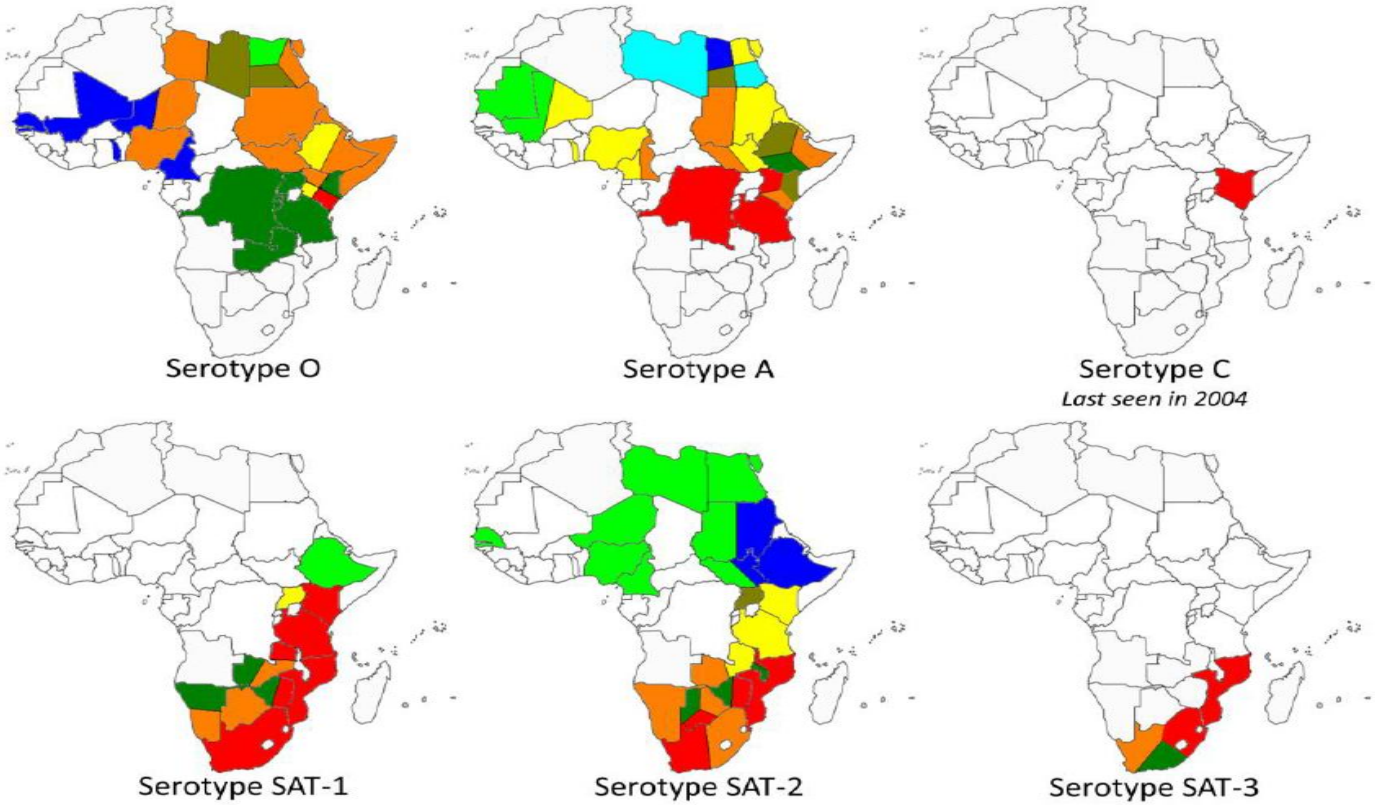

Figure 7: Map with genotyped isolates where the different topotypes and genotypes reported from 2003 to 2013 are shown in different colors. If more than one topotype and/or genotypes were present in a country, it was divided into more than one section, without taking the location of the isolate into account. Adapted from Tekleghiorghis *et al*. (2016).

For type O the topotypes and genotypes were: ● EA-1 ● EA-2 ● EA-3 ● EA-4 ● ME-SA Sharquia-72 ● ME-SA PanAsia-2 ● WA; **for type A the topotypes and genotypes were:** ● AFRICA G-I ● AFRICA G-II ● AFRICA G-III ● AFRICA G-IV ● AFRICA G-VI ● AFRICA G-VII ● AFRICA G-VII ● ASIA Iran-05^{BAR-08}**For type C the topotypes and genotypes were:** ● AFRICA (I) Ken-67; **for type SAT-1 the topotypes and genotypes were:** ● I (NWZ) ● II (SEZ) ● III (WZ) ● IV (EA-I) ● IX; **for type SAT-2 the topotypes and genotypes were:** ● I ● II ● III ● IV ● VII ● X ● XIII and **for type SAT-3 the topotypes and genotypes were:** ● I (SEZ) ● II (WZ) ● V.

2.3.4. Molecular epidemiology

FMD endemic areas in the world are high-risk zones for introducing FMD to countries free of the disease and for the origin of new FMDV lineages. Therefore, a continuing surveillance of FMDV is needed for the early recognition and understanding of emerging risks or changes in the global FMD situation. The detection and characterization of new FMDV lineages and control of possible conformation changes of circulating immuno-relevant epitopes, is of special interest, as it may indicate that a change in the current used vaccine strains is needed (Klein, 2009). As a result, Molecular epidemiologic studies have contributed more in planning FMD control strategies by elucidating historical and current disease transmission patterns within and between countries and it provides information that should be needed when planning FMD vaccination strategies (Mwanandota, 2013; Sangare *et al*., 2004).

Molecular techniques can be useful in defining strains, identifying transmission of events, and characterization of biodiversity (Samuel and Knowles, 2001). At present, DNA sequencing and phylogenetic trees are widely used to illustrate the genetic relationship between viruses (Sahle *et al*., 2004). Phylogenetic analysis of the virus protein 1 (Knowles *et al*., 2016) region of FMD virus has been employed extensively in investigation of molecular epidemiology of the disease worldwide. These techniques have assisted in studies of the genetic relationships between different isolates FMD virus, geographical distribution of lineages, and genotypes. In addition, it was also used for the

establishment of genetically and geographically linked topotypes and in tracing the source of virus during outbreaks (Knowles and Samuel, 2003; Sangare *et al*., 2003). Sequence differences of 30% to 55% of the VP1 gene were obtained between seven serotypes of FMD while different subgroups (genotypes, topotypes) were defined by differences of 15% to 20% (Knowles and Samuel, 2003). Virus isolates from the same epizootic differ by ≤ 1%, viruses belonging to the same epizootics differ by < 7%, viruses of the same genotype differ up to 15% and viruses from different genetic lineage, differ by ≥ 20% (Sahle *et al*., 2004).

2.3.5. Species affected/ host range

Host range always governs the existence of pathogen in environment. With some minor exception, FMD affects all cloven footed domestic and wild ungulate species but the development of the disease is variable depending on the species and virus strain. When considering the host range, it is important to distinguish between animals which (a) play a role in the natural epidemiology of the disease, (b) may play a role under certain conditions or cannot be excluded as being an epidemiological risk and (c) are susceptible to infection, and even may develop disease, under experimental conditions but appear to be without much, if any, importance under field conditions (Alexandersen and Mowat, 2005). Among the domestic species; bovines, water buffalo, swine, ovine and caprine are the most sensitive with more severe disease in bovine and porcine species. African buffalo play an important role as the natural maintenance host in Africa, but more than 70 species of cloven-hoofed wild life, such as deer, antelope, impala and kudu may become infected and also be involved in the natural epidemiology of FMD (Admassu *et al*., 2015; Bastos *et al*., 2000; Michel and Bengis, 2012). However, FMDV does not affect horses, pet animals and birds. Camels also have low susceptibility (Chakraborty *et al*., 2014).

2.3.6. The role of carrier animal in the epidemiology of FMD

Following the acute phase of FMDV infection, some animals may experience a long asymptomatic persistent infection. In addition, some vaccinated animals may also

become persistently infected if exposed to infectious virus. These animals are referred to as carrier animals and the carrier state is defined as an animal from which live virus can be recovered after 28 days following infection (Grubman and Baxt, 2004). Animal considered as persistent FMD Carrier if live virus can be isolated from the oesophageal pharyngeal (OP) area, more than 28 days after infection. FMD virus persists in buffalo (up to 5 years), cattle (up to 3 years), Sheep (up to 9 months), and goats (between 3-6 month), the mechanisms underlying persistence and the immunological pathway that eventually leads to viral clearance are not well understood (Bastos *et al*., 2000; Mwanandota, 2013) but in vitro studies on BHK-21 cell lines revealed that the host cells interfere in the lysis of FMDV (Zhang *et al*., 2013). From domestic animal only pigs are believed to be incapable of establishing a persistent infection. The mechanism of persistence is likely to depend on a number of factors including, viral replication, target cell, host and strain variability (Slager-Bastos, 2001).

2.3.7. Mode of transmission

FMD is a highly transmissible disease in which limited number of infective particles can initiate infection in susceptible host. it is mainly transmitted through direct contact between infected and susceptible animals, although indirect contacts such as contact between a susceptible animal and infected feed products or equipment are effective in transmitting infection (Logan, 2017). The virus can replicate and be excreted from respiratory tract of animals leading to airborne excretion of virus during the acute phase of infection, although, FMD virus may occur in all bodily secretions and excretions including aerosols that infect other animals. Hence, after an animal becomes infected by any means, the primary mode of virus spread is via respiratory aerosols since respiratory tract is the major site of virus replication and large amount of viral particles are secreted from this site (Alexandersen *et al*., 2003; Sutmoller *et al*., 2003). It is the most common mode of transmission in cattle. However, for pigs, eating contaminated food is the major route of transmission than aerosol, which is the least efficient route of transmission (Alexandersen and Donaldson, 2002). Movement of infected livestock has been the main

source of many of the sporadic epidemics in Europe (1990-2001) and many recurrent FMD outbreaks in Africa (Sutmoller and Olascoaga, 2002).

Mechanically the disease can also transmit through fomites contaminated with animal secretions containing virus, contaminated animal products, non-susceptible animals, agricultural tools, farm workers, vehicle, hay or bedding and air borne transmission (Mwanandota, 2013; Nsamba, 2015). When appropriate humidity and temperature are maintained, FMD virus can be carried up to 250 km across the sea and up to 60 km across the land. The prior condition has been held responsible for the FMD outbreak that occurred in France and then spread to UK in 1981 emphasizing the possible windborne spread of the virus under prevailing environmental conditions (Mwanandota, 2013). At present, there are Computer models that can predict the most likely wind-borne spread of the virus from infected herds and allow the examination of a variety of control strategies (Sahle *et al*., 2004).

Outbreaks of FMD can occur also because of viruses escaping from research and vaccine production centers and the semen of infected bull can be a source of infection by artificial insemination (Radostits *et al*., 2000). Personnel handling infected animals can be contaminated on hands, clothes or in nasal passages with live FMD virus and mechanically carry virus to susceptible animals by direct contact. A person in contact with infected animals can serve as a source of infection for 24 hours post infection (Kitching *et al*., 2007).

2.3.8. Morbidity and mortality

Variation in the morbidity rate occurs and may depend on species, age, sex as well as the status of the immunity. Self-recovery in the animals is the result of immunity against the infecting serotype of the virus. Mostly the disease occurs due to one type of virus and development of immunity also remains confined against specific serotype, thus no immunity develops to other serotypes, a reason behind occurrence of the disease in the endemic areas (Chakraborty *et al*., 2014). The morbidity rate of FMD in susceptible

animal can rapidly approach to 100% but some strains are limited in their infectivity to particular species. However, the mortality rate is generally very low in adult animal, about 2% in comparison to 20% in young stock. Calves mostly die due to cardiac involvement and complications such as secondary infection, exposure or malnutrition. Mortality in suckling pigs and lamps ranges from 20-75% in most extreme cases and it is highly age dependent, infect for animals under 4 weeks of age, mortality is high and decrease rapidly as animals get older than 4 weeks (Balemual, 2018).

2.3.9. Immune response

The protection of a susceptible host against FMD virus correlates with the neutralizing antibodies level. Infection with one-serotype produces complete protection against homologous virus, but fails to protect against heterologous viruses (Domingo *et al*., 2003). Cattle which have recovered from infection with one of the seven serotypes of FMD are not immune to the other serotypes but remain protected against the first serotype for a considerable period of time. However, further rounds of infection with other serotypes may result in less severe clinical responses or protection. High-quality immunity, as conferred by a potent vaccine or recent infection (six months post infection), appears capable of preventing development of any clinical signs of disease, regardless of whether the subsequent challenge is made by injection of virus or contact with infected animals. When convalescent animals were challenged approximately one year after exposure to virus, they were found to be protected, although lesions developed at the inoculation sites. However, in the absence of more detailed experimentation, it is probable that the degree of protection after a long convalescent period will depend on the serotype, and possibly subtype, of virus used in the experiment (i.e. on the relative virulence of this strain for cattle), and on variations in the rate of decline of antibody for individual animals (Doel, 1996).

Serotype specific immunity is based on the presence of neutralizing antibodies to one of the viral capsid proten, VP1, develops 7 to 21 days after exposure to the virus. The immunoglobulin M (IgM) is most prevalent in the early convalescent serum and is less

specific to the different serotypes than Immunoglobulin G. Serum immunoglobulin M (IgM) may be detected 3 to 5 days after infection, reaching a peak between 5 to 10 days. IgG is produced in the later stage during the FMD infection and the reaction between the serotype and the homologous antibodies is highly specific. It is appear from 4 days onward and reach maximum levels between 15 to 20 days. It has been reported that healing of lesions and clinical recovery in infected animals would not occur until a few days after the IgG1 antibodies have developed The localized antibody response, specific to anti-FMD IgM and IgA antibodies in the pharyngeal fluid of cattle develops 7 days after exposure to the virus, while IgG activity reaches pick in serum only 14-21 days after infection (Doel, 1996).

The age of individuals has also been shown to influence the antibody response against FMD virus. Calves (age one week to six months) but deprived of maternal antibodies responded as well as, or better than 18 months old cattle to initial vaccination against FMD. Although serum antibody levels play an important role in host protection against FMD virus infection, the cellular responses mediated by T-helper and T- cytotoxic cells also play a role in the immune response to FMD virus infection (Sanz-Parra *et al*., 1998).

2.4. Clinical signs

Clinical signs of FMD are usually develops in 3 to 5 days, although in natural infection, the incubation period may range from 2-14 days. However, it vary with the dose of virus, the viral strain, the portal entry, the husbandry practice and animal species involved (Alexandersen and Mowat, 2005).The clinical signs of the disease ranges from mild to severe based the strain of the virus, the exposure dose, the age and breed of the animal, the host species and its degree of immunity (Balemual, 2018). FMD is more severe in cattle and pigs but the sheep and the goats may even some time undergo undiagnosed. While there is some variability in the clinical signs between species, FMD is typically an acute febrile illness with vesicles (blisters) localized on the dental pad, tongue, muzzle or snout, the hooves, the teats and other site of the skin which rapture within 3 days to leave shallow erosions that heal rapidly (Aftosa, 2015). In acutely infected cattle, initial signs

are rise in temperature (40-40.6^{o}C), dullness, anorexia and marked drops of milk production while within 24hr the appearance of excessive salivation, saliva hanging in long, ropy strings due to oral lesion which leads to nasal discharge are common (Quinn *et al*., 2005). Following a period of initial pyrexia which can last for one to two days, a variable number of vesicles develop on the tongue, hard palate, dental pad, lips, gum, muzzle, coronary band, interdigital spaces and teat (Alexandersen *et al*., 2003). Abortion in pregnant cow, presumably as a consequence of fever (Aftosa, 2015) and young calves up to 6 months of age may die suddenly before the appearance of any clinical signs of the disease due to virus infection of the developing heart muscle (myocarditis) (Radostits *et al*., 2000).

The clinical signs of the disease in sheep and goats are described as less florid and subdued. Up to 25% of affected sheep may not develop clinically apparent vesicles, and 20% have lesions only at one site or develop vesicles visible for less than three days (Kitching and Hughes, 2002). Lameness is often the first observed clinical sign in sheep and goats. Additionally, affected animals may develop fever, show reluctance to walk and may separate itself from the rest of the flock. Vesicles may develop in the interdigital cleft, on the heel bulbs and on the coronary band, but they usually rupture rapidly and their appearance may be hidden by the co-existing presence of foot rot. Vesicles are formed in mouth which ruptures easily leaving shallow erosions, but commonly seen in the dental pad, adjacent to the incisors, also on the tongue, hard palate, lips and gums. Young lambs and kids may die due to heart failure (Rout *et al*., 2015).

FMD in pigs are characterized by fever, inappetence and reluctance to move. Usually, the more severe lesions occur in the feet, starting by lameness and blanching of the skin around the coronary bands. Vesicles then develop on the coronary band and heel, and in the interdigital space. The lesions may become so painful that pigs crawl rather than walk. The horns of the digits are sometimes sloughed. (Leon, 2012).

2.5. Pathogenesis

The respiratory system is the most important route of infection for FMD. Once the virus is inhaled, it can affect the pharynx which is primary multiplication site and then transported by lymphatic and blood circulation to the sites of secondary multiplication in the lymphatic glands, epithelial tissues in and around the mouth, feet and in the mammary glands. Following secondary replication in glandular tissues, the virus appears in different body fluids such as milk, urine, respiratory secretions and semen, before the appearance of frank clinical signs of FMD (Balemual, 2018). In the cattle infected via the respiratory tract, the virus initially replicates in non-cornified epithelial cells of the pharynx (Alexandersen *et al*., 2003) from where it proceeds towards the epithelium of the mucosa associated lymphoid tissue of the nasopharynx, mucous membrane of oral cavity and invades the basal layer of the stratified epithelium of the tongue and produce primary lesions (Arzt *et al*., 2009; Pacheco *et al*., 2010). The pharyngeal area is the usual primary site of virus infection and multiplication except when the virus directly enters into the cornified epithelia or the circulation by damage to the intact integument. The epithelial cells in the pharyngeal region play a special role in primary infection. Most of the oral cavity is covered by cornified/keratinized (i.e. having a layer of dead cells at the surface) stratified squamous epithelia, whereas the roof of the pharynx, dorsal part of soft palate and part of the tonsils are covered by a special non-cornified, stratified squamous epithelia and therefore, in contrast to intact cornified epithelia, have more live cells exposed on the surface and consequently may allow easy access, and provided the right receptors are present, efficient virus entry (Alexandersen and Mowat, 2005). In contact or aerosol exposed animals, virus may be demonstrated in the pharynx for 1 to 3 days before a viraemia or clinical disease can be detected (Alexandersen *et al*., 2002).

Vesicles are formed due to virus multiplication in the stratum spinosum layer where cytolysis takes place and hence giving rise to small cavities in the epithelial layer. Virus also invades the lymphatics and enters into the blood stream resulting in spread of virus to other organs and tissues such as epithelium of mouth, dental pad, coronary band, interdigital space of hoof, mammary gland, teats and snout where the secondary lesions

develop (Alexandersen and Mowat, 2005; Arzt *et al*., 2009; Yang *et al*., 2011). One-two days after infection, fever and viraemia may be observed. In advanced and unaddressed cases, secondary bacterial infection may set up extensive damage of the tissue. Damage of feet may lead to loss of the horny covering and sloughing of the hoofs. Udder lesions may lead to mastitis due to secondary infections. Heart muscles of young animals may show acute degeneration of the myocardial fibers (tiger heart) (Chakraborty *et al*., 2014).

2.6. Diagnosis

An essential component of FMD control strategy includes diagnostic assays to rapidly confirm the initial clinical determination of infection. The diagnosis is mainly rely on the clinical signs, in combination with laboratory examination to establish the serotype of the causal virus (Admassu *et al*., 2015). Diagnosis by clinical signs alone is complicated by other viral disease of livestock; vesicular stomatitis (VS) and swine vesicular disease (SVD) produce lesions which are identical to those of FMD. Lesions induced by bovine papular stomatitis, bovine herpes mammilitis, infectious bovine rhinotracheitis, bovine mucosal disease, malignant catarrhal fever, rinderpest in cattle, bluetongue, parapox-virus, peste des petits ruminants and footroot in sheep may also be mistaken for FMD and all these listed diseases should be considered during the diagnosis (Balemual, 2018).

For laboratory diagnosis, samples include vesicular fluid, epithelium, blood in anticoagulant, serum and esophageal/pharyngeal fluids collected with Probang cup (Quinn *et al*., 2005). Laboratory diagnosis of FMD is achieved by a combination of virus isolation, serological tests and nucleic acid recognition method (Admassu *et al*., 2015; Mwanandota, 2013).

2.6.1. Virus isolation

Virus isolation (VI) remains the definitive proof of the presence of live FMDV. Isolation of the virus can be carried out in primary bovine thyroid cells (Ferris and Dawson, 1988) or primary pig, calf or lamb kidney cells (Ahl *et al*., 1997); BHK-21 or IB-RS-2 cells

(Sakamoto *et al*., 2002) although the cell lines show less sensitivity than the primary cells. From primary cell culture, the most sensitive culture system for virus isolation is primary bovine thyroid cell (Longjam *et al*., 2011). Field samples suspected to contain FMD virus are inoculated into cell cultures (primary pig kidney cells), incubated at 37°C and examined for appearance cytopathic effect (CPE), 24 to 48 hours post infection. If there is no CPE, it confirms the absence of FMDV in the samples (OIE, 2008).

2.6.2. Serological test

Serological tests are essential for supplementary diagnosis of FMD, for certification of animals for import/export, in determining the freedom from infection and for the demonstrating vaccine efficacy. Virus infection can be diagnosed by the detection of specific antibody response. Enzyme linked immunosorbant assay (ELISA), agar gel immunodiffusion test (AGID) and virus neutralization test (VNT) and complement fixation test (CFT) are used for serological diagnosis of FMDV. Previous or current infections can be diagnosed by using antibodies to FMDV structural proteins and include: ELISAs [Solid-phase competition ELISA (SPCE), Liquid-phase blocking ELISA (LPBE)] and virus neutralization tests (VNT) (Gold standard test) which are serotype specific (Deb *et al*., 2013; Ma *et al*., 2011; Singh *et al*., 2008; Verma *et al*., 2009).

2.6.3. Nucleic acid recognition method

The polymerase chain reaction techniques are the most widely used nucleic acid based diagnostic technique for rapid identification of FMD virus and sequence analysis of any PCR positive result (Xu *et al*., 2013). A specific reverse transcriptase polymerase chain reaction (Xu *et al*., 2013) was developed and validated for the detection of the polymerase gene (3D) of FMD with an analytical sensitivity equal to 1000 times higher than that of a single passage virus isolation (Longjam *et al*., 2011). RT-PCR is used as diagnostic tool for FMD virus where specific primers are designed to distinguish seven serotypes. In epidemiological studies of FMD virus, nucleotide sequencing of the VP1

gene has been used extensively to determine the relationships between the field isolates (Mwanandota, 2013).

2.7. Treatment

Treatment is not exists for most of viral disease including FMD (Quinn *et al*., 2002). Instead of specific treatment, symptomatic treatment may be applied depending on the clinical manifestations. Potassium permanganate mixed antiseptic mouth wash, sodium carbonate, boric acid and glycerin may be applied over the lesion. Feet of the affected animals may be also washed with 2% copper sulphate solution. Washing of the wounds with soda ash solution and topical application of honey and finger millet is found suitable in foot lesions (Gakuya *et al*., 2011). Antiviral approaches including 2'-C-Methylcytidine (Lefebvre *et al*., 2014), ribavirin (Yoon *et al*., 2012) are useful for the purpose of prophylaxis in susceptible animals. Additionally, proper animal husbandry practices and treatment of secondary bacterial infection and dressing to inflamed areas to prevent secondary infection is recommended especially in endemic countries where slaughter policy is not practiced. Affected animals will recover however with loss of production based on the infection state of the disease (Hirsh and Zee, 2002).

2.8. Prevention and control

Foot and mouth disease is subject to national and international control and the measure taken depends on whether the country is free from the disease, is subject to sporadic outbreaks or has endemic infection. To control the disease effectively, there is need of good infrastructure, trained veterinary staff, well equipped laboratories, good governance, rapid and accurate diagnostics, rapid response measures, continuous monitoring and surveillance, and compulsory vaccination (Ding *et al*., 2013; Namatovu *et al*., 2013).

2.8.1. Control in endemically affected countries

In countries where the disease is endemic, control and prevention of FMD is mainly relies on repeated vaccination, control of animal movement and also physical separation of wildlife and livestock (Paprocka, 2004; Quinn *et al*., 2015). However, test and slaughter policy shouldn't be implemented due to the economic, social and regional barriers (Dhama *et al*., 2010). For the development of an effective strategy of vaccination, it is important to understand the disease dynamics which indicates the suitable time points to administer vaccine. Vaccination against FMD virus is achieved with inactivated vaccines that should induce protective immunity against each type of antigens incorporated in the vaccine (Asseged, 2005). Therefore, when vaccinating animals, it is important that the vaccine contain the same subtype of virus as is in the area. This necessitates frequent checking of the serotype and subtype during an outbreak because FMD virus frequently changes during natural passage through various species (Chibssa, 2006).

2.8.2. Control in disease free area

2.8.2.1. Stamping out

FMD free countries use 'stamping out' policy, consisting of the slaughter of all affected and in-contact susceptible animals would be instigated. Furthermore, zoosanitory measures including, the imposition of movement's restriction were used to control the outbreaks. Such measures might also extend to preemptively slaughtering other herds in which there is no clinical evidence of the disease, but which have been epidemiologically linked with an outbreak, and may therefore contain infected animals. The stamping out is done with full compensation paid for animals slaughtered (Chibssa, 2006).

2.8.2.2. Emergency vaccination

Emergency vaccination, within an infected area, has gained more preference in recent years, in an attempt to reduce the amount of virus circulating and spreading beyond the restricted area. The use of emergency FMD vaccines has two clear objectives. Firstly, to provide protective immunity, as rapidly as, possible to susceptible stock, and secondly, to reduce the amount of virus released and thereby limit the spread of disease (Asseged, 2005).

2.8.2.9. Protective vaccination

The protective vaccination is used effectively in animals not already exposed to FMD virus. It would therefore be employed outside the 3 km protection zone and outside any predicted aerosol spread of virus from the infected premise. All vaccinates would be naive to FMD antigen, and would require a minimum of 3-4 days to develop protective immunity. This protective vaccination would thus form a ring around the infected area, preventing diseases spread, and allows the outbreak to expire within the protection zone, where infected herds would quickly be identified and slaughtered (Asseged, 2005).

2.9. Economic importance of FMD

FMD is one of the most economically important contagious diseases of livestock in the world. The importance of the disease is observed in terms of its sequelae like contagious nature post outbreak, broad economic impacts on animal wellbeing and productivity, loss of production, cost of veterinary service due to the presence of the disease and the restrictions on the trade of animals and animal's product both locally and internationally (James and Rushton, 2002). The impacts of the disease vary significantly between developed and developing countries, and also within many developing countries depending on the species involved, the genotype of animal, the level of productivity, the significance of livestock to livelihoods, and the effectiveness of indigenous coping mechanisms for controlling the effects of FMD (Perry and Rich, 2007). Generally, the

impact of FMD can be seen into two components as direct losses due to reduced production and changes in herd structure; and indirect losses that relates to the significant costs of FMD control, poor access to markets and limited use of improved production technologies (Knight-Jones and Rushton, 2013).

The direct production effect includes reduced milk production, chronic mastitis due to udder involvement and reduced draught power due to permanent hoof damage (Bayissa, 2009). Overt clinical disease affects animals' performance directly and causes reduction of milk yield in dairy cattle by 20%, growth rate of beef animal by 10-20%, pig meat production up to 20% per annum and inflict fertility impairment due to increased abortion rate up to 10% (Knight-Jones and Rushton, 2013). Moreover, death of very young animals, culling of unproductive and chronically infected animals, and loss of valuable breeding stock and disruption of livestock improvement programs are also attributed to the direct effect of the disease (Catley *et al*., 2004).

The indirect cost is associated with the cost of control carried out by the state veterinary services (e.g. vaccination, Surveillance, outbreak control, culling and compensation and movement control). The costs are enormous with an estimated 2.35 billion doses of FMD vaccine administered in the world every year at a cost of $0.4–3 or occasionally $9 perdose including delivery and application. In FMD free countries there are ongoing costs due to efforts to prevent disease introduction, including import controls. In addition, maintaining FMD early detection and control capability, including vaccine banks, is costly. Other costs include FMD related research and permanent restrictions on the livestock sector (such as post-movement standstills and bans on feeding swill) (Knight-Jones and Rushton, 2013).

The disease is also a major constraint to the international livestock trade. The highest value markets for livestock products are in FMD free countries, and these countries are allowed to restrict or ban imports of livestock products and also of susceptible wildlife that have a potential risk of introducing FMD .The presence of FMD in a country is considered as binding constraint to developing an export sub-sector within the livestock

industry, and, therefore, contributes to unavoidable loss (Gulati *et al*., 2005; Thomson *et al*., 2003). Furthermore, FMD outbreaks have widespread economic and social impacts both in the short and long term, including disruptions of animal feed, veterinary pharmaceutical and tourism associated industries (Kitching *et al*., 2007). In Ethiopia, where the economy is heavily dependent on livestock, the burden of the disease may be severe and local food security impaired. The direct impact of the disease can be a long lasting and it can have effects on livestock output in a number of "hidden" ways (such as delays in reproduction leading to fewer offspring and the consequences of a reduced population) which often exceed the losses associated with clearly visible illness. Additionally, FMD slows economic growth by severely limiting trade opportunities (Balemual, 2018).

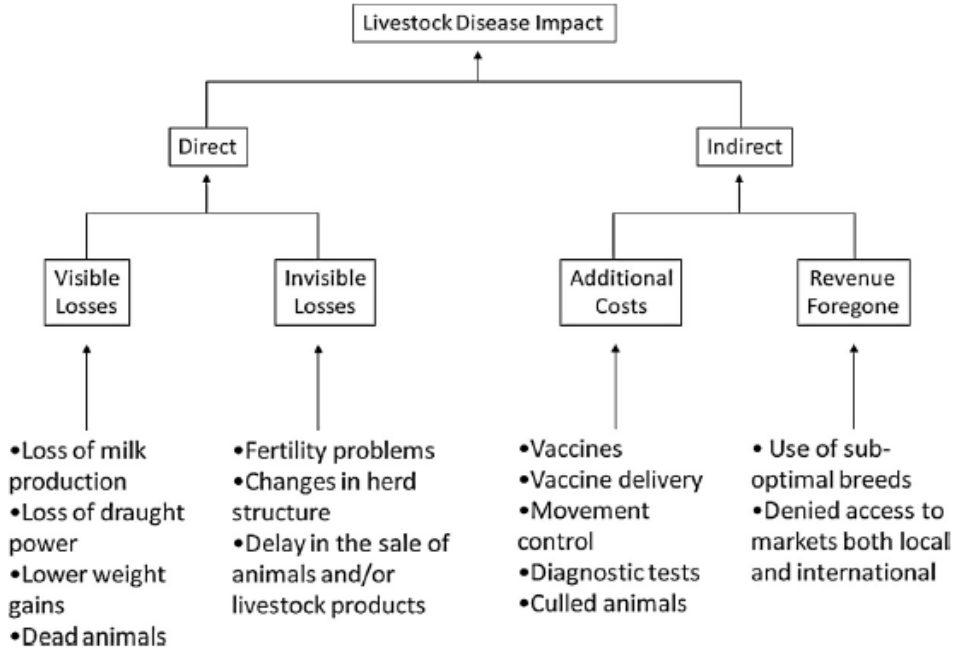

Figure 8: Summary of the economical impacts of FMD. Adapted from Knight-Jones and Rushton (2013).

2.10. Status of Foot and Mouth disease in Ethiopia

2.10.1. National geographical distribution of FMD from 2009-2017GC

Foot-and-Mouth disease is one of the endemic diseases and known for its wider distribution in Ethiopia. Previously, it occurs mostly in the pastoral herds of the marginal lowland areas of the country. However, this has been changed and currently the disease is also frequently noted in the highlands of the country, even though the level of the disease prevalence may show significant variations across the different farming systems and agro-ecological zones of the country (Tefera, 2010). The geographical distribution of 9 years (2009-2017 GC) of FMD outbreaks reports within the regional states is depicted in (Figure 9) as reported to Ministry of Livestock and Fishery (MoLF, 2018).

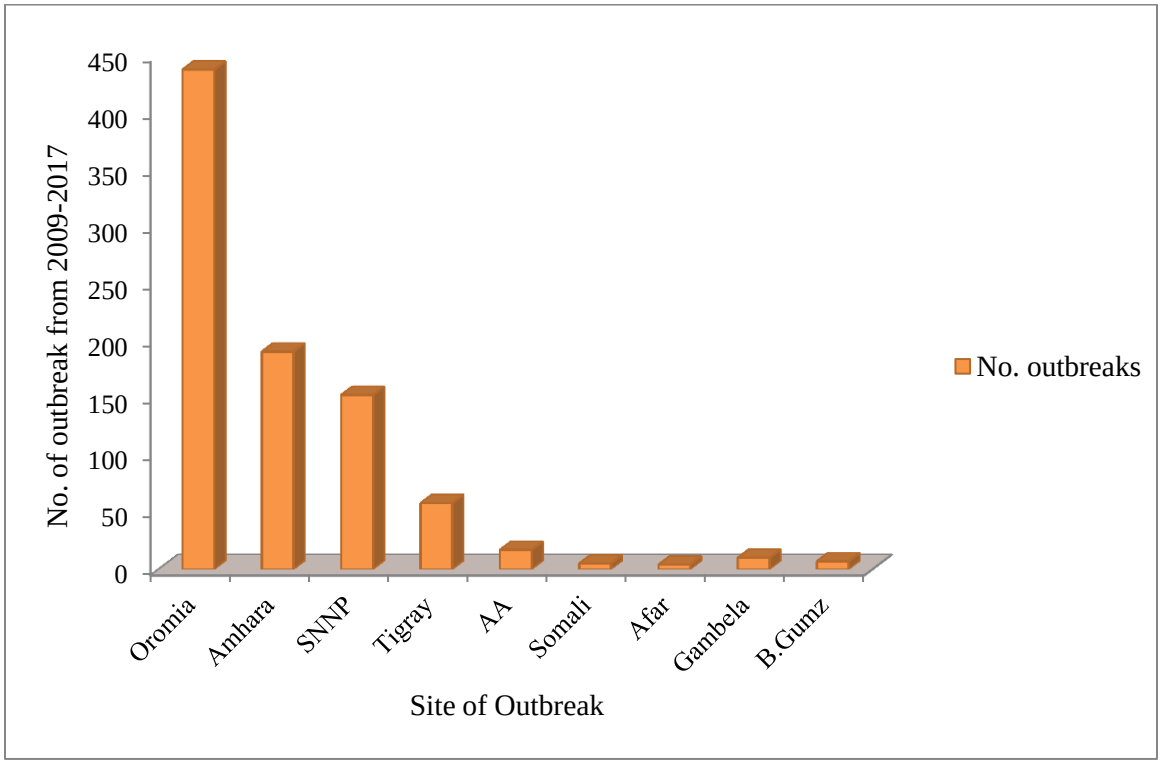

Figure 9: National picture of FMD outbreak from 2009-2017. Adapted from MoLF (2018).

2.10.2. Frequency of disease occurrence

FMD outbreaks are reported frequently throughout the country (Asfaw and Sintaro, 2000). In the past nine years (MoLF, 2018) 884 outbreaks from Addis Ababa, Oromia, Amhara, SNNPRS, Tigray, Gambela, Benshangul Gumuz, Somali, Afar, Harari were reported to MoLF. From this about 438 were reported from Oromia regional state. The outbreaks were reported annually and higher number of outbreak is reported in 2012 which is 205 and the lower is in 2014 which is 39 (Figure 10). However, the figures provided are don't reflect the exact epidemiological situation the disease in the country due to insidious nature of the disease and the unreported cases by farmers (MoLF, 2018).

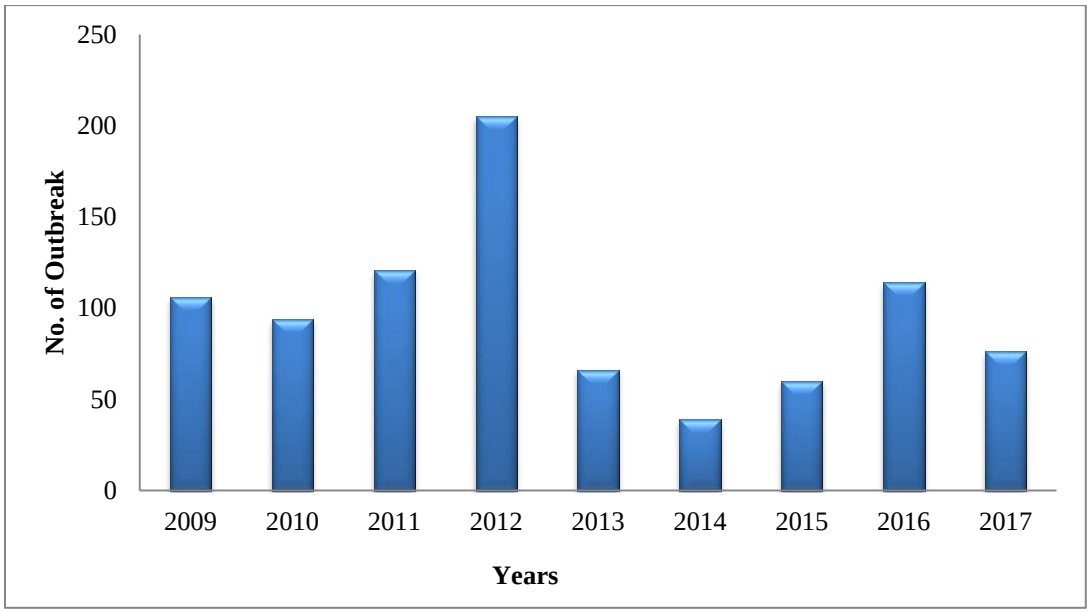

Figure 10: Summary of frequency of disease occurrence from 2009-2017

2.10.3. Seasonal distribution of FMD outbreaks

According to MoLF reports, FMD occurs in any season of the year although highest outbreak is recorded in dry season associated with the drought. During dry seasons especially pastoralists are obliged to move their herds long distances in search of pasture

and water and thereby transmission of highly contagious diseases like FMD exacerbated at herd gathering sites or communal points (Bayissa, 2009; Molla *et al*., 2010).

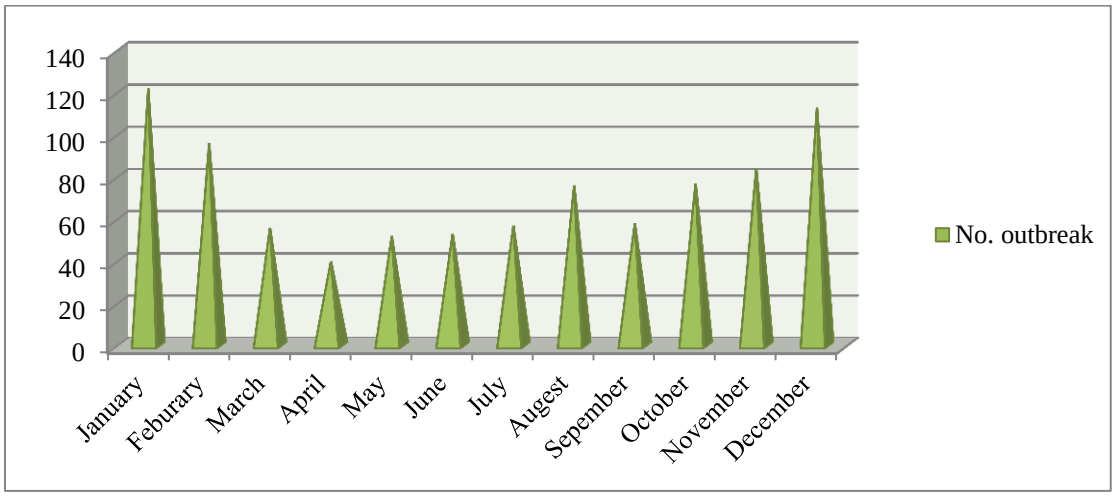

Figure 11: Summary of seasonal distribution of FMD from 2009-2017

2.10.4. Serotype and topotype of FMD circulating in Ethiopia

During 1981-2007, the presence of serotypes O, A, C, SAT1 and SAT2 were reported by Ayelet *et al* (2009). From this report, serotype O was the most predominant strain circulating in the country (73.3%), followed by serotype A (19.5%), SAT 2 (4.1%), SAT 1 (1.8%), and C (1.3%). Serotype C was not isolated in any of the outbreak investigation since 1983 and there is only a single isolation report of SAT 1 in 2007 (Ayelet *et al*., 2009). Ayelet report also showed that, the phylogenetic analysis of virus protein 1 (Knowles *et al*., 2016) sequences on samples from Mizan Teferi indicates the emergence of a new topotype within serotype O, East Africa 4 (EA-4), apart from the dominant topotype within 13 serotype O of EA-3 encountered in Eritrea, Ethiopia and Sudan.

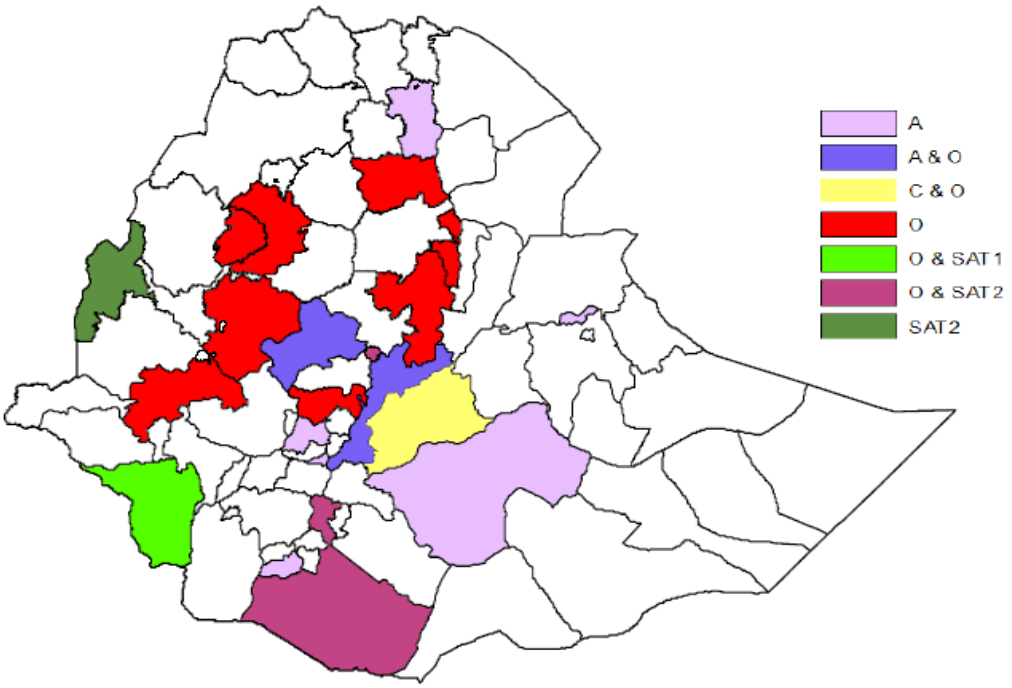

Figure 12: The epidemiology of FMDV serotype isolated in Ethiopia from 1981-2008. Adapted from Ayelet *et al*. (2009).

Table 1: Summary of FMDV topotype detection in Ethiopia

Serotype	Topotype	Genotype/Strain	Reference
O	EA-3		(Ayelet *et al*., 2009; Negussie *et al*., 2013; WRLFMD, 2016)
	EA-4		(Ayelet *et al*., 2009; Urge, 2017; WRLFMD, 2016)
A	Africa	G-VII	(Negussie *et al*., 2013; WRLFMD, 2016)
		IV	(Sulayeman *et al*., 2018)
SAT1	IX		(Ayelet *et al*., 2009; WRLFMD, 2016)
SAT2	VII		(Sulayeman *et al*., 2018)
	XIII		(Ayelet *et al*., 2009; WRLFMD, 2016)
	XIV		(Ayelet *et al*., 2009)

2.10.5. Sero-prevalence of FMD

The sero-prevalence investigation under taken so far in the country indicated that, the prevalence ranges from 5.6% to 42.7% in cattle, 4% to 11% in small ruminants and 30% in wild ungulates (Abdela, 2017). However, the prevalence of the disease is varying from place to place, and the studies conducted so far did not cover all corners of the country. The lack of well-equipped regional laboratories, inaccessibility of certain areas and suboptimal routine surveillance and reporting could hinder to have the overall estimate of the disease magnitude at a national view contrary to its endemicity (Sahle *et al*., 2004).

Table 2: Overall sero-prevalence of FMD in cattle in different parts of Ethiopia

Site	**Study period**	**No.of animal**	**Seropositive**	**Prevalence in %**	**Reference**
Bench-maji	Nov, 2007-Feb, 2008	273	33	12.08	(Ayelet *et al*., 2009)
South omo	Oct, 2009-May, 2009	770	63	8.18	(Molla *et al*., 2010)
Afar	Oct, 2007-April, 2008			5.6	(Jenbere *et al*., 2011)
Somalia	Oct, 2009-Mar, 2010	384	54	14.05	(Mohamoud *et al*., 2011)
Borena	Oct, 2007-Mar, 2008	153	82	53.6	(Mekonen *et al*., 2011)
Dire-Dawa	Nov ,2010-Mar, 2011	986	79	8.01	(Abunna *et al*., 2013)
Kellem-wollega	Nov, 2011-Mar, 2012	384	82	21.4	(Desissa *et al*., 2014)
Tigray	Oct, 2008-Jun, 2009	390	60	15.4	(Zerabruk *et al*., 2014)

Bishoftu	Sep, 2014-July, 2015	634	69	10.88	(Belina *et al*., 2016)
Centeral-Ethiopia	Sep, 2015-May, 2016	574	139	24.22	(Sulayeman *et al*., 2018)
Oromia and AA	Sep, 2016-May, 2017	586	226	38.6	(Urge, 2017)

Table 3: Sero-prevalence of FMD in sheep and goats tested for export certification

Region	**Zone**	**District**	**Species**	**No. tested**	**No. positive**	**% positivity**
Amhara	N.Wollo	Desse	Ovine	2764	53	1.92
Dire Dawa			Caprine	1625	32	1.97
			Caprine	289	7	2.42
			Ovine	139	2	1.44
Oromia	East Shoa	Fentalle	Ovine	1000	22	2.20
		Fentalle	Ovine	9304	103	1.11
		Fentalle	Ovine	226	1	0.44
		Fentalle	Ovine	8468	1187	14.02
		Fentalle	Ovine	21	2	9.52
		Fentalle	Caprine	399	2	0.50

Adapted from Bewket *et al*. (2012).

2.10.6. Risk factors FMD

Several risk factors like production system, geographic location, age of animals, contact with wildlife and season of the year were identified for increasing the incidence of the disease in Ethiopia. This is supported by Jenbere *et al*. (2011) study which indicates the association of herd size, difference in geographical location (district) and age of the animals with seropositivity of FMD. Higher seroprevalence were recorded in pastoral system than sedentary farming due to the fact that in pastoral areas there is unrestricted

high herd mobility, continuous contact and intermingling of different herds at water points, communal grazing areas and exhaustive transportation which increase the spread of the disease (Megersa *et al*., 2009). FMD seropositivity also associated with contact contact between livestock and ungulate wildlife such as buffalo, wild pigs, kudu and warthog (Beyene *et al*., 2015). The significance of the disease for wildlife lies largely in the potential that cloven hoofed wild animals have for transmitting the disease to domestic livestock where disease may be severely debilitating and result in serious economic losses. Some wild ruminants may have the potential to become carriers of the infection, albeit rarely, transmit the infection to cohorts of the same or other species with which they are in close contact (Thomson *et al*., 2003). Season also considered as a risk factor and higher outbreak is recorded in dry seasons which attributed to herd movement to grazing areas. Furthermore, bruising and traumatic wounds by dry grass or bush may increase the severity and disease transmission (Rufael *et al*., 2008). The occurrence of new topotype and uncontrolled animal movement are the other important risk factors in Ethiopia. Hence from 58 FMD outbreak occurred in different regional state of country, all the virus serotypes where O serotype, EA-3 topotypes except the Mekelle outbreak topotype, which was identical with Sudan topotype and its phylogenetic analysis indicated that the isolate was much related to the Sudan 1999, 2004 and 2008 isolates (Ayelet *et al*., 2009).

2.10.7. Control program of FMD in Ethiopia

FMD in Ethiopia is a major obstacle to the development of livestock sector because of its adverse effects on livestock production and export market of the country. Consequently, it requires greater attention and demanding urgent control measures that should result in minimizing its impact. However, the complex nature of the disease, its wider distribution across the country, the presence of multiple host species including wildlife and extreme contagiousness and absence of proper vaccination program with FMD vaccine within Ethiopia demanded that control strategies be implemented progressively on a short and medium to long-term basis (MoARD, 2006).

FMD control measures includes involvement of quarantine, restriction of animal movement, isolation of infected animals, vaccination programs, proper disposal of infected carcass and other methods which are feasible to Ethiopian economy. Currently there is no country-wide vaccination program aimed to control FMD. However, vaccination program should give emphasis to the control of all outbreaks occurring in the country through ring vaccination and vaccination of all export cattle before entering the quarantine stations. To protect export animals from contracting the disease while being kept in quarantine sites cattle found within 10 km radius of these sites could be vaccinated. All dairy animals should also be vaccinated and a ring vaccination is carried out around an infected area. Considering the wide prevalence of the virus, the National Veterinary Institute is producing an inactivated trivalent vaccine against serotype O, A and SAT2 (Admassu *et al*., 2015).

3. MATERIALS AND METHODS

3.1. Study areas

The study was conducted in area where FMD outbreaks occurred from October, 2017 to May, 2018 in three national regional states of Ethiopia: Oromia, Amhara and Addis Ababa city council to determine molecular characteristics of the virus circulating in this area. A total of five outbreaks were investigated throughout the study period. Outbreaks were reported from Meki and Bishoftu from Oromia, Shewarobit and South Wollo from Amhara and Bole subcity from Addis Ababa. In most of study areas animals were reared in extensive management system although semi-intensive and intensive management was practiced especially in farms found in Addis Ababa and Bishoftu.

The first study area was Meki, which is the main capital of Dugda Woreda found in East Shewa Zone of Oromia Regional State. It is located at 134 km to the South East of Addis Ababa on the main asphalt road to Baatuu (Ziway) town. Geographically, Meki is located between 8°9′N latitude and 38°49′E longitude with altitudes between 1500-2300 above sea levels. The total area of the Woreda is 959.45 km^2 and the boundaries are Bora Woreda in the North and North West, Arsi zone in the East, Adami Tulu Jido Kombolcha Woreda in the South and Gurage zone of SNNPRS in the West. The Annual temperature ranges from 17.9^0c to 20.8^0c and December is the warmest month (Setegn, 2015).

Bishoftu is the second areas were FMD outbreak was reported. It is found in Ada'a woreda in east shewa zone of Oromia regional state and located 45 km south east of Addis Ababa. The area is located at 9ºN latitude and 40ºE longitude at an altitude of 1850 maximum above sea level with annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26^0C and 4^0C, respectively, with mean relative humidity of 61.3% (Belay and Muktar, 2015).

Outbreaks of FMD were also reported from Shewarobit, a major town in Kewet woreda found in Semien Shewa zone of Amhara region. The woreda is bordered on the southwest by Termaber, on the northwest by Menz Mam Midir, on the north by Efratana Gidim, and on the east by Afar Region. Shewarobit is located about 225 km north of Addis Abeba with geographic coordinates 10°00′N latitude and 39°54′E longitude with an elevation of 1280 meters above sea level. This city has a tropical climate and when compared with winter, the summers have much more rainfall. The average temperature and precipitation is 23.0 °C and 1199 mm respectively (Mamo *et al*., 2013).

Samples were also collected from South Wollo; which is one of ten Zones in the Amhara National Regional State of Ethiopia located at 410km North East from Addis Ababa. It is located between 10°05´ and 11°45´ North latitude 38°35´ and 40°50´ East Longitude. The elevation of this area is ranging from 900 to 4220 meters above sea level. South Wollo is bordered by North Shewa and Oromia Region on the south, West Gojjam on the west, South Gondar on the northwest, North Wollo on the north, Afar Region on the northeast, and by the Oromia Zone and Argobba special Woreda on the east. The mean annual temperature and mean annual rainfall ranges from 14°C to 20°C and 680 mm to 1200 mm, respectively (Moges, 2014).

The last study area was Bole Sub city of Addis Ababa. Addis Ababa has an altitude of 2300 meter above sea level with a subtropical high land climate. The average annual rainfall and average maximum and minimum temperature for the area are 1180 mm and 22.8ºC and 10.6^{o}C, respectively. It is found between 9°1′48″N latitude and 38°44′24″E longitude. It has a humid subtropical mild summer climate that is mild with dry winters, mild rainy summers, and moderate seasonality. In Addis Ababa, there are about 5,200 dairy farms with some 58,500 cattle, and almost 50% are cross breed (CSA, 2009).

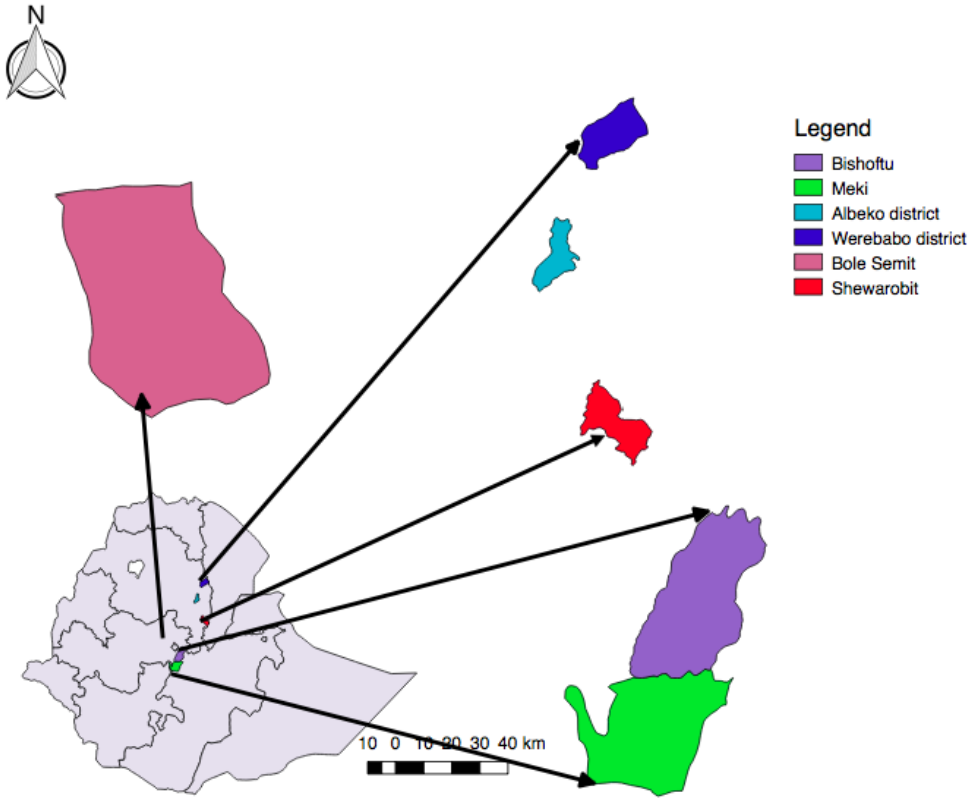

Figure 13: Maps of study areas

3.2. Study animals

The study was conducted in cattle that had experienced outbreaks of FMD and manifesting the clinical signs of the disease such as; vesicles on the dental pad, tongue, muzzle or snout, hooves, teat during outbreak investigation. Cattle of all age groups breed and sex reared under intensive, semi-intensive and extensive production and management systems were included in the study.

3.3. Study Design

Before beginning any outbreak investigation proper information channel to veterinary professionals, district veterinary officers, regional laboratories and National Animal Health Diagnostic and Investigation Center (NAHDIC) were organized and information

about the outbreaks were gathered by telephone. Based on reports of active cases of FMD outbreaks, a cross sectional study design were employed to collect samples for virus isolation and molecular characterization of the isolates from cattle. While an active outbreak of FMD reported, a field investigation was conducted at a particular site of outbreaks within the study districts. Animals of all age, sex and breed that showed clinical symptoms of the disease, history of infection but having healing lesions and any other asymptomatic cattle in the same farm or grazing were used to collect diagnostic samples. The inclusion criteria were cattle with clinical symptoms and the asymptomatic ones in the same farm while exclusion criteria were cattle without any clinical signs or any history the disease.

3.4. Sampling technique

During FMD outbreaks, a field investigation was conducted purposively at the specific site of the outbreak within the study area and animals with the clear signs, symptoms and suspected to be diseased with FMDV were sampled. Throughout field investigation, information about the disease was gathered by interviewing livestock owners and animal health workers of each outbreak site. The information collected were recorded on data collection sheet, and then animals were clinically examined for presence of FMD lesions on the mouth and feet, and specimens were collected for diagnostic testing.

3.5. Ethical consideration

Before starting the research a nine pages request that explain the purpose of this study and the possible managements planned to reduce pain and suffering of animal during sampling was submitted to Addis Ababa University College of Veterinary Medicine and Agriculture Minutes of Animal Research Ethics and Review committee. After the committee evaluated the importance of this study, approval was given on minute number (VM/ERC/05/10/018) with the reference number (VM/ERC/06/05/10/2018) (Appendix 11). Animal owners also asked for their consent before starting sampling and sample was collected only from willingness owners.

3.6. Study methodology

3.6.1. Clinical examination

In each outbreak, animals were first examined for evidence of clinical signs of the disease like, salivation, lameness, dullness, inappetance from distance. Animal with salivation and lameness were restrained for thorough examination and specimen collection. The oral cavity of salivating animals were examined for evidence of any intact and ruptured vesicles, erosions and ulcers on the tongue, dental pad, hard palate, gum and mucosa of mouth cavity. The hooves of lame animals also thoroughly washed with clean water and then carefully examined for similar lesions particularly on the coronary bands and interdigital spaces of the hooves. Animals were also examined their teat and external genitalia for presence of any vesicular lesion. Other animals in the herd without these signs were similarly examined, but sampling was done in animals that showed active clinical sign, healing lesion and those in close contact with the diseased one.

3.6.2. Sample collection

During the study period, a total of 37 specimens were collected from 5 FMD outbreaks sites. From this sample, 25 were epithelial tissue, while 12 were oro-pharyngeal fluid samples. Accordingly, a total of 37 epithelial tissue and oropharengial fluid samples were collected from 5 outbreaks.

Epithelial sample

Epithelial tissues were collected aseptically from unruptured and freshly ruptured oral and foot lesions from suspected FMD infected cattle. The collected sample were immediately placed in a bottle with virus transport medium composed of equal amount of glycerol and 0.04M of phosphate buffer saline solution (PBS) with antibiotics at pH 7.2-7.6 (OIE, 2008). Species, identification number, sex, age, village, and type of tissue were labeled, and samples were immediately placed in ice box containing ice packs for

transport to NAHDIC laboratory, Sebeta, Ethiopia. Once the samples arrived at NAHDIC, it was then stored at -80°C until processed and laboratory investigation**.**

Oro-pharengial fluid (OP) sample

OP samples were collected from previously suspected of FMD infected animals and asymptomatic cattle that were found in close proximity to the affected herd. This sample was collected by inserting a probang cup over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards between the first portion of the oesophagus and the back of the pharynx. The fluid were examined and 2ml of it containing cellular material were poured into a sampling bottle containing equal amounts of transport media (composed of 0.08M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics) with a pH of 7.2-7.4 (OIE, 2008). Then, the samples were labeled and transported to NAHDIC, Sebeta using ice box containing ice and kept at -80^{o}C until further laboratory investigation.

3.6.3. Sample preparation

Sample processing and virus isolation on BHK-21 cell was done following the procedure on OIE manual (OIE, 2012). Briefly, the epithelium samples were first taken from the transport media, and blotted dry on absorbent paper to reduce the glycerol content which is toxic to cell culture. A suspension was prepared by grinding 1 gram of the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium. DMEM medium was added until a final volume of nine times that of added epithelial sample was reached, giving a 10% suspension. The suspension was clarified on a bench centrifuge at 3000 rpm for 10 minutes. The supernatant of the suspension was collected and filtered by Millipore filter paper of 0.22 μm pore size.

3.6.4. Virus isolation

About 1ml of clarified suspensions suspected to contain FMDV were inoculated to baby hamster kidney (BHK-21) cells grown in $25cm^2$ tissue culture flask and incubated at 37^oc for 1 hour for virus adsorption. Finally, the infected cell was added with 8ml of DMEM maintenance media (2% fetal calf serum) and incubated at 37°C and 5% CO2 in a humidified incubator and monitored for cytopathic effect (CPE) for 24-48hours. CPE was observed after 48 hours in positive case although in 24hr in some samples. CPE was characterized by a fast destruction of the monolayer cell, cell rounding and infected cells were disrupted and detached from the flask. Complete destruction of the cell, cell detachment and redistribution of internal cellular membrane sheet was mostly seen within 48 hrs of inoculation. If no CPE was observed after 48 hr, the sample was considered as 'no virus detected' (NVD) and the culture was frozen at -80°C, then thawed and centrifuged at 3000 rpm for 10 min to collect supernatant for second passage (P2); this was repeated for third passage (P3); and if no CPE was observed at 48 hr, then the sample was considered negative for FMDV as described by Souley *et al*. (2018). Normal un infected BHK-21 cell was used for negative control. Finally, isolated samples were labeled according to a system specified by OIE standards (OIE, 2012). The first three letters signify the sample's country of origin i.e. ETH for Ethiopia, followed by a number representing the number of the particular sample in the batch of samples and finally the last two digits of the year in which the sampling was carried out.

3.6.5. Identification of FMDV serotype by antigen detection ELISA

FMDV serotype was detected and identified by using antigen detection sandwich ELISA (IZSLER, Bresica, Italy) with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies. The kit was designed for detecting and typing of six FMD virus serotypes such as type O, A, C, Asia 1, SAT 1 and SAT 2. A pan-FMD test can detect isolates of serotypes O, A, C, Asia 1 and some of the SAT serotypes were also included in the kit to complement the specific typing and to detect FMD viruses which might have escaped binding to selected serotype-specific

MAb. The micro plates were supplied with catched MAbs to detect 10 samples at a time with one positive and negative control for each serotype. The controls were already incorporated into the ELISA miroplate trapped by the respective catched MAb. The test was carried out as the manufacturer recommendation. Briefly, first samples were diluted ½ in diluent buffer and 50µl/well of each sample was distributed to 8 wells of a column (a total of 80 wells of A-H rows). Then, 50µl of diluents per well were added in all wells including column 11 and 12 (positive and negative control, respectively) and plates were incubated at a room temperature for 1hour. After incubation, all the fluid in each wells were discarded and the plate were tap hard to remove all the residual fluid. Then 200µl of washing solution were added and incubated for 3min at room temperature, subsequently wells were emptied and the washing repeated twice (three washing cycles in total). Then all residual fluids were removed by tapping on clean absorbent paper and 50µl/well of conjugate A was added from row A to F and the same volume of conjugate B was added into row G and H (Appendix 7). Plates were covered and incubated at room temperature for 1hour. After incubation 50µl of substrate per well was added to all wells and plates were covered and left at room temperature for 20minutes in the dark. The reaction was stopped by adding 50µl/well of stop solution (sulfuric acid (H2SO4)). Immediately after stopping, reading the optical density (OD) of each well was done at 450 nm wavelength using micro plate reader. The criteria for test validity and result for the samples examined are interpreted as indicated in (Table 4).

Criteria for validity of antigen detection ELISA

The positive inactivated controls were expected to give OD values of 1.0 unit or higher while the negative control for serotype O, A, C, Asia 1 and Pan-FMDV are expected to give OD values lower than 0.1unit and the negative control for serotype SAT1 and SAT2 are expected to give OD value lower or equals to 0.2unit.

Table 4: Interpretation of antigen detection ELISA result

OD values of the samples were interpreted by subtracting the OD value of each negative control from the OD value measured for test sample with the corresponding coated MAb.

Negative For FMDV	OD < 0.1
FMDV positive for type O	OD $\geq$ 0.1 with the type O MAb and with the pan-FMDV MAb; some samples may cross-react with the 1st MAb type A, but OD values with MAB O are higher
FMDV positive for type A	OD $\geq$ 0.1 with at least one of the two type A MAbs and with the pan-FMDV MAb
FMDV positive for type Asia 1	OD $\geq$ 0.1 with the type Asia 1MAb and with the pan-FMDV MAb
FMDV positive for type C	OD $\geq$ 0.1 with the type C MAb and with the pan-FMDV MAb
FMDV positive for type SAT1	OD $\geq$ 0.1 with the type SAT1 catching MAb; some saples could be positive also with the pan-FMDV MAb
FMDV positive for type SAT2	OD $\geq$ with the type SAT2 catching MAb; some samples could be positive also with the pan-FMDV MAb
FMDV positive (untyped)	OD $\geq$ 0.1 with the pan-FMDV catching MAb and < 0.1 with the type specific MAbs

3.6.6. Molecular characterization

3.6.6.1. RNA extraction

Total RNA was extracted from 140 µl original epithelial tissue and oro-pharyngeal fluid suspension using Qiagen RNA extraction kit (Qiagen, Crawley, West Sussex, UK) following the manufactures recommendation as described by Mukasa *et al*. (2016). Briefly, 140 µl of original epithelial tissue suspension was added to 560µl Buffer AVL-carrier RNA in the micro centrifuge tube, vortexed for 15 sec to mix properly and then

incubated at room temperature (25^{o}C) for 10 minutes. The solution was briefly centrifuged to remove drops from the inside of the lid, and then 560µl of ethanol (96%) was added to the sample and mixed by vortexing for 15 seconds. Then 630µl of the solution were applied to the QiAmp Mini column in a 2ml collection tube and centrifuged at 8000rpm for 1minute and the filtrate was discarded. This procedure was performed twice. Then 500µl of Buffer AW1 was added and centrifuged again at 8000 rpm for 1 minute. The filtrate was discarded and the column was placed in a fresh 2ml collection tube. Then 500µl of buffer AW2 were added to the column then centrifuged at 14,000 rpm for 3 min and the filtrate was discarded. Then 65µl of Buffer AVE was added to the column, equilibrated at room temperature for 1 minute then centrifuged at 8000 rpm for 1 minute. Finally, the nucleic acid of the virus was obtained.

3.6.6.2. Real time RT-PCR

The qualitative one step real-time polymerase chain reaction (rRT-PCR) assay was applied for primary identification of the FMDV using primers and probe described by Callahan *et al*. (2002) that detects the 3D RNA polymerase encoding gene. The nucleotide sequence of forward primer (5'-ACTGGGTTTTACAAA CCT GTGA-3'), reverse primer (5'-GCG AGT CCT GCCACGGA-3') and the 3D probe (5'-6-FAM-TCC TTT GCA CGC CGT GGG ACTAMRA-3') were used in this assay. The probe labeled with 6- (FAM) at the 5' end and the quencher tetramethylrhodamine (TAMRA) at the 3'end in Real-time RT-PCR reaction detects the 3D^{pol} gene sequence in all the FMDV serotypes. The reaction was based on procedure combined with reverse transcription and Real-time assay. Therefore Real-time assay was carried out by Superscript III/Platinum Taq one-step rRT-PCR kit. The composition of the 25 µl Master Mix for the one-step rRT-PCR included the following: 12.5 µl 2x- reaction buffer, 2.0 µl of each of the forward and reverse primer, 1.5 µl of the probe, 5.0 µl extracted RNA, 0.5 µl Superscript III RT/Platinum Taq mix, 1.5 µl of molecular grade H2O. The amplification carried out in the thermal cycler at a temperature cycle as follows: Reverse transcription (one cycle), 48 ^{o}C for 30 minutes, the initial denaturing, at 95 ^{o}C for 10 minutes; then 50 cycles

consisting of 95^{o}C for 15 seconds and 60^{o}C for 1 minute and 72^{o}C for 30 seconds. Negative and positive controls were included in each run (Mukasa *et al*., 2016).

Interpretation of real time PCR result

The cycle threshold or crossing point (Ct value) corresponds to the number of cycles required for a given sample to reach the threshold above is considered as positive. Using a positive cut-off cycle threshold (Ct) value of $\leq$ 40, FMDV genome was detected by rRT-PCR. The PCR cyclical cut-off point lies $\leq$ 40 were FMDV positive sample while > 40 indicates negative samples (Ludi *et al*., 2016).

3.6.6.3. DNA sequencing and phylogenetic analysis

Eighteen clinical samples collected from five different outbreaks (Table 5) was sent to World Reference Laboratory for FMD (WRLFMD), The Pirbright Institute, Pirbright, United Kingdom, for confirmatory diagnosis and molecular characterization of the causative FMDV strain according to recommended international standard (Callahan *et al*., 2002). Sequencing was conducted in WRLFMD and all the sequences were edited in the same laboratory. The edited sequences were used for molecular characterization of the virus by phylogenetic tree reconstruction. For this multiple sequence alignments were performed using the Clustal W algorithm implemented in MEGA6 software (Tamura *et al*., 2013) package to compare the VP1 sequence of outbreaks isolate with other reference isolates retrieved from GenBank. Alignment of VP1 nucleotide sequence was also done by using Clustal W applied in BioEdit software (Thompson et al., 1994). For comparative studies, BLAST search as conducted PUMED using MEGA6 software (Tamura *et al*., 2013) was used to collect additional FMDV serotype O and A sequences from GenBank NCBI (National Centre Biotechnology Information). Phylogenetic tree of FMDV VP1 sequences from Ethiopia and Africa, and from those other countries were constructed by using minimum-evolution methods of analysis as impelemented in MEGA6. The robustness of tree topology was assessed with 1000 bootstrap replicates and bootstrap values of $\geq$70 are shown at the relevant major nodes (Gorna *et al*., 2014).

Table 5: List of Representative Sample Summited to WRLFMD

Site of outbreak	Kebele	Country code	Date of sampling	Type and no. of sample
Meki	Sera wekele	ETH-02-18, ETH-06-18, ETH-10-18	05/01/18	3 Tissue
	Tuchi	ETH-11-18, ETH-13-18, ETH-14-18	12/01/18	3 Tissue
Bishoftu	K05	ETH-92-17, ETH-95-17, ETH-86-17	29/12/17	1 Pooled OP
Addis Ababa	Bole-semit	ETH-30-18, ETH-31-18	17/02/18	1 Pooled tissue
Shewarobit	Yelen	ETH-17-18, ETH-20-18, ETH-23-18	24/01/18	3 Tissue
	Kewet	ETH-24-18, ETH-26-17, ETH-27-17	24/01/18 20/12/17	3 Tissue
South Wollo	Albeko	ETH-35-18, ETH-36-18	04/02/18	2 Tissue
	Werebabo	ETH-37-18, ETH-39-18	05/02/18	2 Tissue

ETH, Ethiopia

3.7. Data management and analysis

Data generated from field and laboratory work were recorded in Microsoft Excel spreadsheet and descriptive statistics were used to calculate the proportion. The generated sequence data were aligned by multiple sequence alignments using the Clustal W algorithm as implemented in BioEdit and MEGA6 software packages to compare the VP1 sequence of outbreaks sequences with other reference sequences. The aligned sequence were used to reconstruct phylogenetic tree using minimum-evolution methods of analysis and maximum likelihood method imbedded in MEGA6 (Tamura *et al*., 2013) and confidence levels were assessed by 1000 bootstrap replications. Serotypes were distinguished on the basis of nucleotide sequence differences of 30-50% and high bootstrap support (> 70%) while a divergence of 15% distinguishes topotypes (Souley *et al*., 2018).

4. RESULTS

4.1. Clinical examination

Out of 125 cattle physically examined in four outbreaks, 56 (44.8%) cattle showed a typical clinical signs of FMD. The principal signs observed in clinically affected cattle were fever more than 40ºC, ropy salivation, lameness, vesicles and erosions in gums, dorsum of the tongue and in the inter-digital spaces and coronary band. Lesions like erosion and ulcer on the oral cavity were the most encountered signs during the clinical examination of affected cattle in all study areas except in Bishoftu and Addis Ababa. In those two areas, lesions encountered were on the hoof and it was so severe that the hoof tended to separate from the coronary band and complicated with bacterial infection (Appendix 9). One animal from Shewarobit also showed severe lesion on the hoof. The interview result about the vaccination history showed that animals were not vaccinated against FMD in all areas including the intensive farm in Bishoftu and Addis Ababa. The farm owner complains for shortage of vaccine supply.

Table 6: Clinical examination results of cattle during the filed study

Sites of outbreak	**Kebele**	**No. of animal examined**	**No. animal clinically infected**
Bishoftu	k 05	17	5
Meki	Sera wekele	30	14
	Tuchi	22	11
Addis Ababa	Bole-Semit	11	4
Shewarobit	Yelen	30	14
	Kewet	15	8
Total		**125**	**56/125 (44.8%)**

No.: number

4.2. FMD Virus isolation

Out of 37 samples collected from different outbreaks, 22 (59.46%) representative samples from each outbreak were subjected for virus isolation using Baby Hamster Kidney (BHK-21) cell culture. The rest 15 (40.54%) samples were not inoculated to the BHK-21 cell because two and more samples were collected from each outbreak so that representative samples were used for virus isolation. From 22 samples cultured on BHK-21 cell, cytopathic effect (CPE) was observed on 15 (68.18%) samples (Table 7), while virus did not grow (CPE was not observed) on the rest of the 7 (31.82%) samples even at third blind passage. From the total samples inoculated, representative samples from Meki, Shewarobit and South Wollo showed CPE on BHK-21 cells; while samples from Bishoftu and Addis Ababa didn't show CPE on BHK-21 cell; as a result virus were not isolated from those two sites. The CPE (morphological alteration) observed in BHK-21 cell were fast destruction of mono-layer cell and the infected cell appeared as singly and round in shape (Figure13). Additionally, the CPE was characterized by complete destruction of the cell and cell detachment which was mostly seen within 48 hrs of inoculation.

Table 7: FMDV isolated from cattle from different outbreaks

Site of outbreak	**Kebele**	**No. of sample inoculated**	**Type of samples**	**Date of collection**	**Virus isolated**
Meki	Sera Wekele	3	2Tissue and 1OP	5/01/18	ETH/02/18, ETH/07/18
	Tuchi	3	Tissue	12/01/18	ETH/11/18, ETH/13/18, ETH/14/18
Bishoftu	K05	2	OP	29/12/17	__
Addis Ababa	Bole Semit	1	Tissue	17/02/18	__
Shewarobit	Yelen	4	3Tissue and 1OP	24/01/18	ETH/17/18, ETH/21/18, ETH/23/18
	Kewet	3	Tissue	24/01/18	ETH/24/18, ETH/27/17
South Wollo	Albeko	2	Tissue	4/02/18	ETH/35/18, ETH/36/18
	Werebabo	4	Tissue	5/02/18	ETH/38/18, ETH/39/18, ETH/41/18
Total		**22**			**15/22 (68.18%)**

ETH, Ethiopia; OP, Oro-pharengeal fluid

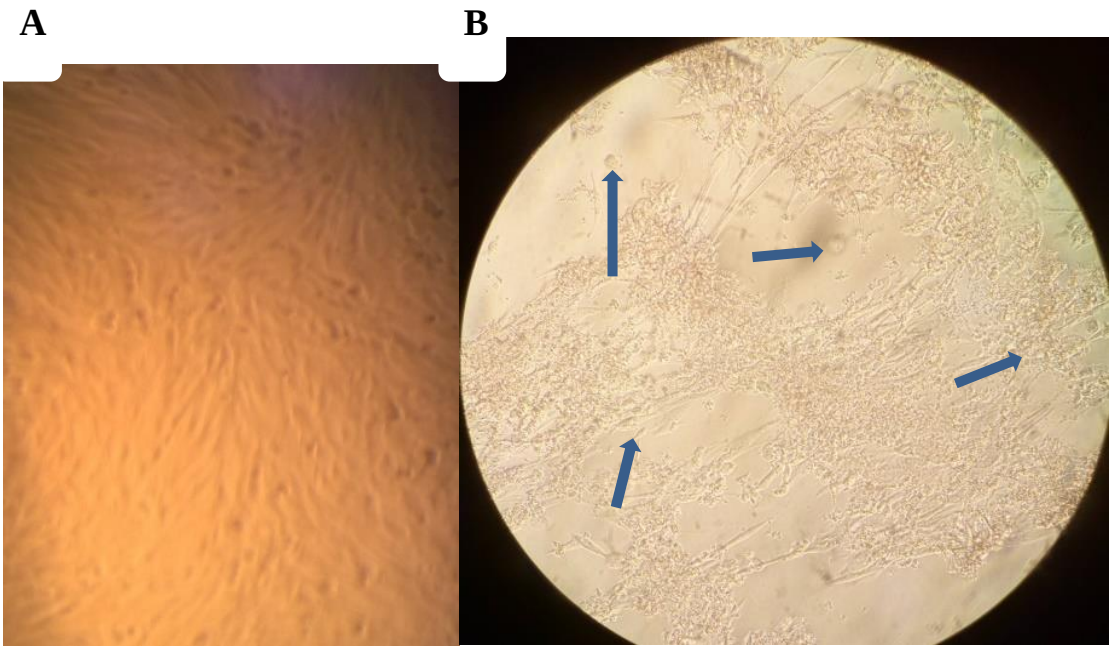

Figure 14: Picture showing FMDV isolation on BHK-21 cell line. A: BHK-21 cell control (Virus was not inoculated), B: showed morphological change (CPE) after FMDV inoculation as indicated by the arrow. Arrow shows rounded cells as a result of FMD virus infection.

4.3. FMDV serotype identification

Sixteen out of 37 (43.24%) the clinical samples were typed as serotype O using serotype differentiating antigen detection sandwich ELISA, while in 6 (16.22%) samples serotype A was detected. In the rest 15 (40.54%) sample, on virus was detected (Table 8). The detected serotypes showed regional difference in that serotype O was identified in samples collected from Shewarobit and South Wollo areas of Amhara regional state but, serotype A was detected in samples collected from Meki district of Oromia regional state.

Table 8: Serotypes of FMDV identified in the study areas

Site of outbreak	Kebele	No. of sample collected	No. of sample isolated	No. serotype identified	
				O	A
Meki	Serawekele	7	2	-	3
	Tuchi	4	3	-	3
Bishoftu	K05	3	-	-	-
Addis Ababa	Bole semit	2	-	-	-
Shewarobit	Yelen	7	3	5	-
	Kewet	4	2	3	-
South Wollo	Albeko	5	2	3	-
	Werebabo	5	3	5	-
Total		**37**	**15/22 (68.18%)**	**16/37 (43.24%)**	**6/37 (16.22%)**

4.4. Molecular characterization

4.4.1. FMDV gene detection by real time reverse transcriptase polymerase chain reaction (rRT-PCR)

The PCR amplification was carried out in the thermal cycler to amplify and detect genome fragments of FMD virus in clinical materials including epithelium and Oro-pharyngeal samples. The extracted RNAs from clinical samples were tested by rRT-PCR method targeting the 3D regions of FMD virus genome to determine the presence of viral RNA in clinical samples. The successfully amplified target gave an amplification curve and the cycle threshold (Ct), at which the target amplicon was initially detected above the background fluorescent levels as determined by the imbeded software. The Ct cut-off value is $\leq$ 40.0 and any Ct value <40.0 indicates positive result and Ct values > 40 indicated negative result. Out of 27 samples tested by rRT-PCR, 12 (44.44%) samples

were found positive having Ct value ranging from 20 to 40 (Figures 15 and 16). The lowest Ct value (=20) was recorded in epithelial samples collected from Shewarobit district, while the highest Ct value (= 40) was obtained from bovine oro-pharyngeal fluid sample collected from the same site. In most of epithelial sample tested, the Ct value was lower than oro-pharyngeal fluid samples.

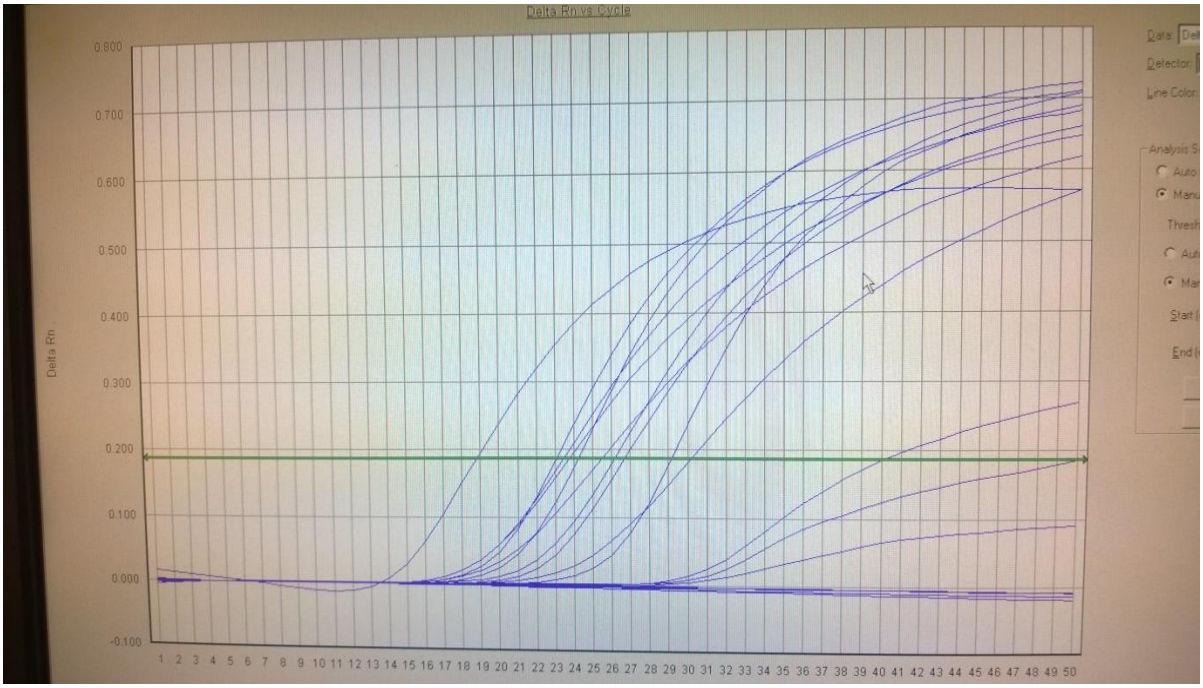

Figure 15: Real time RT-PCR result showing amplification curve

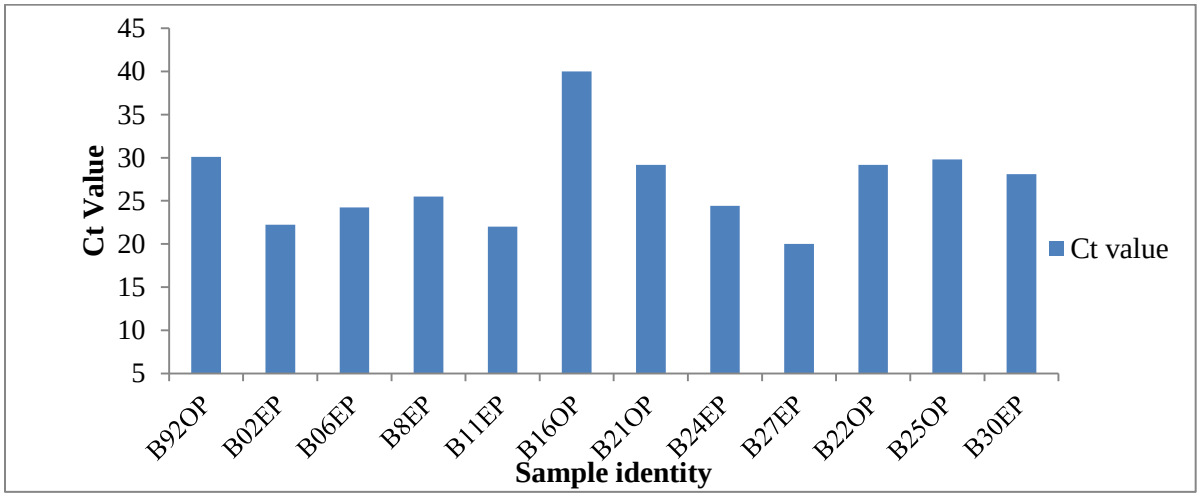

Figure 16: Real time PCR result with Ct value showing a positive sample. B, Bovine; EP, Epithelial sample; OP, Oro-pharyngeal fluid sample

4.4.2. Phylogenetic analysis

From the FMDV isolated in this study, amplicons corresponding to the complete VP1 coding region were generated by RT-PCR and sequence data for 13 (9 for serotype O and 4 for serotype A) virus isolates in WRLFMD, Pirbright, UK. The sequence data were used for comparison with other available viral sequences downloaded from NCBI, GenBank to reconstruct phylogenetic relationships of the isolate.

4.4.2.1. Phylogenetic analysis of FMDV serotype O

Assessment of phylogenetic analysis of 639 nucleotide sequence of VP1 gene of FMDV serotype O isolates showed that, both Shewarobit and South Wollo strains were clustered with other strains belonging to the East African topotype-3 (EA-3). The isolates from Yelen (O/ETH/23/2018,O/ETH/17/2018 and O/ETH/20/201) and Kewet (O/ETH/24/20 18 and O/ETH/27/2017) kebele of Shewarobit shared 99.5% nucleotide identity. Similarly, isolates from Albeko (O/ETH/36/2018 and O/ETH/35/2018) and Werebabo (O/ETH/37/2018 and O/ETH/39/2018) kebele of South Wollo had only 0.6% nucleotide diversities. Furthermore, isolate from both outbreak sites (Shewarobit and South Wollo) had also 99.3% nucleotide similarity with each other. The VP1 sequences FMDV serotype O isolates in this study were also compared with other Ethiopian and African isolates available in the GenBank database (Figure 17). Accordingly, the isolate O/ETH/17/2018 and O/ETH/24/2018 from Yelen and Kewet kebele of Shewarobit had 7.8% nucleotide divergence with Ethiopian isolates of O/ETH/3/96 and O/ETH/30/94. However, only 3.4% nucleotide diversities were recorded when compared to Sudan isolates (O/SUD/3/2008, O/SUD/5/2008 and O/SUD/4/2008 with an accession number KR149728, GU566061 and KJ831704, respectively) which are genetically homologues based on the 639 nucleotide sequence of VP1 gene and this is supported by 99% bootstrap value. The phylogenetic analysis of those isolates also showed that, they are differing by 15.7% with EA-1 (O/UGA/5/96, O/KEN/83/79), 17.1% with EA-2 (O/KEN/5/2002, O/UGA/3/2002), 21.5% with EA-4 (O/ETH/58/2005, O/ETH/59/2005) and 22.1% with WA (O/BKF/1/92, O/BKF/2/92) virus group.

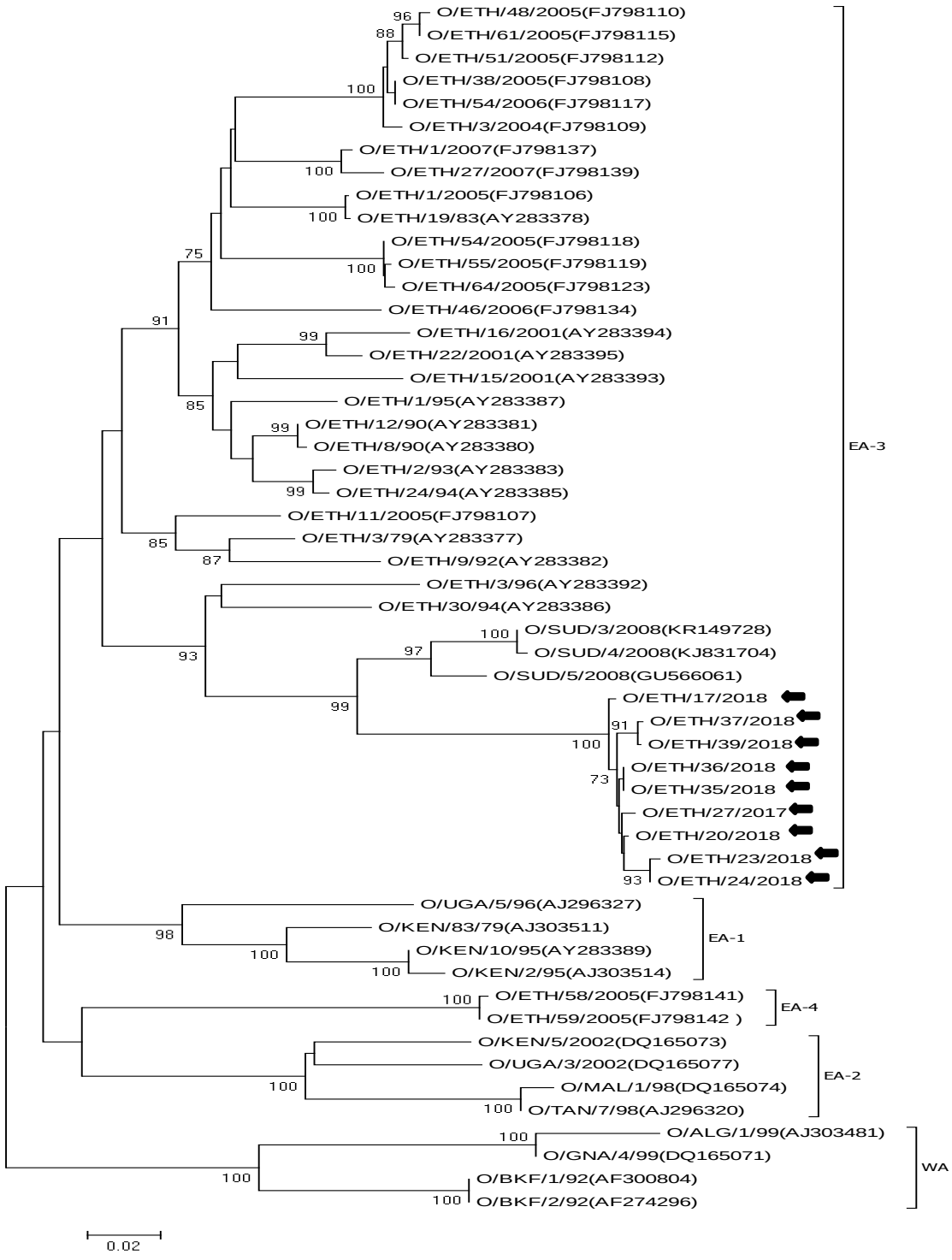

Figure 17: Minimum-Evolution tree showing the relation between the serotype O isolates from Ethiopia and reference viruses. The arrow (⬅) indicates the new isolates obtained from the outbreak cases of Shewarobit and South Wollo. Bootstrap value <70% were not shown in the picture. EA, East Africa; WA; West Africa.

4.4.2.2. Phylogenetic analysis of FMDV serotype A

Four FMDV serotype A isolates from Sera wekele (A/ETH/02/18) and Tuchi (A/ETH/13/2018, A/ETH/11/2018, A/ETH/14/2018) kebeles of Meki were belonged to genotype G-I within the Africa topotype as determined by phylogenetic analysis. Those viruses shared above 97% sequence similarity with viruses grouped under this genotype. This is the first report for the existence of G-I in Ethiopia. The viruses isolated from both kebeles (Sera wekelle and Tuchi) were closely related and sharing 99.8% nucleotide identity. The phylogenetic analysis of the VP1 coding sequences of A/ETH/13/2018 also showed, it was highly similar with the neighboring Kenyan isolate of 2008 and 2009 (A/KEN/28/2008 and A/KEN/22/2009 with an accession number KF561702 and KF561703, respectively) and Tanzanian isolate of 2009 (A/TAN/47/2009 and A/TAN/42/2009 with an accession number KF561697 and KF561695, respectively) having >97% nucleotide similarity, which supported by 100% bootstrap value. However, it was dissimilar with Ethiopian isolate (A/ETH/7/92 with an accession number EF208765) with 17% nucleotide level which is previously grouped under genotype G-VII. The genetic relationship of the current isolates with the other serotypes A are displayed on phylogenetic tree (Figure 18).

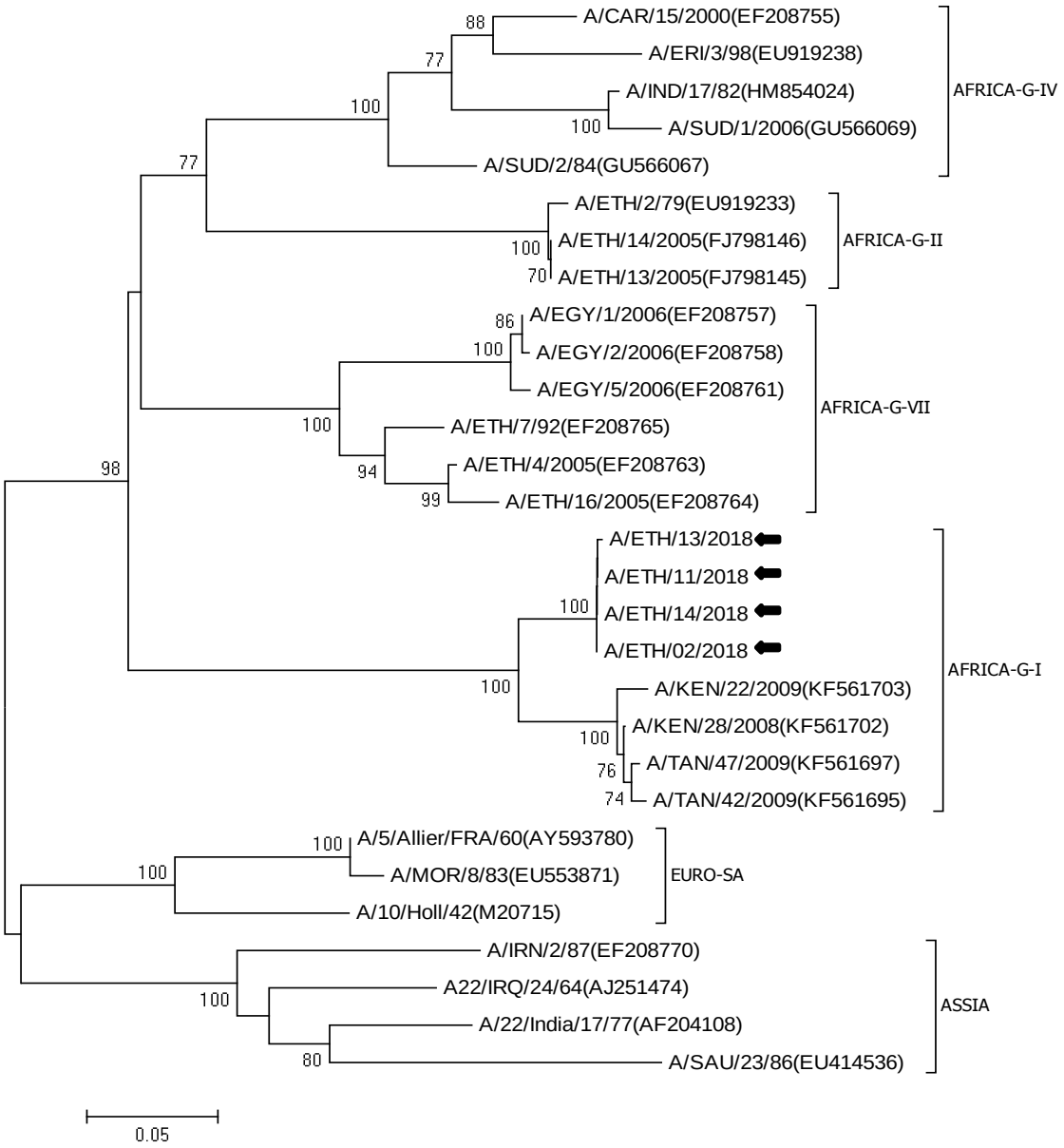

Figure 18: Minimum-Evolution tree showing the relation between the serotype A isolates from Ethiopia and reference viruses. The arrow (⬅) indicates the new isolates obtained from the outbreak cases of Meki. Bootstrap value <70% were not shown in the tree. G, Genotype; EURO-SA, Europe - South America.

4.4.3. Sequence based comparison of Serotype O isolates with the vaccine strain (O/ETH/38/2005)

The VP1 sequence of serotype O isolates identified in this study were compared with the VP1 sequence of vaccine strain currently used for vaccine production in National Veterinary Institute (NVI), Ethiopia to determine their genetic relationship. Accordingly, 107 (17%) nucleotide variable sites were detected between VP1 sequence of the vaccine strain (O/ETH/38/2005 with an accession number FJ798108) and serotype O isolate from Shewarobit and South Wollo across the 639 nucleotides while 532 (83%) were conserved over the region. Most of these nucleotide variations were occurred at 3rd codon (71.03%) positions, while some in the 1st (18.69%) and 2nd (10.28%) codon positions. The deduced amino acid sequences were also aligned and investigated in an attempt to determine the amino acid variations. A total of 22/ 213 (10.3%) amino acid variations were observed in different sites of the VP1 gene while the rest 191/213 (89.7%) amino acids (aa) were conserved with reference to the vaccine strain. Those variations were predominantly occurred in two distinct regions, comprising the G-H loop (aa positions 133–158) and the C-terminus (aa positions 194–213), known to be the main immunogenic sites of VP1 (Ayelet *et al*., 2014; Sahle *et al*., 2004). For example, the aa substitutions at different positions are: 133 (N→S), 138 (V→E), 139 (T→A), 140 (S→R), 141 (V→A), 142 (T→A), 158 (P→T), 197 (S→A) and 198 (E→G) amino acid replacement was documented in all current isolates compared to vaccine strain. In position 134 most of the isolate including the vaccine strain, contained a C while the recent isolate O/ETH/23/2018 and O/ETH/24/2018 had a G. However, amino acid change also observed in other antigenic sites for example at antigenic site three at position 45 (Samuel, 2000) threonine (T) was replaced by glutamine (Q) (Figure 18). Interestingly, the RGD cell attachment sites within the G–H loop of the gene at position 145-147 were conserved in all isolate including the vaccine strain.

```
                                        10        20        30        40
                                ....|....|....|....|....|....|....|....|
O/ETH/38/2005 VP1/FJ798108      TTSPGESADPVTATVENYGGETQVQRRQHTDVSFILDRFV
O/ETH/27/2017                   ..............D..............A.......
O/ETH/17/2018                   ............................A.......
O/ETH/20/2018                   ............................A.......
O/ETH/23/2018                   ............................A.......
O/ETH/24/2018                   ............................A.......
O/ETH/35/2018                   ............................A.......
O/ETH/36/2018                   ............................A.......
O/ETH/37/2018                   ............................A.......
O/ETH/39/2018                   ............................A.......

                                        50        60        70        80
                                ....|....|....|....|....|....|....|....|
O/ETH/38/2005 VP1/FJ798108      KVTPTDQINVLDLMQTPAHTLVGALLRAATYYFADLEVAV
O/ETH/27/2017                   ....QS.T.......I.S.........S............
O/ETH/17/2018                   ....QS.T.......I.S.........S............
O/ETH/20/2018                   ....QS.T.......I.S.........S............
O/ETH/23/2018                   ....QS.T.......I.S.........S............
O/ETH/24/2018                   ....QS.T.......I.S.........S............
O/ETH/35/2018                   ....QS.T.......I.S.........S............
O/ETH/36/2018                   ....QS.T.......I.S.........S............
O/ETH/37/2018                   ....QS.T.......I.S.........S............
O/ETH/39/2018                   ....QS.T.......I.S.........S............

                                        90       100       110       120
                                ....|....|....|....|....|....|....|....|
O/ETH/38/2005 VP1/FJ798108      KHEGNLTWVPNGAPESALDNTTNPTAYHKAPLTRLALPYT
O/ETH/27/2017                   ..............T.........................
O/ETH/17/2018                   ..............T.........................
O/ETH/20/2018                   ..............TS........................
O/ETH/23/2018                   ..............T.........................
O/ETH/24/2018                   ..............T.........................
O/ETH/35/2018                   ..............T.........................
O/ETH/36/2018                   ..............T.........................
O/ETH/37/2018                   ..............T.........................
O/ETH/39/2018                   ..............T.........................

                                        130       140       150      160
                                ....|....|....|....|....|....|....|....|
O/ETH/38/2005 VP1/FJ798108      APHRVLATVYNGNCKYGVTSVTNVRGDLQVLAQKAARPLP
O/ETH/27/2017                   ...........S....EARAA............V.T..
O/ETH/17/2018                   ...........S....EARAA..............T..
O/ETH/20/2018                   ...........S....EARAA..............T..
O/ETH/23/2018                   ...........SG...EARAA..............T..
O/ETH/24/2018                   ...........SG...EARAA..............T..
O/ETH/35/2018                   ...........S....EARAA..............T..
O/ETH/36/2018                   ...........S....EARAA..............T..
O/ETH/37/2018                   ...........S....EARAA..............T..
O/ETH/39/2018                   ...........S....EARAA..............T..
```

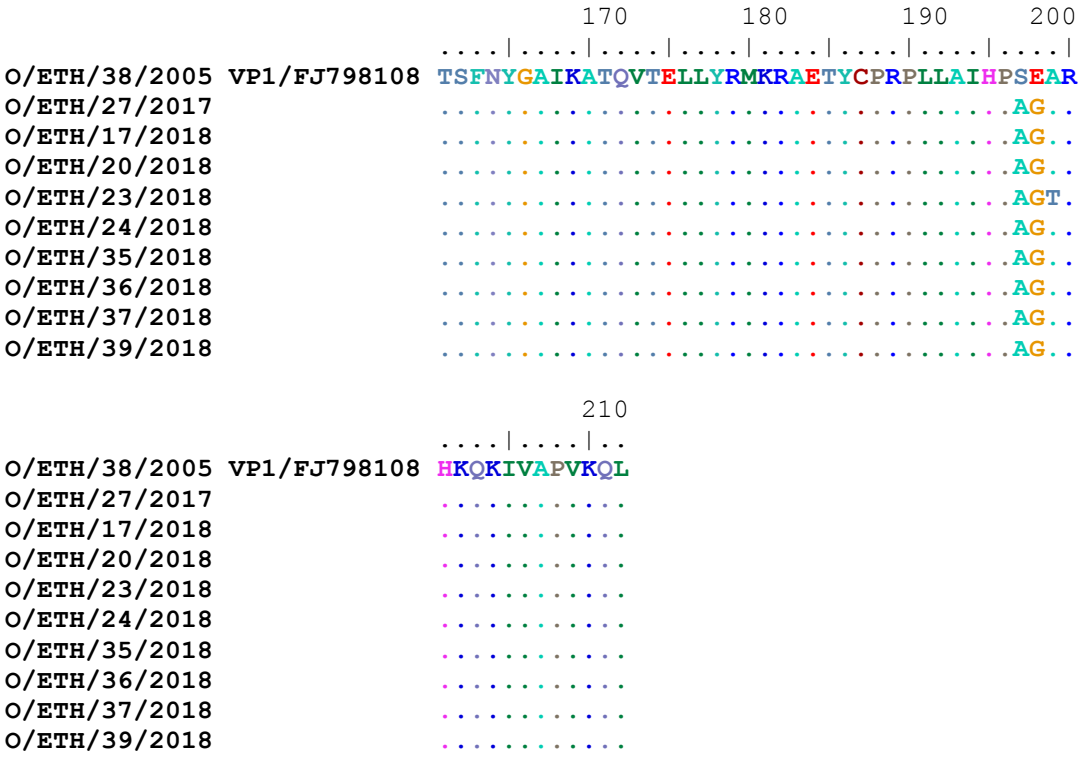

Figure 19: Amino acid sequence alignments of VP1 gene of serotype O FMD viruses investigated in this study with the vaccine strain (O/ETH/38/2005) used in Ethiopia. The amino acids sequence of vaccinal strain (O/ETH/38/2005) is written on top row and the conserved amino acids are indicated by dots of different colors.

5. DISCUSSION

The FMD outbreak investigation and etiological agent characterization indicated that two serotypes namely serotypes O and A FMD viruses were circulating in the two regional states. Upon molecular characterization based on VP1 sequence data the serotype O FMDVs were grouped into topotype 3 while the serotype A FMD viruses were grouped into genotype I of the African topotype. In addition, the serotype O FMD viruses were identified from Amhara region while the serotype A viruses were identified from Oromia regional state suggesting the distributions of the FMD virus serotypes seems regionally specific in the current study. The observation of genotype I of serotype A FMD virus in Ethiopia for the first time need more detailed investigation concerning its sources and means of introduction of the virus to country.

FMD is a very recognizable disease from its clinical sign although some diseases are confusing the diagnosis. In present study, a total of 125 cattle from different sites of outbreaks were examined clinically for the presence of suggestive lesion of FMD. Out of these 56 (44.8%) showed the clinical signs (like excessive salivation, formation of vesicles and erosion on the tongue, gum and dental pad, as well as inter-digital spaces and coronary bands on the feet) which typical of the disease (Appendix 9). The signs and lesion observed in the diseased cattle were similar with those reported by other researchers like Aftosa (2015), Alexandersen *et al*. (2003) and Quinn *et al*. (2005). However, there is variation in clinical severity and manifestation of the disease which could be associated with cattle breed affected, individual susceptibility of the cattle and the farming system (McLaws *et al*., 2006).

In this study, BHK-21 cell was used to isolate the virus from clinical samples. Out of 22 samples propagated on BHK-21 mono layer cell, 15 (68.18%) showed cytopathic effect (CPE) after 24 hrs incubation in a humidified incubator at 37°C and 5% CO2. But, in some sample CPE was observed after 48hrs. In the rest 7 (31.82%) samples, virus growth was not observed on the cell (no CPE was observed) even after third passage. Most of the

sample that didn't showed CPE were oro-pharyngeal fluid samples collected from Bishoftu, Meki and Shewarobit probably because the samples were collected from animals that had previous history of the disease but at early stage of recovery during sample collection. This could suggest that the chances of virus isolation from oro-pharyngeal fluid samples were lower as a result of lower concentration of virus. In support of this observation, OIE (2009) reported that the preferred sample for virus isolation is epithelial tissue sample from unruptured or freshly ruptured vesicle. In isolated virus the CPE observed were characterized by rounding, detachment as well as destruction of mono-layer cell. In agreement with this, Menda *et al*. (2014), Negusssie *et al*. (2011) and Sulayeman *et al*. (2018) reported FMDV growth on BHK-21 cell which was characterized by fast destruction of monolayer cells and infected cells were appeared singly and the cell was found round in shape.

The serotype identification in the present study confirmed that, FMDV serotype O was the predominant serotype circulating in different sites of outbreaks of Ethiopia. From 37 sample serotyped by sandwich ELISA, 16 (43.24%) were identified as serotype O while 6 (16.22%) were identified as serotype A. In agreement with this finding, Ayelet *et al*. (2009), Menda *et al*. (2014), Negussie *et al.* (2011), Sulayeman *et al*. (2018) and Urge (2017) reported serotype O as the most prevalent and the dominant serotype causing outbreaks in different parts of the country. Klein (2009) and Rweyemamu *et al*. (2008) also indicated that, the two serotypes are the prevailing serotypes worldwide. In the current study, serotype O was isolated from sample collected from Shewarobit and South Wollo districts of Amhara Regional State. In line with this, Assefa *et al*. (2017) previously reported serotype O FMDV from south wollo and other districts around it. Furthermore, the work of Alemu (2014) and Jemberu *et al*. (2016) also revealed that serotype O was mostly isolated in different parts of the region.

In this study, serotype A was identified from samples collected from Meki districts of Oromiya Regional State. Previously this serotype was reported from samples collected from Hadiya and Yabello areas (Ayelet *et al*., 2013) Sinana and Yabello area (Negussie *et al*., 2013) and Konso area (Alemu, 2014); similarly serotype A FMDV were reported

from bovine and swine samples obtained from different outbreak areas of Ethiopia (Gelaye *et al*., 2007). In addition, Sulayeman *et al*. (2018) also reported serotype A from Arsi districts of oromiya region which is neighboring to Meki district. Thus, because of the presence of unrestrected animal movement, transmision of the disease and distribution of the serotype from one site to other could happen easily. This observation was in agreement with Mersie *et al*. (1992) who reported, the high incidence of the disease in Ethiopia may be associated with uncontrolled animal movement and the high rate of contact between animals at marketing and common grazing places as well as at watering points.

From 27 samples tested by rRT-PCR targeting the 3D region, in 12 (44.44%) samples genome was detected. The Ct value in positive sample showed the cycle threshold, at which the target amplicon was initially detected above the background fluorescent levels. From the result, the lowest (20) and the highest (40) Ct value was recorded in epithelial and oro-phryngeal sample collected from shewarobit area, respectively. The Ct value from epithelial sample was lower than oro-pharyngeal fluid sample. This might indicates higher level of viral RNA was found in freshly collected epithelial samples than oro-pharyngeal fluid samples. The lower concentration of FMDV in oro-pharyngeal fluid sample might be due to sampling time; since they are collected from convalescent cattle in later stage of the outbreaks.

Sequence coding for the FMDV VP1 protein has been used extensively to investigate the relationship between different isolates of the virus worldwide (Gorna *et al*., 2014). On basis of VP1 gene sequence comparison, existence of 8 serotype O topotypes has been confirmed within samples collected around the world. Among those, 2 topotypes were found in Africa, one in East Africa, and the other in West Africa (Samuel and Knowles, 2001). FMD viruses with <5% of nucleotide differences in the VP1 sequence are considered as closely related (Gorna *et al*., 2014) and in those viruses serotype (O, A, C) with >85% nucleotide similarity were clustered in a single genetic lineage (Ayelet *et al*., 2009). It is also assumed that, FMD viruses that differ between 2–7 % from each other are generally believed to originate from the same epizootic (Slager-Bastos, 2001). Thus,

VP1 sequences analysis of the current serotype O isolates from Shewarobit and South Wollo revealed that, they were clustered in East Africa lineage III (EA-3) and sharing above 92% nucleotide identity with virus O/ETH/3/96 and O/ETH/30/94 which grouped under this lineage. Similarly, Ayelet *et al*. (2009), Menda *et al*. (2014), Negussie *et al*. (2011) and WRLFMD (2016) reported EA-3 lineage in different parts of Ethiopia based on comparison of VP1 gene sequence. The isolates from this two outbreak site had 99.3% nucleotide similarity which is <5% indicating the viruses in both areas were closely related and they probably originated from the same. In addition, isolate O/ETH/17/2018 and O/ETH/24/2018 shared 96.6% nucleotide identity with Sudan isolates of 2008 (O/SUD/3/2008, O/SUD/5/2008 and O/SUD/4/2008). In support of this finding, Kassaw and Berihun (2013) showed that serotype O FMDV isolates of Ethiopia and Sudan had 95.31% nucleotide similarity between isolate O/ETH/59/2011 from Ethiopia and O/SUD/3/2008, O/SUD/4/2008 and O/SUD/3/2008 isolates from Sudan.

Serotype A identified in sample collected from Sera wekele and Tuchi kebeles of Meki district in the current study were clustered to genotype I (G-I) of African topotype. This is the first report for existence of G-I in Ethiopia. Thus, further detailed genetic analysis is needed to be conducted to have more insight about this genotype. The viruses from both kebeles were closely related with only 0.2% nucleotide divergence indicating the viruses originated from the same sources. The isolates A/ETH/13/2018 from Tuchi kebele shared >97% nucleotide similarity with isolates in Kenya (A//KEN/28/2008 and A/KEN/22/2009) and Tanzania (A/TAN/47/2009 and A/TAN/42/2009) which are grouped previously under G-I indicating virus from Ethiopia, Kenya and Tanzania shares a common epidemiological link due to the presences of free cattle movement at border. In support of this, Kasanga *et al.* (2015) reported, 97.5-99.2% nucleotide identity between the Tanzanian virus (A/TAN/47/2009 and A/TAN/42/2009) and the Kenyan isolates (A/KEN/28/2008 and A/KEN/22/2009).

Generally, in the current study both serotype O and A are highly similar with isolates of the virus in neighboring countries like Sudan for serotype O and Kenya and Tanzania for serotype A. This suggested that the outbreaks due to those isolates were most probably

spread by uncontrolled trans-boundary animal's movement, and live cattle are traded from Borna to Meki and Adama for fattening purposes. Such uncontrolled cattle movements have a big risk on the transmission of the virus across the border in both directions because there is no strong animal movement regulation across the border besides the ability of the virus to transmit via wind. This statement is supported by Samuel *et al*. (1999) who reported that, closely related viruses could either be from the same outbreak or from viruses temporally closely related. Sangare (2005) also described the presence of trans-boundary and trans-continental transmission of viruses in west and southern Africa.

A total of 107/639 (17%) nucleotide and 22/213 (10.3%) amino acid changes were noted at the VP1 gene of field isolate when compared to the vaccine strain (O/ETH/38/2005). Most of these variations were occurred in G-H loop at positions 133–158 and the C-terminus region at positions 194–213, known to be immunodominat region of VP1. In support of this Aggarwal and Barnett (2002) reported a peptide vaccine from VP1 at amino acid position 141-160. As he added this amino acid position is immunodominant region in serotype O of FMDV. Amino acid change was also noted in antigenic site 3 at position 45, were threonine (T) was replaced by glutamine (Q). The presence of very limited genetic variation in the immunodominant regions can alter the antigenic specificity of FMD viruses isolates (Sahle *et al*., 2004). Thus, in this study, the existence of significant level amino acid variation especially at immunodominant region indicates the need for in-vitro vaccine matching studies to establish the level of protection conferred against the currently circulating viruses by the available vaccine strains. These changes have implications in vaccine strain selection as these changes occurred in G-H loop as compared with the vaccine strain in use in the Ethiopia.

6. CONCLUSION AND RECOMMENDATIONS

In present study, serotype O and A was identified and isolated on BHK-21 cell culture from the different outbreaks, and serotype O was the dominant one. The molecular characterization of these serotype revealed, the current isolate of serotype O was clustered with East African topotype-3 (EA-3) while serotype A was grouped in genotype I of African topotype. Genotype-I (G-I) of serotype A FMD virus is observed in Ethiopia for the first time and the viruses shared >97% nucleotides identity with Kenyan and Tanzanian isolates. Thus suggests the emergence of new topotype probably from neighboring countries. During comparing serotype O isolates with the vaccine strain currently in use, a number of amino acid variation were noted at different site of VP1 gene including the immmunodominant region indicating the presence of genetic heterogenecity of the current isolates from vaccine strain.

Based on the finding of this study, the following recommendations are forwarded

- Research on continuous surveillance, serotyping and molecular characterization on FMDV should be conducted to check the introduction and circulation of new serotype and topotypes coupled emerging strains of the virus in the country.
- As genotype I of the serotype A FMD viruses newly observed in Ethiopia, vaccine matching test were should be conducted on these isolates and also serotype O isolate in order to assess the protection potential of the vaccines in use.
- Restriction of free movement of livestock (and probably livestock products) with neighboring countries and establishment of quarantine station around the border area is necessary to contain and detect the disease before being spread to susceptible populations since the emergence of new genotype was recorded.
- Government FMD control strategy through regular vaccination should be started and carried out in all susceptible animals and/or selected areas to control the disease and access export market.

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8. APPENDICES

Appendix 1: Sample collection format

No	Sample Code	Site of Outbreak			SPP	Breed	Sex	Age	Type of sample			Date of collection	Remark
		Region	Districts	Kebele					Tissue	VF	OP		
1													
2													

Appendix 1: Data collection format for clinical examination during outbreak investigation

No	Site of outbreak	Species	Breed	Sex	Age	No. of animal examined	No. of clinically affected animal	Remark
1								
2								

Appendix 3: Animals age determination based on teeth eruption

Species	Eruption of teeth	Age estimation
Bovine	One incisor	$\leq$ 2 years
	Two incisors Three incisors	From 2-3 years
	Canine teeth Wear of teeth	>3 years

Source: (Merck Veterinary Manual, 1998).

Appendix 4: Summary of sample collected in the present study from different outbreak site

Sample code	Area of outbreaks	Kebele	SPP	Breed	Sex	Age	Type of sample	Date of collection
B92	Bishoftu	Kebele 05	Bov	EX	F	Young	OP	29/12/17
B95	Bishoftu	Kebele 05	Bov	EX	F	Adult	OP	29/12/17
B86	Bishoftu	Kebele 05	Bov	EX	F	Adult	OP	29/12/17
B2	Meki	Sera wekele	Bov	LC	F	Young	TE& VF	5/1/18
B3	Meki	Sera wekele	Bov	LC	M	Adult	OP	5/1/18
B4	Meki	Sera wekele	Bov	LC	F	Adult	OP	5/1/18
B6	Meki	Sera wekele	Bov	LC	M	Adult	TE	5/1/18
B7	Meki	Sera wekele	Bov	LC	M	Young	HE	5/1/18
B8	Meki	Sera wekele	Bov	LC	M	Adult	TE	5/1/18
B10	Meki	Sera wekele	Bov	EX	M	Adult	Gum	5/1/18
B11	Meki	Tuchi	Bov	EX	M	Adult	TE	12/1/18
B12	Meki	Tuchi	Bov	LC	F	Adult	OP	12/1/18
B13	Meki	Tuchi	Bov	LC	M	Young	TE	12/1/18
B14	Meki	Tuchi	Bov	LC	F	Adult	TE	12/1/18
B16	Shewarobit	Yelen	Bov	LC	M	Young	OP	24/01/18
B17	Shewarobit	Yelen	Bov	LC	F	Adult	Gum	24/01/18
B19	Shewarobit	Yelen	Bov	EX	F	Adult	TE	24/01/18
B20	Shewarobit	Yelen	Bov	LC	F	Adult	HE	24/01/18
B21	Shewarobit	Yelen	Bov	LC	M	Young	OP	24/01/18
B22	Shewarobit	Yelen	Bov	LC	M	Adult	OP	24/01/18
B23	Shewarobit	Kewete	Bov	LC	M	Adult	TE	24/01/18
B24	Shewarobit	Kewete	Bov	EX	M	Young	TE	24/01/18
B25	Shewarobit	Kewete	Bov	LC	F	Adult	OP	24/01/18

B26	Shewarobit	Kewet	Bov	LC	M	Adult	TE	20/12/17
B27	Shewarobit	Kewet	Bov	LC	M	Adult	TE	20/12/17
B30	Addis Ababa	Bole-semit	Bov	EX	F	Adult	HE	17/02/18
B31	Addis Ababa	Bole-semit	Bov	EX	F	Adult	HE	17/02/18
B32	South wollo	Albeko	Bov	LC	M	Adult	Gum	31/01/18
B33	South wollo	Albeko	Bov	LC	F	Adult	OP	31/01/18
B34	South wollo	Albeko	Bov	LC	F	Young	HE	31/01/18
B35	South wollo	Albeko	Bov	LC	F	Adult	HE	31/01/18
B36	South wollo	Albeko	Bov	LC	M	Adult	TE	31/01/18
B37	South wollo	Werebabo	Bov	LC	M	Young	HE	31/01/18
B38	South wollo	Werebabo	Bov	LC	F	Young	TE	31/01/18
B39	South wollo	Werebabo	Bov	LC	M	Adult	TE	31/01/18
B40	South wollo	Werebabo	Bov	LC	M	Adult	OP	31/01/18
B41	South wollo	Werebabo	Bov	LC	F	Adult	Gum	31/01/18

Bov, Bovine; OP, Oro-pharaengeal fluid; TE, Tongue epithelium; HE, Hoof epithelium; VF, vesicular fluid; LC, Local; EX, Exotic

Appendix 5: Preparation of BHK-21 cell for virus inoculation

- First prepare a complete media containing 1x minimal essential media, 10% fetal calf serum, tryptose phosphate broth, 100x antibiotics and 1000x antimycotic solution.
- Pre warm the trypsine 0.25% and the phosphate saline buffer (PBS).
- Disinfect all the material used for sub culturing the cell by 70% ethanol and placing them into biosafety cabinet.
- Take the cell culture flask with confluent monolayer of BHK-21 cell from incubator to biosafety cabinet after spraying with 70% ethanol.
- Decant the old media and wash the cell briefly with PBS (which must be free from Ca and Mg if versine is used as dispersant) to remove the residual serum and the residual bivalent ion.
- Add trypsine and mixing gently and incubate for 10 minute at 37^{o}c.

- Add growth complete media and pipette gently to mix thoroughly until monolayer cell dispersed to single cell.
- Add the final amount of the complete media and dispense to other 3 TC-25 flask and then incubate at humified incubator with 5% CO2 at 37^{o}c.
- Then follow the growth of the cell line and for any contamination using inverted light microscope.

Appendix 6: Virus isolation procedure on BHK-21 cell

- The tissue suspension virus sample is thawed.
- 2% MEM were thawed in water bath.
- Material used and the sample bottle were disinfected by 70% ethanol and blotted dry by sterile gauze.
- Put the cell, media and other materials that are used in inoculation into biosafety cabinet.
- Discard the old media from the monolayer cell and wash the cell gently with 2-3ml of pre warmed PBS.
- Inoculate 1ml of virus sample to the cell in $25cm^2$ tissue culture flasks and rock the flask gently to distribute inoculum evenly over the monolayer cell.
- Incubate inoculated culture in incubator at 37^{o}c for 1hr to allow virus adsorb.
- Add 7ml of 2% MEM to the inoculated cell and incubate at 37°C and 5% CO2 in a humidified incubator.
- Monitor the inoculated tissue flask daily for the development of CPE and any other contamination.

Appendix 7: Plate layout for antigen detection sandwich ELISA

Catching MABs		SP 1	SP 2	SP 3	SP 4	SP 5	SP 6	SP 7	SP 8	SP 9	SP 10	P.con	N.con	
Type O	A													**Conjugate A**
Type A (1st MAB	B													
Type A (2nd MAB)	C													
Type Asia 1	D													
Type C	E													
Pan O-A-C-Asia 1	F													
Type SAT 2	G													**Conjugate B**
Type SAT 1	H													

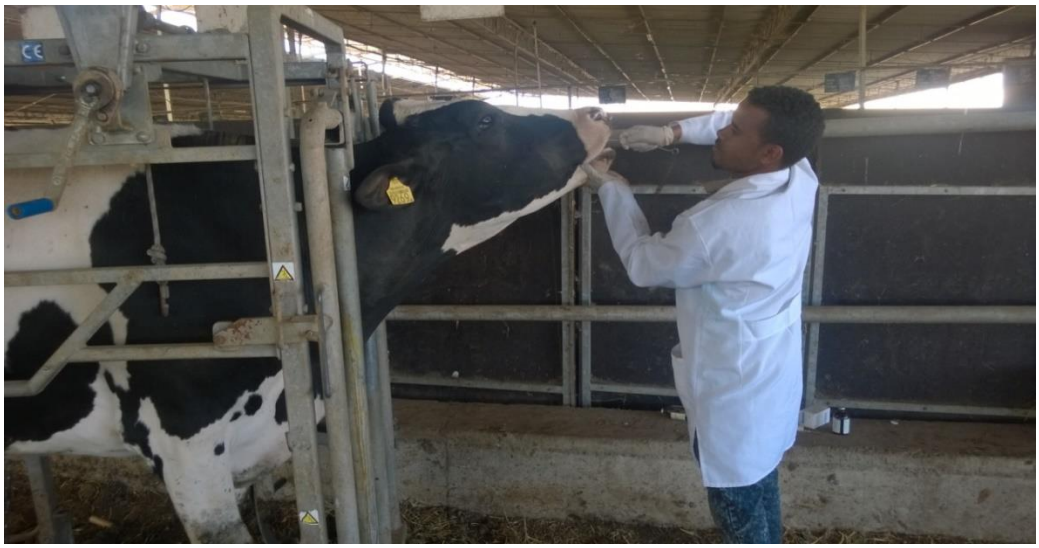
SP, Sample; MAB, Monoclonal antibody; P.con, Positive control; N.con, Negative control

Appendix 2: Nucleotide sequence of primer used for sequencing VP1 protein of FMDV in WRLFMD

Serotype	Primer name	Sequence (5'-3')	Genome direction	Gene
O	O–1D296aF	ATAACACCACTAATCCAAC	Forward	VP1
	O–1D628aR	GTTGGATTAGTGGTGTTAT	Reverse	VP1
A	A–1D202aF	TCAGCCACCTACTATTTCTCTGA	Forward	VP1
	A–1D478aR	CAGTGCTCCGTAGTTAAAGGATGA	Reverse	VP1

Appendix 9: Miscellaneous photo during study period

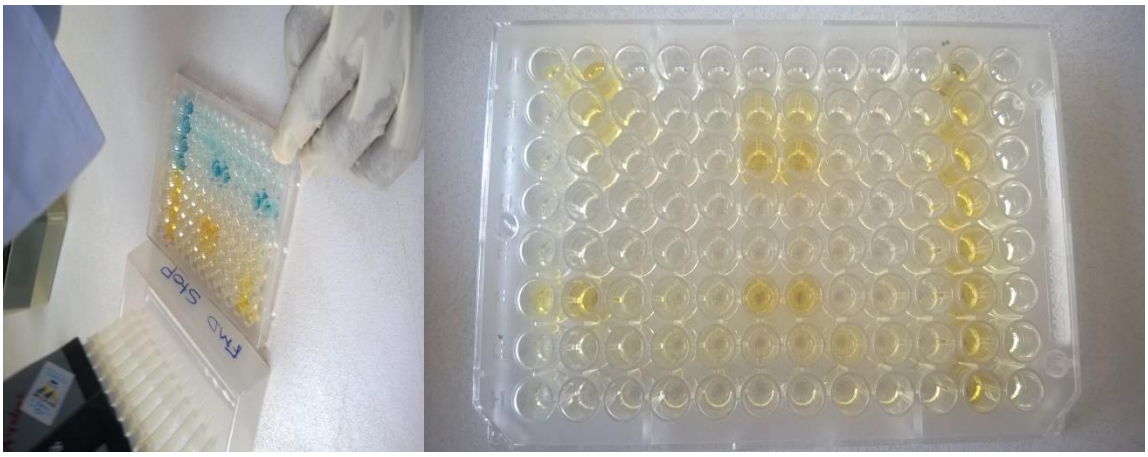
1. Photo during sampling

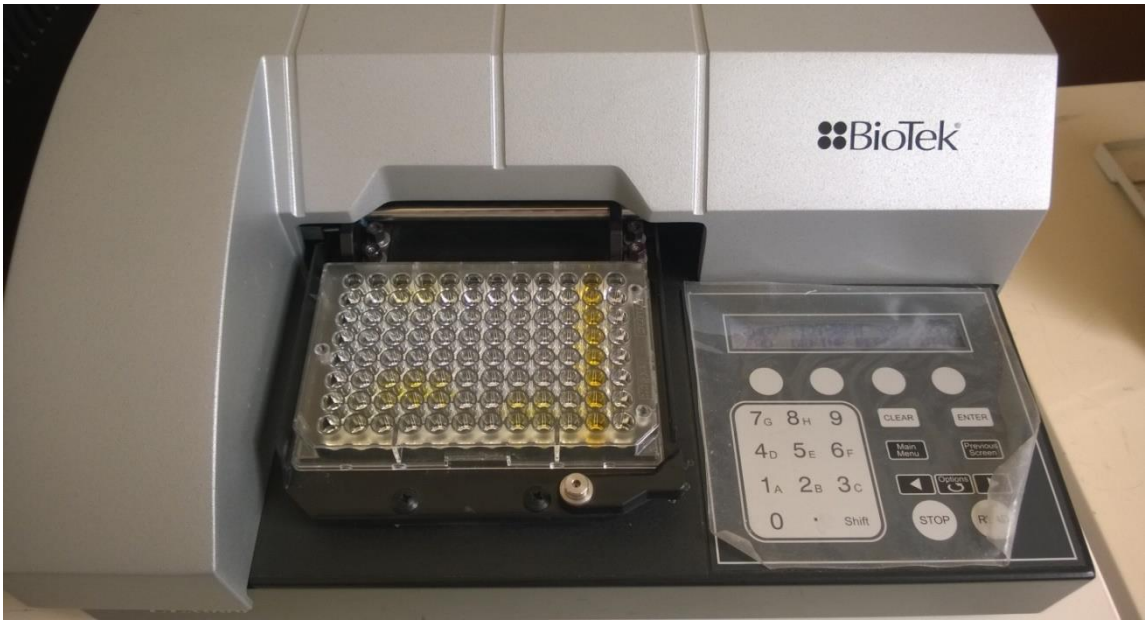


2. Photo during sample processing in lab.

3. Photo in cell culture

4. Photo during serotype detection by ELISA





Appendix 10: Ethical clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/06/05/10/2018

Name of Applicant: Metages Yirgalem (DVM, MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: ***Detection and molecular characterization of foot and mouth disease virus from outbreaks occurred in different parts of Ethiopia***

Date of application:	**15/10//2017**
Nature of the project:	**mildly invasive**
Target animal species:	**cattle**
Number of animals involved:	**based on cases available**
Study area:	**Ethiopia**

Minutes No. and date of review: **VM/ERC/05/10/018, 03/01/2018**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When replying

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