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ANTIGEN DETECTION AND MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUS FROM OUTBREAK CASES IN ETHIOPIA

MVSc Thesis

BY

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MVSc Thesis

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By

Shimels Tikuye Yalew

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TABLE OF CONTENTS

CONTENTS **PAGES**

TABLE OF CONTENTS (*continued*)

TABLE OF CONTENTS (*continued*)

TABLE OF CONTENTS (*continued*)

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LIST OF TABLES

PAGES

LIST OF FIGURES

PAGES

LIST OF APPENDIXES

PAGES

LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
AA	Addis Ababa
UTR	Untranslated Region
BHK-21	Baby Hamster Kidney 21 day
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CFT	Complement Fixation Tests
CO_2	Carbon Dioxide
CPE	Cytopathic Effect
Cre	Cis-acting Replication Element
CSA	Central Statics Agency
DMEM	Dulbecco's Modified Eagle Medium
dNTPs	Deoxy Nucleoside Triphosphates
DNA	Deoxyribose Nucleic Acid
EA	East Africa
ELISA	Enzyme-Linked Immunosorbent Aassays
ETB	Ethiopian Birr
FAO	Food and Agriculture Organization
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
G	Genotype
GDP	Gross Domestic Product
IRES	Internal Ribosome Entry Site
Kb	Kilo Base Pair
MAbs	Monoclonal Antibodies
MEGA	Molecular Evolutionary Genetics Analysis
MEM	Minimum Essential Medium
NSP	Non Structural Protein

LIST OF ABBREVIATIONS *(Continued)*

Nt	Nucleotide
NAHDIC	National Animal Health Diagnostic and Investigation Centre
NCBI	National Center for Biotechnology Information
NVD	No Virus Detected
NVI	National Veterinary Institute
OD	Optical Density
OIE	World Animal Health Organization
ORF	Open Reading Frame
PAs	Peasant Association
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RdRp	RNA Dependent RNA Polymerase
RNA	Ribonucleic Acid
rRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAT	South Africa Territories
SNNPs	Southern Nations, Nationalists and peoples
SP	Structural Protein
UK	United Kingdom
US$	United States Dollar
VNT	Virus Neutralization Test
VP	Virus Protein
VPg	Viral Genomic Protein
WRLFMD	World Reference Laboratory for Foot and Mouth Disease

ABSTRACT

Foot and mouth disease is a highly contagious viral disease of cloven-hooved animals with significant economic impact. Outbreak investigation was conducted in Tigray, Oromia and SNNPs regional states of Ethiopia from September 2018 to May 2019. Purposive sampling was conducted in the respective districts and kebeles where the outbreaks occurred. A total of 215 animals were examined for the presence of typical clinical signs, 55 animals (25.58%) showed clinical signs and lesions suggestive of FMD. Totally 55 epithelial tissues and 8 oral swab samples were collected from suspected cases and submitted to the NAHDIC, Sebeta, Ethiopia for virus isolation, serotype identification and molecular characterization. Culture positive FMDV isolates were sent to WRLFMD, Pirbright, UK for sequencing. Of the 63 collected samples, 53 samples (84.13%) were positive for the FMDV genome by rRT-PCR with C_t values ranging from 15.06 to 31.19. Out of 34 cultured samples, 76.47% (n = 26) exhibited cytopathic effect in BHK-21 cell and the viruses were isolated. In the current study, serotypes of O (53.85%) and A (46.15%) were identified by antigen detection ELISA. Phylogenetic analysis of VP1 sequences from these viruses were used to determine the relationships from Ethiopia and other viruses retrieved from GenBank. Phylogenetic analysis of the VP1 nucleotide sequences showed that the type O viruses belonged to the EA-3 and EA-4 topotypes. Serotype A isolates belonged to genotype IV of African topotype. Amino acid substitutions were observed at critical residues of antigenic sites of serotype O at position 45 and 48 of VP1. Amino acid variations also identified in the main antigenic sites between the vaccine strain and field isolates of FMDV serotype A at positions 45, 140, 141, 143, 149 and 157. Similarly a total of 12.68% and 15.96% amino acid variations were observed in serotype O and A, respectively, in different sites of the VP1 gene with reference to the vaccine strain of the country. Therefore, regular investigation of FMD outbreaks to have more detailed information about the serotypes and topotypes circulating in Ethiopia is important for development of effective vaccine for the controlling of the disease.

Key words: *Ethiopia, FMDV, molecular characterization, Outbreak investigation, Serotype O and A, Vaccine strain*

1. INTRODUCTION

Ethiopia has the largest livestock population in Africa, the country is not benefiting from this sector as development of this sector is hampered by different constraints that include wide spread endemic disease, lack of appropriate disease control policy and lack of appropriate veterinary services and lack of attention from government (Abdela, 2017). Among the livestock diseases hampering productivity of the sector, foot and mouth disease (FMD) is considered as a bottleneck for livestock production and productivity and is prompting trade embargos for livestock and livestock products (Mansley *et al*., 2011; El-Khabaz and Al-Hosary, 2017; Sulayeman *et al*., 2018). It is an acute and highly contagious disease of domestic and wild cloven-hoofed animals and it is a transboundary disease (Kasambula *et al*., 2012; Dyirakumunda *et al*., 2017). The disease is characterized by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats, and sudden death of young stock (AbuBakar *et al*., 2013). Generally, it is mild in adult animals although severe in young animals due to myocarditis (Arzt *et al*., 2011). Unrestricted animal movements are an important mechanism by which FMD is spread from one place to the other (Habiela *et al*., 2010).

The foot and mouth disease virus (FMDV), is a positive sense, single stranded RNA virus, a small non-enveloped RNA virus belongs to genus *Aphthovirus* of family *Picornaviridae* (Al-Shawkany *et al*., 2012) . FMDV has a high mutation rate because the viral RNA dependent RNA polymerase lacks proofreading ability and exists in seven immunologically distinct serotypes, O, A, C, Asia 1, SAT (Southern African Territories) 1, SAT 2 and SAT 3, each with a wide range of antigenically distinct subtypes (Maree *et al*., 2011; Kasambula *et al*., 2012; Valdazo-González *et al*., 2012; Maree *et al*., 2014). Infection with one serotype does not confer immunity against another (Lloyd-Jones *et al*., 2017). The viral genome is about 8.3 kb long and enclosed in a protein capsid. It contains a single open reading frame (ORF) encodes the four structural proteins which form the capsid (VP1, VP2, VP3, VP4); the VP1-3 proteins are located on the surface, while VP4 is internal (Bari *et al*., 2015) and ten non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1-3, 3C, and 3D) (Gao *et al*., 2016). Within each serotype, many strains can be identified by genetic and immunological tests (OIE, 2012). They also differ in distribution across the globe (FAO, 2007).

FMDV is endemic in Ethiopia causing several outbreaks every year (Ayelet *et al*., 2012). Previous studies have provided evidence for the presence of five FMDV serotypes from the seven serotypes

(O, A, C, SAT1, SAT2) were reported in Ethiopia samples collected from different outbreaks occurred from 1981-2018 (Roeder *et al*., 1994; Sahle, 2004; Ayelet *et al*., 2009; Negusssie *et al*., 2011; Kassaw *et al*., 2013; Legesse *et al*., 2013**;** Menda *et al*., 2014; Belay and Muktar, 2015*;* Jemberu *et al*., 2016**;** Beksisa, 2017; Metages, 2018; Sulayeman *et al*., 2018). Topotype is used to reflect the presence of genetically and geographically distinct evolutionary lineages (Samuel and Knowles, 2001). East Africa 1 (EA-1) (Sahle, 2004), EA-3 (Ayelet *et al*., 2009; Negusssie *et al*., 2011; Menda *et al*., 2014; Kassaw *et al*., 2013; Metages, 2018) and EA-4 topotypes (Ayelet *et al*., 2009; Motuma**,** 2017**;** Beksisa, 2017; Wondwossen, 2017) of FMDV serotype O were circulating in the country. From serotype A, Africa topotype of genotype I (G-I) (Metages, 2018), G-IV (Sulayeman *et al*., 2018), G-VII (Negusssie *et al*., 2011; Bari *et al*., 2014), and African topotype of serotype C (Ayelet *et al*., 2009)**,** topotype of IX from serotype SAT 1 (Ayelet *et al*., 2009) and topotypes of XIV, IV, XIV, XIII (Ayelet *et al*., 2009) and VII (genotype Alx-12) from SAT 2 were identified (Sulayeman *et al*., 2018).

Effective control and prevention of FMD depends largely on the implementation of strategies such as physical separation of wildlife and livestock, vaccination of cattle herds, control of animal movements and careful assessment of the risk of FMDV introduction into disease-free areas (Thomson *et al*., 2003; Jori *et al*., 2009).

A detailed knowledge of molecular epidemiology of the FMDV is helpful to define strains, to characterize diversity, to effective quarantine measures against reintroduction and the development of specific diagnostic tests and protective vaccine (Samuel and Knowles, 2001). These requirements are achieved via determination of the nucleotide sequence of the viral RNA that allows unequivocal characterization of the genetic relationships between strains, and given precise evidence. A phylogenetic tree based on the VP1 (1D) region of FMDV is widely used for genetic characterization because of its significance for virus attachment, entry, protective immunity and serotype specificity (Burman *et al*., 2006)**.** This technique has helped in defining genetic relationships between FMDV isolates and geographic distribution of lineages and genotypes; it has also helped establish genetically and geographically linked topotypes and trace the source of outbreaks (Samuel and Knowles, 2001; Knowles and Samuel, 2003; Sangare *et al*., 2003).

Outbreak investigations are continuously required in the disease endemic areas coupled with submission of specimens to reference laboratories to increase the chances of detecting new emerging diverse strains of FMD virus. Hence, the availability of updated information on genetic characteristics of FMDV field isolates and strains helps to know circulating strains in the field (Beksisa, 2017).

Currently the occurrence of FMD outbreaks in Ethiopia is increasing from time to time and cattle were under risk of infection, however, there is no government strategy in FMD control. Lack of vaccination strategies, presence of free animal movement, high rate of contact among animals at commercial markets, in communal grazing areas and watering points, poor surveillance and diagnostic facilities were among the reasons forwarded for increasing incidence of the disease. Effective control strategies of this disease needs sensitive, specific and rapid diagnostic tools like molecular detection (real time RT-PCR), virus isolation and antigen detection by ELISA. However, in Ethiopia the information and knowledge of molecular characterization and serotyping is scarce particularly in remote areas of the country. Serotyping and molecular characterization of the FMDV isolates from suspected outbreaks in cattle, which is useful for vaccine strains selection, tracing the source of outbreaks, implementation of good control programmers and the eradication of the disease in the country.

Therefore, the main objectives of the study were:

- ✓ To isolate and characterize the serotypes and topotypes of FMD virus causing outbreaks in cattle
- ✓ To determine recent FMDV strains circulating in the study areas
- ✓ To reconstruct phylogenic (evolutionary) relationships between virus strains
- ✓ To determine the relationship between field isolates against current vaccine strain based on VP1 sequence.

2. LITRATURE REVIEW

2.1. Definition

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven-hooved animals with significant economic impact, in cattle and swine as well as sheep and goats (Rodriguez and Gay, 2011; Upadhayay and Ewam, 2012; Ding *et al*., 2013; Xu *et al*., 2013). The disease is characterized by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats and sudden death of young stock (Arzt *et al*., 2010; Knight-Jones and Rushton, 2013; Admassu *et al*., 2015). It is one of the most globally important notifiable diseases of livestock due to its high infectious and transboundary distribution nature (Longjam *et al*., 2011). The disease was identified for the first time by Friedrich Loefffler in 1898 (Lefevre, 2010) and has different names in different regions of the world which include: Aphtous fever, Epizotic aphtae, Infectious aphtous stomatitis, Aftosa (Italian and Spanish), fievere aphtheuse (French), Maul and Klavenseuch (German), (Timoney *et al*., 1988; Di Nardo *et al*., 2011).

2.2. Etiology

Foot and mouth disease is associated with foot and mouth disease virus (FMDV), is classified within the *Aphthovirus* genus as a member of the *Picornaviridae* family, being small, a non-enveloped, single stranded RNA virus, icosahedral and is 26 nm in diameter (Arzt *et al*., 2010; Knowles *et al*., 2011), which occurs as seven major serotypes, over 60 subtypes have been described. There is also extensive genetic heterogeneity within individual serotypes with many distinct virus subtypes occurring within each serotype (Knowles and Samuel, 2003). The genome contains one open reading frame (ORF) encoding Lpro and capsid proteins as well as nonstructural proteins (Mason *et al*., 2003).

2.2.1. Genomic structure of FMDV

The FMDV genome is an 8.3kb single stranded positive sense RNA. It is divided into three sections; 5'untranslated region (UTR), a single open reading frame (ORF) and 3'UTR (Yang *et al*., 2014; Ashfaq *et al*., 2015; Bari *et al*., 2015). The organization of the FMDV genome is shown in figure

(1). A small non-structural protein (NSP) of about 24 bases, named VPg (viral protein linked genome), is linked to the 5'end of the genome. This protein is the primer for genome replication (Longjam *et al*., 2011).

Following this protein is the 5'UTR which consists of an S fragment, poly C tract, pseudoknot structures, a cis-acting replication element (cre) and the internal ribosome entry site (IRES) (Naveed *et al*., 2018). Poly C tract is of variable length and evidence has shown that the length is linked to virulence of the virus (Longjam *et al*., 2011). The cre element is used as a template for the uridylylation of the VPg and is therefore involved in the initiation of the genomic replication (Mason *et al*., 2002). The IRES is responsible for cap-independent initiation of viral protein synthesis. The roles of the other elements are unknown albeit functional (Grubman and Baxt, 2004).

The viral genome is enclosed in a protein capsid (Ashfaq *et al*., 2015; Bari *et al*., 2015; Ludi *et al*., 2017). Downstream the 5'UTR is a single ORF which is about 7000 residues long. The FMDV particle is roughly spherical in shape and about 25–30 nm in diameter. It consists of the RNA genome and the capsid is composed of 60 copies of the capsomers (Jamal and Belsham, 2013). The viral genome encodes the four structural proteins which form the capsid (VP1-VP4); the VP1-3 proteins are located on the surface, while VP4 is internal (Ashfaq *et al*., 2015; Bari *et al*., 2015; Gao *et al*., 2016; Mahapatra *et al*., 2017) and ten non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1-3, 3C, and 3D) (Jamal and Belsham, 2013; Gao *et al*., 2016).

These four proteins form the capsid of the virus and are coded for by 1D, 1B, 1C and 1A coding sequences respectively. The 1D is 627-639 nucleotides long and codes for VP1 which is a capsid protein containing 209-213 amino acid residue depending on the serotype. The most important antigenic site of FMDV is in the VP1 (Feng *et al*., 2003). It has multiple roles including receptor recognition, neutralization and antigenic diversity (Bastos *et al*., 2003). The VP1 coding sequence analysis can be used to determine the relatedness of FMDVs and thus evaluate the likelihood that a vaccine will provide protective immunity to the vaccinated animals since most FMDV vaccines are developed targeting the VP1 (Feng *et al*., 2003; Xiao *et al*., 2007). The genome is subject to a high rate of mutation because the FMDV RNA-dependent RNA polymerase lacks proof reading ability (Lordwin, 2011).

Lastly, the 3'UTR follows the ORF termination codon. It is involved in the replication of the RNA and consists of a stem-loop structure and a poly A tract which plays a role in the translation process (Figure 1) (Grubman and Baxt, 2004; Jamal and Belsham, 2013).

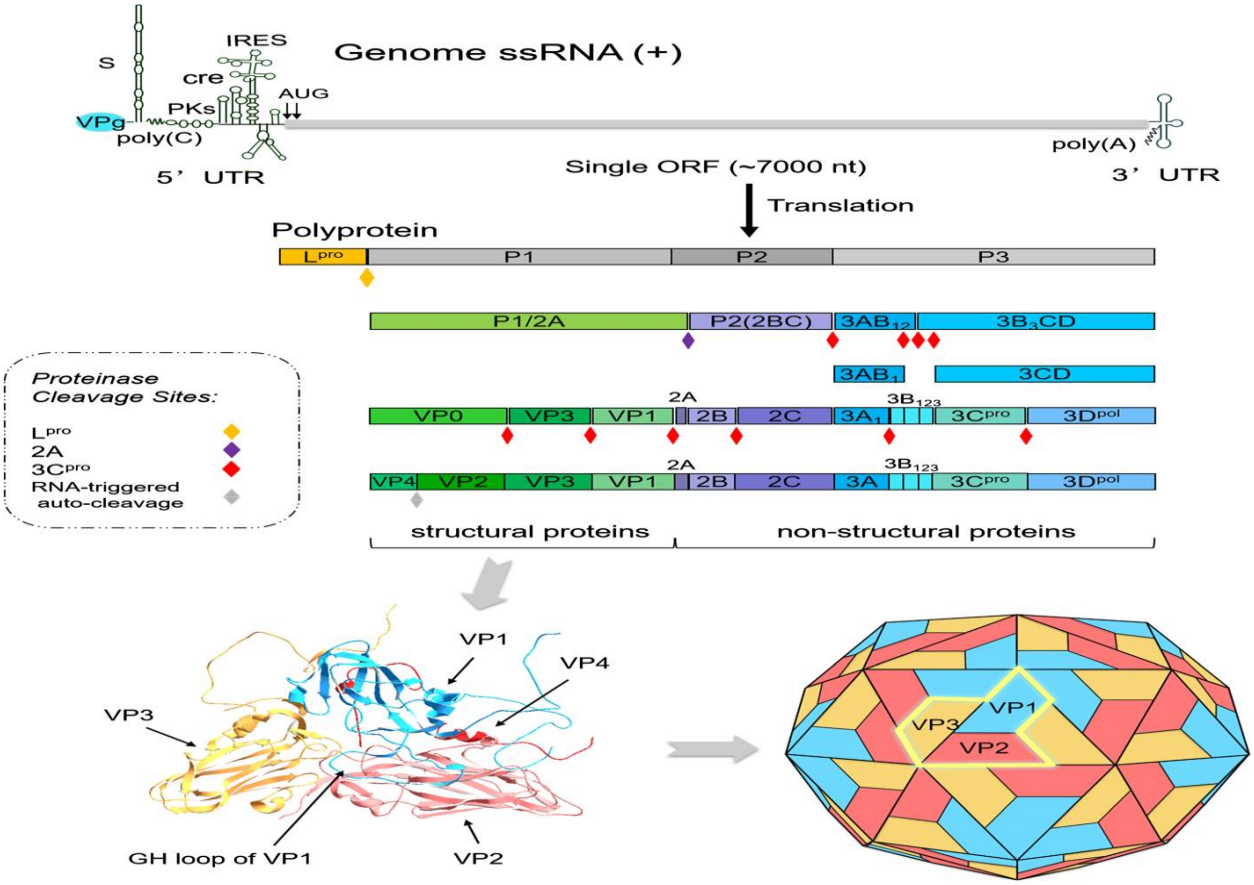


Figure 1: Schematic diagram of FMDV genome, processing of viral polypeptide and conformations of the structural proteins.

Source: (Gao *et al*., 2016)

2.2.2. Antigenic variation in FMD virus

The microorganism changes its surface proteins to provoke the host immune response. The immune response is associated with the mutation rate that causes amino acid replacement. Mostly, it occurs at hyper variable sites of the G-H loops in the variable protein 1 (VP1). The main factor of antigenic variation is an abrupt antigenic change which also causes mutation in B-cell epitope. The basic alteration in the antigenic profile follows the antigenic diversity in which the virus pass through the host population and hold the system of the original infected host cell (Naveed *et al*., 2018). Changes

to the genes encoding capsid proteins through mutation can result in antigenic variation and evolvement of new subtypes (Haydon *et al*., 2001). Genetic variation also produced by those RNA viruses that exhibit an error prone RNA replication process. FMDV continue to adapt and evolve new environments during RNA replication process to give high mutation rates (Naveed *et al*., 2018).

Antigenic variation is the most remarkable character of the FMD virus. The infectious virus alters its surface proteins by a special process to dodge a host immune response that is specifically related to replacement of amino acid causing mutation (Mahy, 2005). Nucleotide sequencing is routinely used to identify the genetic relationships between different isolates and historical strains. In this way the origin of a virus can be traced in outbreak conditions (Bari *et al*., 2015). Antigenic characteristics of a virus related to emergence of new strains, the severity of outbreaks and vaccine selection is common. This is defined by a complex interplay of viral and host factors as phylogenetic measures of viral similarity are poorly correlated to antigenic relationships (Reeve *et al*., 2016).

2.2.3. Physical characteristics of FMDV

Picornaviruses are small RNA viruses that are enclosed within a non-enveloped protein shell called capsid. The capsid consists of polypeptides, which are devoid of lipoprotein and is stable to lipid solvents like ether and chloroform (Awan, 2009). The effects of acid and heat on virus capsid structure and identification of interactions that may correlate with enhanced with heat or acid stability (Mateo *et al*., 2008; Maree *et al*., 2013).

The virus may persist for days or weeks in organic matter under moist and cold conditions. It can survive in frozen bone marrow, lymph nodes and also in cheese during its processing. FMD virus can be inactivated by acetic acid (2%), citric acid (0.2%), sodium hydroxide (2%), sodium carbonate (4%), sodium chloride (2%) and sodium hypochlorite (3%). It is destroyed in muscles at pH above 6. The virus in milk and meat can be inactivated by heating at the temperature of 70°C for at least 30 minutes (Awan, 2009). But, the virus can survive in aerosols and micro-droplets in the environment with humidity levels greater than 55% and cool temperatures. The virus also protected from degradation in the environment in neutral or slightly alkaline conditions in fomites like hay, dry faces, animal hair and winter slurry and may also have implications on the spreading of the virus (Alexandersen *et al*., 2003b).

2.2.4. Error-prone replication of FMDV

The virus particle has an icosahedral capsid made of protein, without an envelope outer layer, containing a single strand of ribonucleic acid with a positive encoding of its genome (Martinez-Salas *et al*., 2008). FMDV RNA replicates within the cytoplasm of infected cells and requires the virus encoded RNA-dependent RNA polymerase. This enzyme catalysis the synthesis of a negative strand copy of the positive strand viral genome and then the nascent negative strand is used as the template for the production of new positive strand RNA molecules (Paul and Wimmer, 2015). The positive strand RNAs can be packaged by the virus capsid proteins to make new virus particles. The VPg acts as a protein primer for the synthesis of the viral RNA (Paul and Wimmer, 2015). The replication process is error prone, i.e. incorrect nucleotides are incorporated into the RNA copies. On average the error rate suggest that about 1 error is made for every 10,000 nucleotide that are synthesized (Castro *et al*., 2005). There is no known mechanism of proof reading activity in picornaviruses and thus the viral RNA represents a pool of closely related sequences; this pool is known as a quasi-species (Domingo *et al*., 2012). Modifications to the fidelity either to increase or decrease the error rate the $3D^{pol}$ from picornaviruses reduce the "fitness" of the virus (Korboukh *et al*., 2014). Because of this continuous generation of errors, the virus population is always evolving. During the process of RNA replication, it is possible for the RNA polymerase ($3D^{pol}$) to switch from copying one positive strand template to another (Jackson *et al*., 2007; Jamal *et al*., 2011).

When the virus comes in contact with the membrane of a host cell, it binds to a receptor sites and triggers a folding-in of the membrane. The exact receptors for FMDV are not quite known, but beta integrins that are used for adhering to other cells and the cell matrix as well as interacting with extracellular proteins to perform physiological processes have been identified as FMD receptors; a receptor known as a heparan sulfate, a glycosaminoglycan, also shows affinity for FMDV (Ruiz-Sáenz *et al*., 2009). Once the virus is inside the host cell, the capsid dissolves and the RNA gets replicated, and translated into viral proteins by the host cell's ribosomes using a cap-independent mechanism driven by the internal ribosome entry site element. The synthesis of viral proteins includes 2A 'cleavage' during translation. They include proteases that inhibit the synthesis of normal cell proteins, and other proteins that interact with different components of the host cell. The infected cell ends up producing large quantities of viral RNA and capsid proteins, which are assembled to

form new viruses. After assembly, the host cell lyses (bursts) and releases the new viruses as shown in figure (2) (Martinez-Salas *et al*., 2008).

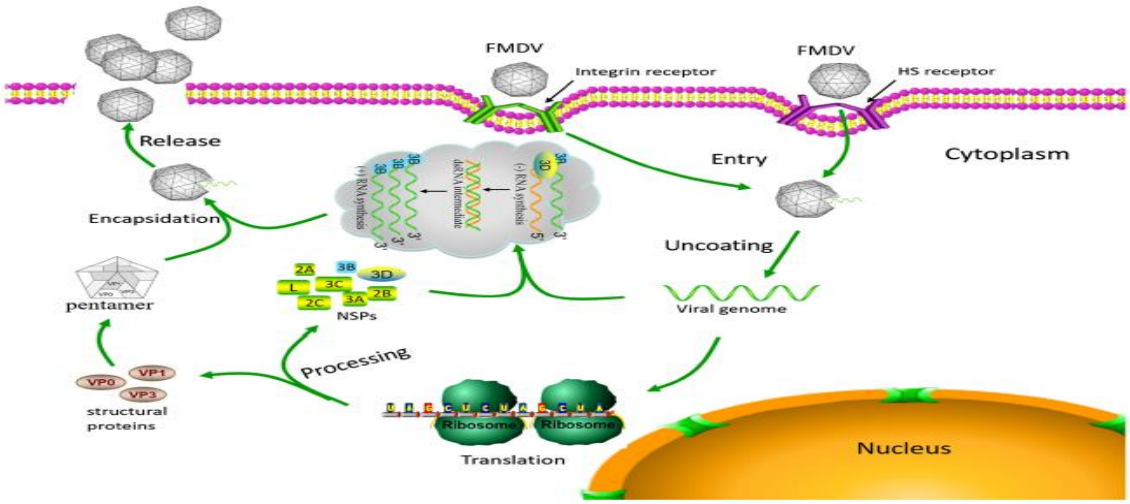


Figure 2: Life cycle of FMDV in host cells.

NSPs- nonstructural proteins, HS- heparan sulphate, Green line- viral positive – strand (+) RNA, Orange line- viral negative – strand (-) RNA

Source: (Gao *et al*., 2016)

2.2.5. Serotypes of foot and mouth disease virus (FMDV)

Globally, there are seven immunologically distinct serotypes of FMDV namely O, A, C, SAT (Southern African Territories) 1, 2 and 3, and Asia 1, which infect cloven hoofed Animals (Rweyemamu *et al*., 2008; Di Nardo *et al*., 2011; Kasambula *et al*., 2012; Jamal and Belsham, 2013). Examination of an extensive number of samples from across the world have failed to reveal the existence of another serotype although there are many different "sub-types", some of which are quite distinct from other strains of the same serotype (Jamal and Belsham, 2013).

In 1922 Vallée and Carré demonstrated that the existence of serotype O, they named these Vallée O after the regions they originated from department of Oise in France but latter shortened to O (Vallée and Carré, 1922). It allows the classification of the viruses into many distinct lineages and the lineages are Cathay, ME-SA (Middle East-South Asia), SEA (South-East Asia), Euro-SA (Europe-

South America), ISA-1 (Indonesia-1), ISA-2 (Indonesia-2), EA (East Africa) and WA (West Africa) (Knowles *et al*., 2001).

Serotype O is the pandemic and the most prevalent serotype worldwide, although there is no precise genetically explanation for this higher prevalence (Mason *et al*., 2003). Five neutralizing antigenic sites (1–5), involving three of the capsid proteins (VP1–3), have been identified on the surface of serotype O FMDV. Antigenic site 1 is linear and trypsin-sensitive, whereas the rest of the identified antigenic sites are conformation-dependent and trypsin-resistant. 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. Antigenic site 2 is located on the B-C loop of VP2 that contributes this site at amino acid residues 67-79 and 131. Antigenic site 3 involves residues 43, 44, 45 and 48 found on B-C loop of VP1, and antigenic site 4 is located on VP3 of amino acid residues at positions 56 and 58 of VP3 that have been critical for this site whereas antigenic site 5 is located at amino acid residue at position of 149 of VP1 and characterized by interaction of the VP1 G–H loop region with other surface-located amino acids. Antigenic site 1 has been considered to be immune dominant and its linear structure has made it an obvious target for the development of vaccines (Aktas and Samuel, 2000).

In 1922 Vallée and Carré demonstrated that the existence of serotype A, they named these Vallée A (Allemagne in French) but latter shortened to A (Vallée and Carré, 1922). FMDV serotype A is the most genetically and antigenically diverse among the seven FMDV serotypes and more than thirty subtypes have been identified (Kitching, 2005; Abubakar *et al*., 2012). There is also often no cross-protection between them (Klein *et al*., 2006; König *et al*., 2007; Bai *et al*., 2011). There is also evidence that recombination in serotype A occurs much more than in the other serotypes (Simmonds, 2006; Jackson *et al*., 2007; Bai *et al*., 2011). 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. However, it is still unclear why so many new lineages of serotype A regularly appear and disappear (Kitching, 2005).

The serotype C was identified by Waldmann & Trautwein in 1926, they named these Waldmann C but latter shortened to C (Waldmann and Trautwein, 1926). Serotype C has a very limited distribution compared with most of the other FMDV serotypes (Kitching, 2005). During the last 20 years there have been no major outbreaks reported of this serotype (only sporadically in South America, East Africa and Pakistan between 2000 and 2006) (OIE, 2008) and there is no explanation for the apparent disappearance (Kitching, 2005).

The serotype Asia 1 was identified in the early 1950s in viruses isolated from India and Pakistan (Brooksby and Rogers, 1957; Dhanda *et al*., 1957). The members of this serotype seem to be the less virulent and are usually restricted to Asia (Biswas *et al*., 2006; Chang *et al*., 2007; Bai *et al*., 2011). Serotype Asia 1 has never circulated within Africa. However, in recent years an increase of Asia 1 caused outbreaks in Asia has been reported, as well as further spread of viruses of this type to Turkey and Greece in 2000 (Valarcher *et al*., 2005).

Two new serotypes were identified in 1948 in samples from Bechuanaland (Botswana) and Northern Rhodesia (Zambia) (Brooksby, 1958). Retrospective testing of samples from Southern Rhodesia (Zimbabwe) from the 1930s found both of these new serotypes and a third new serotype as well. These new serotypes were named Southern African Territories 1 to 3 (SAT 1, SAT 2 and SAT 3). The SAT serotypes are usually found in Africa but there have also been sporadic outbreaks in Saudi Arabia and Kuwait in 2000 (East, 2002). The natural host for the SAT viruses is the African buffalo which is persistently infected with multiple serotypes. Several studies in Southern Africa have confirmed the African buffalo can sustain FMD virus in their body as a carrier for serotypes SAT1, SAT2 and SAT3 (Vosloo and Thomson, 2017). The SAT serotypes of FMDV are not well characterized as there have been very limited studies (Grazioli *et al*., 2006). There is a higher sequence variation within each of the three SAT types than in serotype O (Bastos *et al*., 2001; Bastos *et al*., 2003).

There are four independent antigenic sites on the surface exposed proteins of SAT1 serotypes. Site I is located on VP1 and has two sub-sites namely site Ia residues at 146 or 154 or 157 of VP1 and site Ib residue at 146 alone or together with residue position 148 on VP1. The second site (site VII) has two different sites on different structural proteins residue at 135 alone or together with residue 71 and residue 76 on VP3 and another site at position 179 or 181 of VP1. The third site (site VII)

involves residue at VP2 72. Fourth site (site VIII) involves one residue at position 111 of VP1 (Grazioli *et al*., 2006).

In SAT2, three different antigenic sites were reported: two of the antigenic sites are located on VP1 at positions 210 and 154 while the third one was identified on VP2 at position 72 indicated that antigenic sites on the SAT2 viruses at residues 147 to 149 and 156 or 158 of VP1 that might match with the second antigenic site reported by (Grazioli *et al*., 2006; Bari *et al*., 2015).

Serotype SAT 3 was identified for the first time from Uganda in East Africa in 2013 (Dhikusooka *et al*., 2015). SAT 3 FMDV is the least well–characterized serotype; the most recent incidence of SAT 3 reported by the FMD World Reference Laboratory (Pirbright Institute, UK) was in buffalo within the Kruger National Park (South Africa) in 2006 (Ahmed *et al*., 2012).

2.2.6. Genotypes of FMDV

Nucleotide sequence analysis compares the nucleotide sequences of the capsid protein genes from many FMD viruses from different geographical sites of isolation showed that there was good correspondence with serotype differences, the sequences clustering into serotype-specific lineages upon phylogenetic analysis. The seven serotypes of FMDV cluster into distinct genetic lineages with approximately 30%–50% differences in the VP1 gene (Knowles and Samuel, 2003). FMD viruses are classified into geographically restricted clusters also known as topotypes (Knowles and Samuel, 2003; Vosloo *et al*., 2004). Topotypes generally refers to isolates that has <15% genetic variations for serotypes O, A and C and <20% for SAT serotypes (Knowles and Samuel, 2003; Di Nardo *et al*., 2011). Distinct genetic variants exist within these serotypes, with the serotypes being divided into topotypes based on genetic differences (Knowles and Samuel, 2003). Serotype SAT1 can be grouped into eight topotypes I to VIII. This is based on nucleotide differences (Samuel and Knowles, 2001). SAT2 viruses appear to be particularly diverse, with the largest number of topotypes, whilst serotype C, probably as a result of being the rarest serotype in the continent, has the fewest (Bastos *et al*., 2001; Reid *et al*., 2001; Sangare *et al*., 2001).

2.3. Mode of transmission

Susceptible animals are infected through direct or indirect contact with infected animals or other objects exposed to live virus. The most common route of infection of susceptible animals is by

direct contact, either by mechanical transfer or by aerosol infection. Oral transmission is also possible especially when the animal has damaged skin in and around the mouth as well as on pre-existing abrasions on animals (Alexandersen *et al*., 2003b). Some cases of airborne transmission as far as 300km from source of infection have been described (Sørensen *et al*., 2000; Sørensen *et al*., 2001).

Inhalation of aerosolized virus is also common mode of transmission for cattle (Alexandersen *et al*., 2003b). Pigs are more likely to get infected by eating contaminated food (Alexandersen and Donaldson, 2002). Pigs can be infected by FMDV if placed in premises previously housing infected animals and like cattle, they are at risk of infection due to direct contact with infected animals (Grubman and Baxt, 2004).

2.4. Incubation period

Incubation period, which represents the time from infection to disease, depends on the dose of the virus, portal of entry, animal husbandry practices and animal species involved (Alexandersen *et al*., 2003; Grubman and Baxt, 2004; Alexandersen and Mowat, 2005) in cattle it varies from 2-14 days in pigs it is usually 2 days (or more) but can be even short (18-24 hours) and in sheep normally it is 3-8 days (Chakraborty *et al*., 2014)). The incubation period is most likely to be 2-6 days (Alexandersen *et al*., 2003).

2.5. Clinical signs and lesions

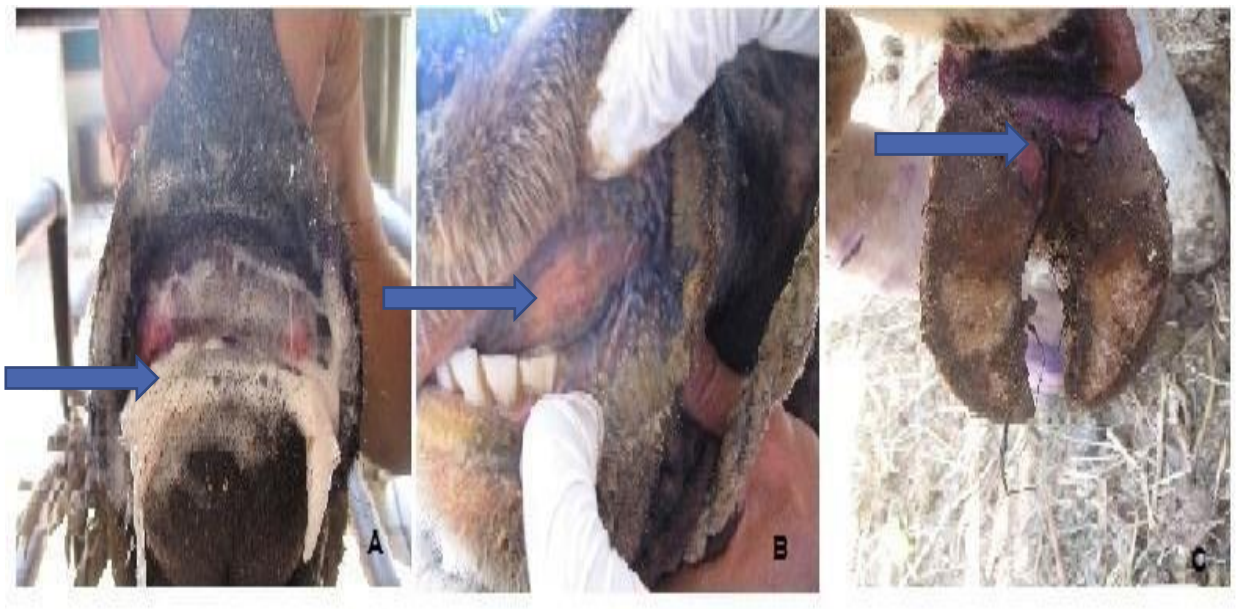
Animals that can be affected include cattle, swine, sheep, goats, buffaloes and wild ruminants (Alexandersen and Mowat, 2005). When susceptible animals are in contact with clinically infected animals, clinical signs usually develop (Kitching, 2002). The severity of clinical signs of the disease varies with the strain of the virus, the exposure dose, the age and breed of the animal, the host species, and its degree of immunity. The signs can range from a mild or in apparent in sheep and goats to a severe disease occurring in cattle and pigs (OIE, 2004).

FMD in Bovine is systemic vesicular disease characterized by fever (above 40 ^{o}C), excessive salivation, lameness, depression and decreased milk production, which requires a differential diagnosis from other vesicular diseases (Longjam *et al*., 2011). The mucosa of the lips, dorsum of the tongue and the dental plate are most severely involved. Lesions are often observed initially as

blanched areas, which subsequently develop into vesicles (Figure 3). The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof (OIE, 2012).The mortality rate is low and mostly young animals are affected due to myocardial necrosis (Kitching, 2002; Alexandersen *et al*., 2003a; Gulbahar *et al*., 2007).

FMD in pigs primarily affects the feet. It is dominated by rather painful formation of vesicles in the epidermis of the feet (coronary band, interdigital clefts and bulbs) associated with severe lameness. Complications are seen, such as detachment of the hoof and secondary infection of disrupted aphthae (Kitching and Hughes, 2002).

Clinical signs of FMD in sheep and goats are less severe; often only lameness through aphthae and inflammation at the cloves (Alexandersen and Mowat, 2005). Lameness is usually the first indication of FMD in sheep and goats. An affected animal develops fever, is reluctant to walk and may separate itself from the rest of the flock. Vesicles may develop in the interdigital cleft, on the heal bulbs and on the coronary band but they usually rupture rapidly (Kitching and Hughes, 2002).



A B C

Figure 3: Clinical signs of FMD

(A) Salivation, (B) Erosion of oral mucosa and (C) erosion in interdigital space

Source: (El-Khabaz and Al-Hosary, 2017)

2.6. Pathogenesis

The predominant entrance of virus is most commonly through the upper respiratory tract by inspiration of infected aerosols, but infection may also occur through a skin injury (Lyytikäinen *et al*., 2011). After inhalation, the virus can affect the pharynx and primary multiplication of the virus in the mucous membrane is 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 (Lefevre, 2010). Secondary replication in other glandular tissues, the virus appears in different body fluids such as milk, urine, respiratory secretions and semen before the appearance of clinical signs of FMD. The virus can also persist in oral cavity of infected animals for long periods after the acute infection (Alexandersen *et al*., 2003b).

The clinical outcome of the disease may vary among the host species considered and the infecting virus strain. The acute phase of disease lasts about 1 week and declines gradually coinciding with the emergence of a strong humoral response. In sheep and goats symptoms are frequently less severe and may make the detection of the disease difficult (Knowles *et al*., 2001). In fatal cases, death is caused either by dehydration or by ventricular fibrillation during cardiac attacks or as a result of bacterial complication (Lefevre, 2010).

2.7. Morbidity and mortality rate

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 in cattle is 100% but mortality rate is very low (2%) (Seyoum and Teshome, 2018). However, fatality in calves may reach up to 20% (Radostits *et al*., 2007; Seyoum and Teshome, 2018). The death in young animals may be due to myocarditis (Gulbahar *et al*., 2007).

2.8. Molecular epidemiology of FMDV

The molecular epidemiology of FMDV is based on the comparison of genetic differences between viruses (Knowles and Samuel, 2003).The molecular epidemiology of FMDV has been extensively studied using the VP1 coding region of the virus genome. Phylogenetic analysis of VP1 can be applied to categorize field strains into discrete topotypes and lineages which, despite the tendency for the virus to spread, frequently show geographical clustering based on the historical distribution of the virus (Di Nardo *et al*., 2011). VP1 is the most variable capsid protein includes a major immunogenic site of the virus has been used to genotype the seven serotypes of FMDV into geographically distinct groups called topotypes. In VP1 protein only 26% of its amino acids are universally conserved between serotypes (Jackson *et al*., 2007). Furthermore, comparison of VP1 coding sequences from isolates obtained during outbreaks provides evidence of relatedness between individual FMDV strains and hence the tracing of the spread and transmission of the virus from one region to another or across national borders (Knowles and Samuel, 2003). The evolutionary changes of virus are determined by comparing genomic material from more than one virus with each other. At present, sequencing and phylogenetic trees are widely used to illustrate the genetic relationship between viruses (Sahle, 2004).

2.9. Diagnosis techniques of FMD

The accurate diagnosis of infection with FMDV is of prime most importance for both control and eradication campaigns in FMD endemic areas (Longjam *et al*., 2011). The disease is diagnosed based on clinical signs, including high temperature, excessive salivation, and formation of vesicles on the oral mucosa, on the nose plus the inter-digital spaces and coronary bands on the feet. However, the clinical signs can be confused with other diseases and thus laboratory based diagnosis is necessary (Jamal and Belsham, 2013).

For laboratory diagnosis, the sample of choice is tissue epithelium or vesicular fluid. Ideally, epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. When epithelial tissue is not available, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of oropharyngeal fluid is collected by means of a probang cup (or in pigs by swabbing the throat) for diagnosis. Serum samples are used for FMD diagnosis based on spiking of antibody against a particular serotype (Musser, 2004; OIE, 2009).

Diagnosis of FMD in the laboratory is conducted by virus isolation, demonstration of the FMD viral antigens or nucleic acid in a sample tissue or fluid. Detection of virus specific antibodies can also be used. Additionally, antibodies to viral nonstructural protein can be used as indicators of infection irrespective of vaccination status (Grubman and Baxt, 2004).

2.9.1. Virus isolation

The isolation and characterization of the virus is the "golden standard" for the diagnosis of viral diseases (Admassu *et al*., 2015). Virus isolation (VI) remains the definitive proof of the presence of live FMDV. Virus isolation requires the presence of infectious virus, which depends on sample quality. Up to 4 days may be required to demonstrate the presence of virus, especially when the levels of virus are low (Jamal and Belsham, 2013). Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IBRS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV (Damena *et al*., 2017). Pig cell lines were not always suitable for isolation of FMDV coming from other species (Bouma *et al*., 2001). The BHK-21 cell line seems to be less species dependent. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. In the case of OP fluids, pretreatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes (Damena *et al*., 2017). A small proportion of samples may give negative results for infectivity but positive results by ELISA or RT-PCR (Alexandersen *et al*., 2003b).

2.9.2. Serological approaches

The virus infection can be diagnosed by the detection of specific antibody response (Admassu *et al*., 2015). Serological tests are widely used to monitor the immune status of animals exposed to FMDV or FMDV vaccines. Approaches used include enzyme linked immunosorbent assays (ELISAs) and virus neutralization tests (VNTs), although complement fixation tests (CFT) are still used in a limited number of laboratories. 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 (Singh *et al*., 2008; Verma *et al*., 2009; Ma *et al*., 2011; Deb *et al*., 2013).

One particular application of these serological assays is to identify animals in a vaccinated herd that have been infected with FMDV. This so called DIVA (differentiating infected from vaccinated animals) principle exploits differences in the antibody (humoral) responses generated in vaccinated animals compared to those animals naturally infected with FMDV (whether or not they have been vaccinated). High quality FMDV vaccines are purified to contain structural protein (SP) viral capsid components from which most of the viral non-structural proteins (NSP) have been removed. In contrast, during natural infection with FMDV, viral NSP are expressed that elicit a corresponding immune response that can be detected using diagnostic approaches (Figure 4) (Ludi *et al*., 2017).

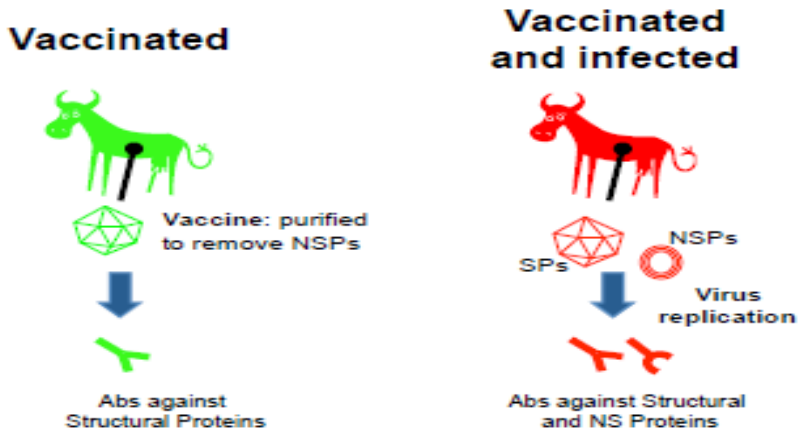


Figure 4: The principle of using NSPs tests to differentiate between vaccinated and infected animals.

Source: (Ludi *et al*., 2017)

2.9.3. Nucleic acid recognition method

The polymerase chain reaction (PCR) can be used to amplify the genome fragments of FMDV in diagnostic material (Lomakina *et al*., 2004). The PCR techniques are the most widely used nucleic acid based diagnostic technique for rapid identification of FMD virus (Xu *et al*., 2013). A specific reverse transcriptase polymerase chain reaction was developed and validated for the detection of the polymerase gene (3D) of FMD with an 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.

Real time PCR assays recommended by the World Organization for animal health (OIE) for detection of FMDV incorporate universal primers and fluorescent labeled probes that recognized conserved region within the 5' UTR or conserved gene regions within the RNA-dependent RNA polymerase gene ($3D^{pol}$) (Reid *et al*., 2014). TaqMan technology has combined the 5- nuclease activity of the Taq DNA polymerase and foster resonance energy transfer to detect and quantify amplification product. Using this technology real time PCR has been developed to detect the nucleic acid (Oleksiewicz *et al*., 2001). This is most sensitive and rapid method to detect the nucleic acid. The viral RNA can be consistently detected over a seven-log range, the lowest of which corresponded to as few as 10-100 RNA per volume tested. The test can be performed in 2 hours or less on a portable instrument and sample can be held at ambient temperatures. Real time chemistry allows for the detection of PCR amplification during the early phases of the reaction and real time PCR monitors the progress of a PCR reaction in the real time. At the same time, a relatively small amount of PCR products (DNA or cDNA) can be quantified (Shaw *et al*., 2007). In epidemiological studies of FMD virus, nucleotide sequencing of the VP1 gene has been used extensively to determine the relationships between the field isolates (Genchwere and Kasanga, 2014).

2.10. Differential diagnosis

Clinical signs of FMD have got species variation but feet vesicles and erosions or those in the oral cavity or teats suggest the presence of disease. Clinical signs of excessive salivation (except in pigs and sheep) and laminitis with the history of high rise of temperature are always suggestive of FMD (Chakraborty *et al*., 2014). Clinically, it is impossible to distinguish foot and mouth disease from the other vesicular diseases of the viral origin. Vesicular stomatitis, Vesicular exanthema and Swine vesicular disease required laboratory studies to differentiate them from foot and mouth disease (Leforban, 2005; Teifke *et al*., 2012).

2.11. Epidemiology

2.11.1. Host range

Affected Host range always governs the existence of pathogen in environment. Larger host range always supports fast spread of disease with more chance of the antigenic diversity and hence makes the control program a tedious task (Teifke *et al*., 2012). FMD is highly contagious and affects over 70 domestic and wild *Artiodactyl* species (Michel and Bengis, 2012). Of the domesticated species, it causes disease in cattle, pigs, sheep and goats. In addition, many species of cloven-hoofed wild life such as deer, antelope and wild pigs may become infected though their involvement in the epidemiology of FMD in the domesticated species is not certain (OIE, 2004). The susceptibility of these animals can vary with breed of animal and strain of virus. African buffalo are the only wildlife species to play a significant role in the epidemiology of FMD (Awan, 2009). Cattles and pigs are more susceptible and show greater severity of signs than sheep and goats (Alexandersen *et al*., 2003b). Sheep, pigs and cattle are maintenance, amplifier and indicator hosts of FMD respectively. Human health is not affected by FMDV. Although Horses, dogs and cats can spread the virus by carrying it on their hair, they are not susceptible for the disease (Radostits *et al*., 2007).

2.11.2. The role of carrier animals

In FMD, Carrier is defined as an animal from which FMD virus can be isolated from the oropharyngeal (OP), more than 28 days after infection. Although it is well established that 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) (Bastos *et al*., 2000). This may provide a mechanism for the maintenance of the virus in nature and may contribute to the emergence of new antigenically variant viruses (Sahle, 2004).

2.11.3. The role of wild life

FMD can infect other wildlife species apart from African buffalo (*Syncerus caffer*) these include, Impala (*Aepyceros melampus*), Kudu (*Tragelaphus strepsiceros*) species, Warthog (*Phacochoerus aethiopicus*) and elephants that has a role in epidemiology of the disease. African Buffalo can harbor the infection up 24 years whilst an individual animal can maintain the infection for up to five years. Buffalo have unequivocally been shown to be a source of infection for cattle under both natural and experimental conditions (Sangare, 2002).The SAT-type virus transmission is mainly facilitated by close contact between the two species during the acute stage of infection and shedding of virus in large amount. Strong spatial associations between molecular types from outbreaks in cattle and virus

recovered from buffalo suggest that any control strategy for FMD in cattle must address control in buffalo (Thomson *et al*., 2003). Impala is the most frequent infected species and act as intermediaries in disease transmission. But, impala do not become carriers, it appears that the disease can persist in impala populations for between 6 and 13 months (Vosloo *et al*., 2002).

2.12. Geographical distribution

The disease has been present in almost every part of the world where livestock are kept. More than 100 countries are still affected by FMD worldwide and distribution of the disease roughly reflects economic development (Jamal and Belsham, 2013). Globally and across multiple serotypes, there are distinct genetic and antigenic strains of FMDV circulating and evolving in defined geographical regions that are hence grouped into seven regional pools. Pools 1 and 2 occur in Asia, while pool 3 occurs in Asia, Middle East and North Africa. Virus pool 7 is found in South America while Africa contains 3 different pools roughly spread across East, West and South Africa respectively (Figure 5) (Rweyemamu *et al*., 2008b; Tully and Fares, 2008). Each of these contains at least three serotypes of virus and because virus circulation is mainly within these regional reservoirs, strains have evolved which are specific to the region (Paton *et al*., 2009). FMDV has an essentially global distribution, with the exception of North America and Central America, New Zealand, Australia, Greenland, Iceland and Western Europe. The FMD status of any particular country or region can be defined as endemic, epidemic or free. The USA, Canada, Mexico, Australia and Scandinavia haven't had the disease for many years (Samuel and Knowles, 2001). At present, most developed countries have successfully controlled or eradicated the infection and have implemented strict control measures especially regarding imports of animals and animal products to prevent re-emergence of the disease.

The seven serotypes display different geographical distributions and epidemiology (Bastos *et al*., 2003; Knowles and Samuel, 2003; Bronsvoort *et al*., 2004). The cumulative incidence of FMD serotypes showed that six of the seven serotypes of FMD (O, A, C, SAT1, SAT2, SAT3) have occurred in Africa (Bastos *et al*., 2001; Valarcher *et al*., 2004; Rweyemamu *et al*., 2008).

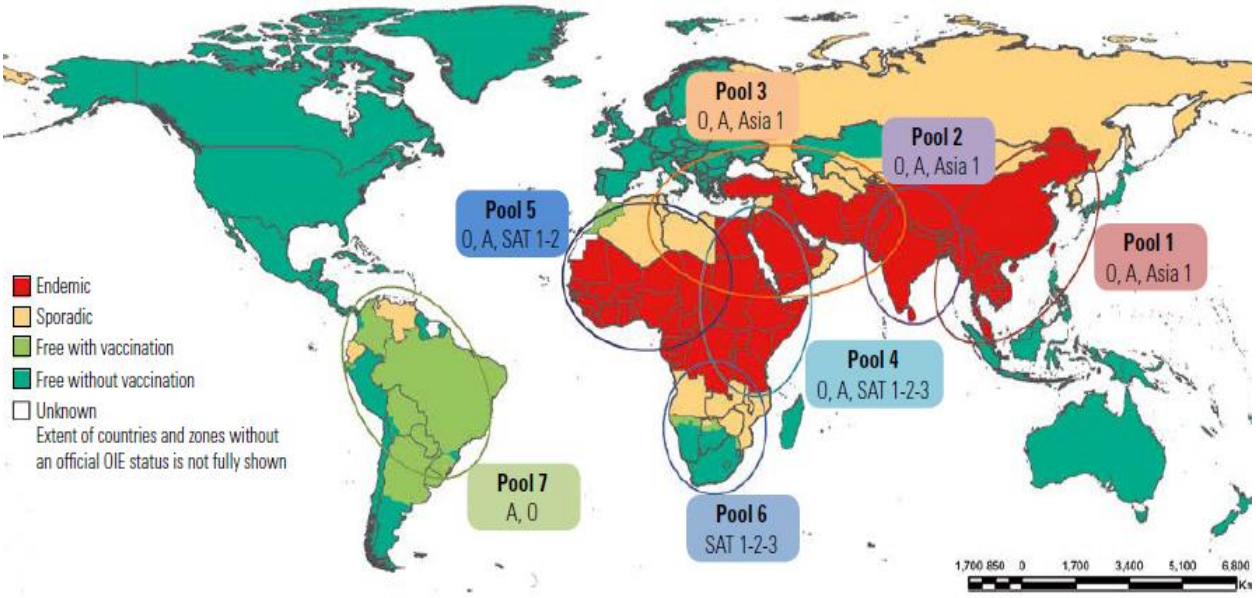


Figure 5: Global circulation of foot and mouth disease virus

Source: (Freimanis *et al*., 2016)

2.13. Distribution of FMDV serotypes and topotypes in Africa

Foot and mouth disease was first described in Africa in 1780 but the disease may have been present in the continent for centuries. Foot and mouth disease is endemic in Africa and the epidemiology of the disease is more complicated than in other parts of the world (Vosloo *et al*., 2006). In Africa, the FMDV serotypes are not uniformly distributed and each serotype results in different epidemiological patterns. The cumulative incidence of FMDV serotypes show that six of the seven serotypes of FMD (O, A, C, SAT1, SAT2, and SAT3) have occurred in Africa with the exception of Asia-1 (Figure 6) (Rweyemamu *et al*., 2008; Maree *et al*., 2014).

Based on the antigenic relationship of the virus and genetic characterization of FMDV in Africa, the virus distribution has been divided into three virus pools: namely, pool 4 covering East and North Africa, with predominance of serotypes A, O, SAT1, and SAT2; pool 5 restricted to West and northern Africa, with serotypes O, A, SAT1, and SAT2; and pool 6 restricted mainly to South Africa, with SAT1, SAT2, and SAT3 serotypes (Figure 5). Recent studies in East and southern Africa have revealed genetic differences between viruses isolated at different times and places (Sangula *et al*., 2010; Kasanga *et al*., 2012; Maree *et al*., 2014; Kasanga *et al*., 2015).

The last known cases of FMD caused by serotype C FMDV within Africa occurred in Kenya, in East Africa, during 2004 (Sangula *et al*., 2011). In the wider East African region, type C was last reported in Uganda in the 1970s and Ethiopia in 1983 (Roeder and Knowles, 2008; Ayelet *et al*., 2009). No serotype C FMDV has been detected anywhere in the world since 2004 and it is now possible that this serotype is extinct (Roeder and Knowles, 2008). Serotypes O, A, and the South African Territories (SAT) FMDVs are endemic in Africa; serotype O is the most widely distributed in eastern and western Africa, whereas SAT viruses are mostly found in sub-Saharan Africa (Brito *et al*., 2017). SAT 1 and SAT 2 serotypes are much more prevalent than SAT 3 (Vosloo *et al*., 2002). Serotype SAT 3 FMDV has been isolated from cattle and buffalo in sub-Saharan Africa (Dhikusooka *et al*., 2015).

In North Africa, incursions of FMDV from sub-Saharan Africa and the Middle East are mainly by livestock trade and the virus maintenance is very likely within the domestic animals (Knowles *et al*., 2007; Hall *et al*., 2013). Northern Africa is a bridge for FMDV exchange between Africa (pools 4, 5, 6) and western Asia (pool 3) (Brito *et al*., 2017). The West, Central and East Africa region is divided into two virus pools (4 and 5) (Paton *et al*., 2009). FMDV serotypes of A, O, SAT1 and SAT2 are endemic in most countries of Eastern Africa. Serotypes that are known to be endemic in western Africa are O, A, SAT1 and SAT2 (Brito *et al*., 2017). Of the four serotypes, serotype O is the least prevalent in southern Africa (Sebhatu, 2014). The SAT serotypes are maintained by large numbers of African buffalo in the region, which provides a potential source of infection for both domestic livestock and other wild, cloven-hoofed animals, such as impala, which can be transiently infected and transmit the disease to susceptible livestock (Thomson *et al*., 2003; Hargreaves *et al*., 2004; Phologane *et al*., 2008).

Serotype SAT1 is limited to southern and eastern Africa and SAT1 has not been isolated in West and Central Africa since 1981, although serological studies reported antibodies to SAT1 in cattle and sheep against the virus (Ehizibolo *et al*., 2014). Serotype SAT2 is also limited to southern and eastern Africa, but SAT2 topotype VII has spread to the North and West Africa. Serotype SAT3 is restricted to South Africa, Namibia and Mozambique since 2006 (Sebhatu, 2014).

Figure 6: Occurrence of FMD serotypes in Africa from 1990 to 2013.

Countries colored with light grey: North Africa, white: West, Central and East Africa, dark grey: Southern Africa.

Source: (Sebhatu, 2014)

The term topotype is used to reflect the presence of genetically and geographically distinct evolutionary lineages (Samuel and Knowles, 2001). In Africa, six topotypes have been identified for serotype O, two for serotype A, three for serotype C, nine for serotype SAT1, fourteen for serotype SAT2 and five topotypes for serotype SAT3 (Figure 7) (Di Nardo *et al*., 2011; Sebhatu, 2014).

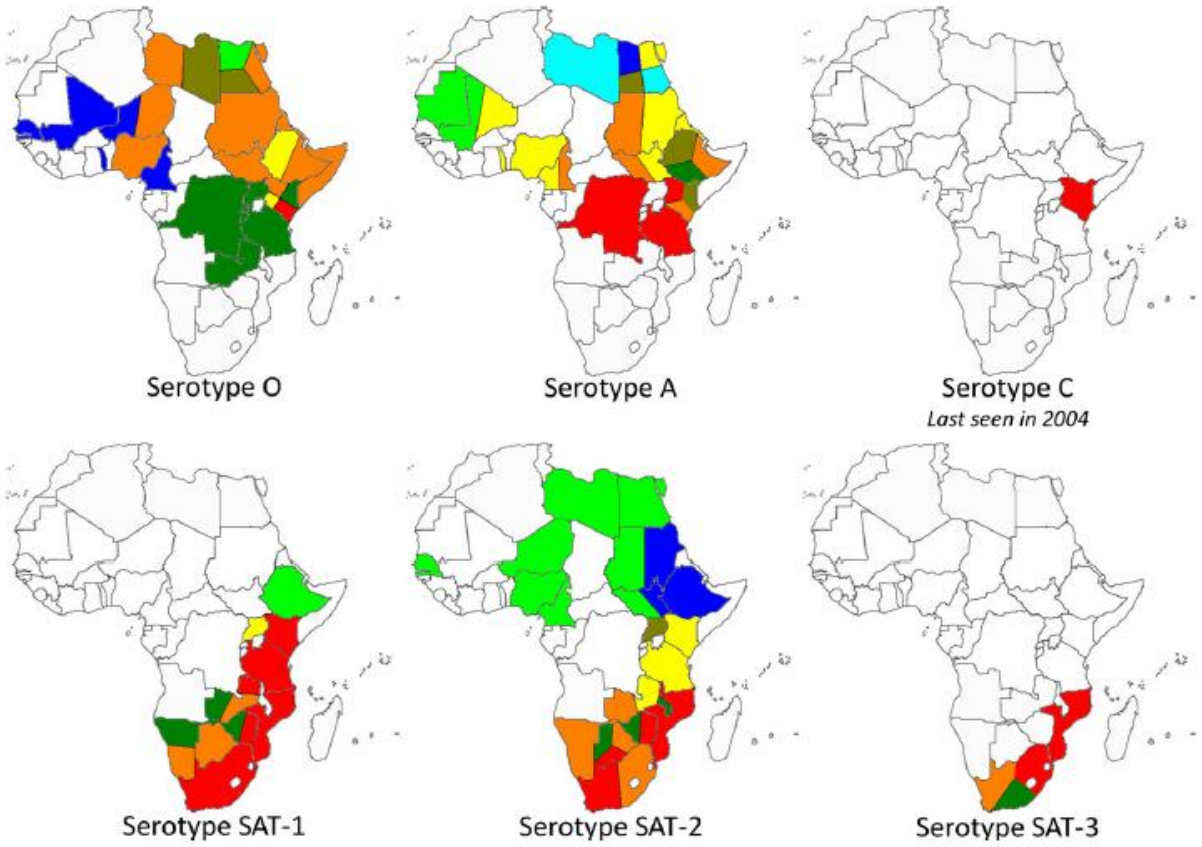


Figure 7: Map of Africa with different topotypes and genotypes from 2003 to 2013.

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 SAT1 the topotypes and genotypes were:
● I (NWZ) ● II (SEZ) ● III (WZ) ● IV (EA-I) ● IX
For type SAT2 the topotypes and genotypes were:
● I ● II ● III ● IV ● VII ● X ● XIII
For type SAT3 the topotypes and genotypes were:
● I (SEZ) ● II (WZ) ● V

Source: (Sebhatu, 2014)

2.14. Treatment

Currently, there is no specific drug, which can be recommended to treat foot and mouth disease (Grubman and Baxt, 2004). Instead of specific treatment, symptomatic treatments may be applied

depending on the clinical manifestations. 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 is found suitable in foot lesions (Gakuya *et al*., 2011). Antiviral approaches including 2'-C-Methylcytidine and ribavirin are useful for the purpose of prophylaxis in susceptible animals (Lefebvre *et al*., 2014; Yoon *et al*., 2012) .

However, proper animal husbandry practices and treatment of secondary bacterial infection of lesions and dressing to inflamed areas to prevent secondary infection is recommended in endemic countries where slaughter policy is not enforced. Sick animals may be treated by applying broad-spectrum antibiotics parentally, tetracycline in particular, in order to control the consequences of secondary bacterial infections (Radostits *et al*., 2007). Affected animals will recover however with loss of production based on the infection state of the disease. The recovery in animals may take around 15 days (Grubman and Baxt, 2004).

2.15. Prevention and control measures of foot and mouth disease

Foot and mouth disease is subject to national and international control and the measures taken depend on whether the country is free from the disease, is subject to sporadic outbreaks or has endemic infection (Rweyemamu *et al*., 2008a). In countries free of FMD that have naive livestock populations, great attention is paid to reducing the possibility of incursions of the virus (Paton *et al*., 2009). In disease free counties, strict movement controls and slaughter of infected and contact animals when outbreaks occur is applied (OIE, 2004). In endemic areas, the disease is generally controlled by vaccination and movement restriction of animals (Asseged, 2005).

Preventive measures in the absence of disease should be implemented as follows: Control of national borders to prevent significant movement of animals and livestock products from non-free neighbors or trade partners. For officially free countries, prohibition of imports of animals and livestock products from endemic countries in accordance with the OIE standards. Emergency measures in the event of outbreaks through: Rapid slaughter of infected animals, in contact animals and herds considered to have received infection by contact, to reduce the quantity of virus released policy of “stamping-out”, followed by cleaning and disinfection to reduce the risk of re-infection, strict movement controls, extending to movement on and off farms of livestock products. And also

possible emergency vaccination is important (Radostits *et al*., 2007; Lefevre, 2010; Ding *et al*., 2013).

In Ethiopia context the control of FMD is practiced by involvement of quarantine, isolation of infected animals, restriction of animal movement, vaccination programs, proper disposal of infected carcass and other methods which are feasible to Ethiopian economy (Admassu *et al*., 2015). Currently there is no country-wide vaccination program aimed to control FMD and ring vaccination is carried out around an infected area. Considering the wide prevalence of serotypes the National Veterinary Institute (NVI) is producing an inactivated trivalent vaccine which contains O, A and SAT 2 serotypes (Tesfaye, 2014).

2.16. Economic impacts of FMD

Foot and mouth disease is one of the most important livestock diseases in the world in terms of economic impact (Garner *et al*., 2002; James and Rushton, 2002; OIE, 2004). FMD hinders the trading of milk, meat, animals and other agricultural products. The global impact of the disease can be separated in to direct and indirect loses (Figure 8) (Knight-Jones and Rushton, 2013). The direct and indirect losses associated with FMD are in terms of mortality, morbidity and milk production, import-export losses, growth rate and abortion (Chakraborty *et al*., 2014). The disease also interferes with agriculture (Longjam *et al*., 2011; Lordwin, 2011; Lee *et al*., 2017). Additional costs include application of control measures such as quarantines, slaughter, compensation, vaccination as well as conducting scientific surveillance after an outbreak in order to prove that the disease and the virus have been eliminated (Prempeh *et al*., 2001). FMD is a greater threat to animal industry throughout the world (Reller *et al*., 2009). It has been estimated that the costs associated with FMD in endemic areas ranges between 6.5 to 21 billion USD annually (Paarlberg *et al*., 2002; Knight-Jones and Rushton, 2013). Outbreaks in previously FMD-free countries are estimated to cost more than 1.5 billion USD per year (Paarlberg *et al*., 2002). Estimates of indirect costs due to lost exports for the U.S. have been estimated at $6.3 billion in beef exports and about $5.6 billion in pork exports each year (Buetre *et al*., 2013).

In Ethiopia FMD is posing a major threat thereby causing substantial economic losses through morbidity and mortality (Abdela, 2017). The country lost more than 14 million USD in consequence of the Egyptian trade ban in 2005/2006 (Leforban, 2005). In 2011 the total annual economic loss

due to bulls rejection from international market was estimated to be 3,322,269 USD (Alemayehu *et al*., 2014). According to Wagari (2016) about 71,026.8 USD losses was recorded in terms of economic impact on export earnings. Jemberu *et al*. (2016) reported that the estimated economic losses of FMD outbreak in cattle, arising from milk loss, mortality and draft power loss, average 76 USD per affected herd, 9.8 USD per head in crop-livestock mixed system, and 174 USD per affected herd and 5.3 USD per head in the pastoral system. In another study, the overall short-term farm level direct loss due to FMD outbreak in an urban dairy farm was estimated to €1962 (Beyi, 2012).

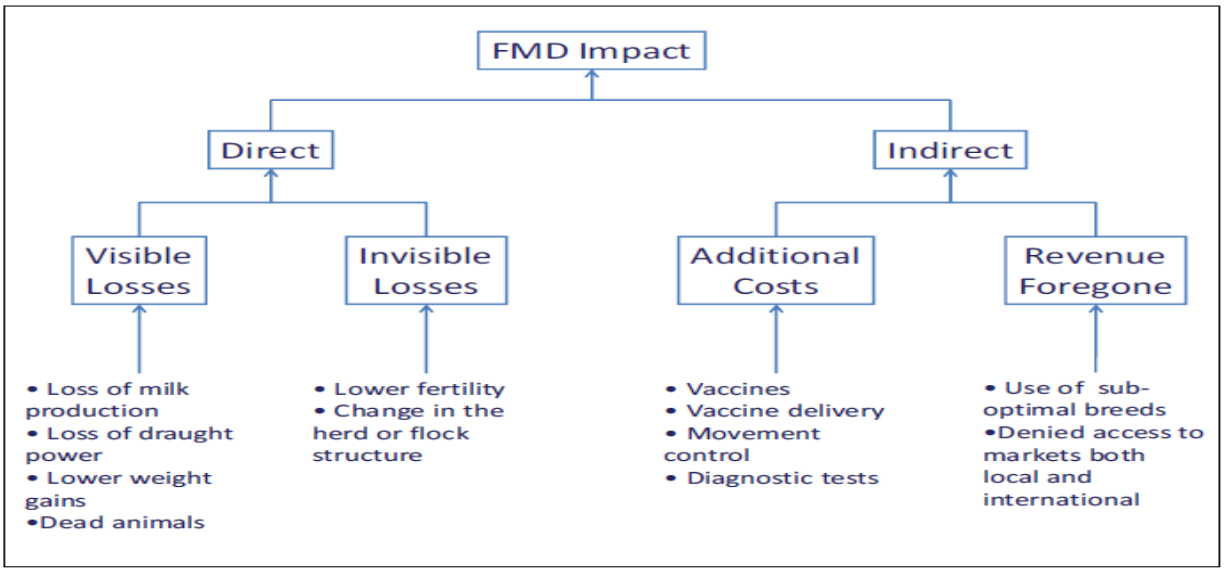


Figure 8: The impacts of foot and mouth disease

Source: (Rushton, 2009)

2.17. FMD in Ethiopia

2.17.1. Disease status

FMD is endemic to Ethiopia as it is in all the bordering countries like Eritrea, Sudan, Kenya and Somalia and restriction of animal movement is limited. A large number of wildlife, including African buffalo (particularly in the Mago and Omo national parks) could act as FMDV reservoirs. The association of SAT serotypes with wildlife, particularly African buffalo has been indicated (Bastos *et al*., 2000). The disease is widely prevalent and previously used to occur frequently in the

pastoral herds of the marginal lowland areas of the country. However, this trend has been changed and currently the disease is frequently noted in the highlands of the country (Abdela, 2017).

2.17.2. FMD virus serotypes identified

In Ethiopia foot and mouth disease was first recorded in 1957 when serotypes O and C were found while serotype A was identified in 1969 (Martel, 1974; Martel, 1975). The first isolation of SAT2 in Ethiopia was in 1989 in a sample collected from Awassa, Sidamo and Negelli Borena (Roeder *et al*., 1994). The presence of FMDV serotype SAT1 in Ethiopia was isolated and reported for the first time in 2008 from three species of animals; cattle, sheep and goats (Legesse *et al*., 2013). Currently FMD is endemic and widely prevalent and distributed in all areas of the country (Abdela, 2017). The presence of foot and mouth disease in the country is a major obstacle for the development of agriculture because of its adverse effects on livestock production and exports (Negusssie *et al*., 2011).

Endemic distributions of five of seven serotypes of FMDV are maintained in the country and serotypes O, A, C, SAT1 and SAT2 were responsible for FMD outbreaks during 1981–2018 as shown in table (1). From the report, serotype O was the most predominant serotype circulating in the country (Jemberu *et al*., 2016). The serotype C has not been reported in the country since 1983 (Ayelet *et al*., 2009; Abdela, 2017).

Table 1: Serotypes of foot and mouth disease virus isolated in Ethiopia from 1981 to 2018

Year	Serotypes	Sample origin	References
1988-1991	O and SAT 2	Addis Ababa, Eritrea, Wellega, Hararge, Dire Dawa, Borena	(Roeder *et al*., 1994)
1981-2007	O, A, C, SAT 1 and SAT 2	Addis Ababa, Ahmara and Tigray, Dire Dawa, Beneshangul-Gumuz, and Southern Nations Nationalities and Peoples Region	(Ayelet *et al*., 2009)
2007/08	O and SAT 1	Girar Jarso, Yabello, Surma, Maji, Ankesha Guagusa,	(Legesse *et al*., 2013)
2007-2012	O, A, SAT 1 and SAT 2	Koka, Surma, Sheka, Yeki, Benshangul Gumuz, Debre Zeit, Addis Ababa, Bahir Dar Harar, Debre Birhan Sululta East Shoa, Arbaminch Abaya, Borena, Dama, Guji, Adama, Mekelle, Jille Timuga, Kombolcha, Wollayta Sodo, Sidama, Mekele	(Jemberu *et al*., 2016)
2008/09	O and A	Bahirdar Zuria, South Achefer, Yilmana Densa, and Dangela, East Harereghe (Haremaya University dairy farm), Borena (Yabello District), and Bale (Sinana District)	(Negusssie *et al*., 2011)
2009/10	O and SAT 2	AA, Debre-Birhan, Debre-Zeit,	(Deribie, 2017)

		Sululta	
2010/2011	O and SAT 2	Debrezeit (Ada clinic)	(Belay and Muktar, 2015)
2011/12	O	Mekelle University Farm, Aynalem, Shibta, Cholekot, Debri **"continued"**	(Kassaw *et al*., 2013)
2011/2012	O	Alage Dairy Farm, Alaba, Adamitulu Jido kombolcha, Debre Zeit, Malga, Adama, Akaki-Kality, Mekele Universty Farm, Enderta, Debre Berehan	(Menda *et al*., 2014)
2015/16	A, O and SAT2	Guna, Ludehitosa, Adama, Boset, Kolfe	(Sulayeman *et al*., 2018)
2016/17	O, A, SAT 1 and SAT 2	Wolmera, Adea Berga Kolfe keranyo, Mulo, Kimbit	(Beksisa, 2017)
2016/17	O and A	Mulo woreda, Aleltu, Kimbibit and Wochale woredas, Adea woreda and Kolfe Koraneyo subcity	(Damena *et al*., 2017)
2016/17	O	Addis Ababa, Bishoftu, Adama	(Motuma, 2017)
2016/17	O and SAT 2	Akaki, Bole, Yeka sub city, Mojo, Koka, Alemtena, Angolela, Birbersa and Godoberet	(Wondwossen, 2017)
2016/17	O and A	Mulo, Aleltu, Kimbibit, Wochale, Adea woredas and Kolfe Koraneyo sub city	(Getachew, 2017)
2017/18	O and A	Meki, Bishoftu, Shewarobit, Bole sub city	(Metages, 2018)

Topotype is used to describe the presence of genetically and geographically distinct evolutionary lineages (Samuel and Knowles, 2001). Previous studies have provided evidence for the presence of

different topotypes of FMDV serotypes from the five serotypes (O, A, C, SAT1, SAT2) reported in Ethiopia samples collected from different outbreaks occurred from 1979-2018 as shown in table (2).

Table 2: Summary of topotype distribution of FMDV serotypes in Ethiopia from 1979 to 2018

Serotype	**Topotype**	**Genotype/ strain**	**Country/year**	**References**
O	EA-1		Ethiopia (1979–2001)	(Sahle, 2004)
	EA-3		Ethiopia (1981–2007)	(Ayelet *et al*., 2009)
	EA-4		Ethiopia (1981–2007)	(Ayelet *et al*., 2009)
	EA-4		Ethiopia (2016-2017)	(Beksisa, 2017)
	EA-3		Ethiopia (2017-2018)	(Metages,2018)
	EA-3		Ethiopia (2009/2010)	(Deribie, 2017)
	EA-3		Ethiopia (2011/2012)	(Menda *et al*., 2014)
	EA-3		Ethiopia (2008/09)	(Negusssie *et al*., 2011)
	EA-3		Ethiopia (2011/2012)	(Kassaw *et al*., 2013)
	EA-4		Ethiopia (2016/17)	(Motuma, 2017)
	EA-4		Ethiopia (2016/17)	(Wondwossen, 2017)
A	Africa		Ethiopia (1981–2007)	(Ayelet *et al*., 2009)
	African	IV	Ethiopia (2015/2016)	(Sulayeman *et al*., 2018)
	Africa	G-VII	Ethiopia (2008/09)	(Negusssie *et al*., 2011)
	African	G-I	Ethiopia (2017/2018)	(Metages, 2018)
C	Africa		Ethiopia (1981–2007)	(Ayelet *et al*., 2009)
SAT 1	IX		Ethiopia (2007)	(Ayelet *et al*., 2009)
	IX		Ethiopia **(**1981–2007)	
SAT 2	XIV		Ethiopia (1991)	(Ayelet *et al*., 2009)
	IV		Ethiopia (1989)	
	XIV		Ethiopia (1991)	
	XIII		Ethiopia (2007)	

	XIII		Ethiopia (2009/10)	(Deribie, 2017)
	VII	Alx-12	Ethiopia (2015/2016)	(Sulayeman *et al*., 2018)

2.17.3. Vaccine type

In Ethiopia trivalent inactivated vaccine manufactured from locally isolated FMDV serotypes O, A and SAT2 is produced by the National Veterinary Institute (NVI) (Tesfaye, 2014). The virus is propagated from cell culture and absorbed into aluminum hydroxide gel and inactivated with 0.3% formaldehyde and adjuvinated with saponin. The recommended dosage for cattle is 4ml per head and administered subcutaneously, preferably in the dewlap region. In order to protect the cattle, two injections at 6 months interval are recommended. Immunity develops 2-3 weeks after vaccination and may last for one year (DACA, 2006).

3. MATERIALS AND METHODS

3.1. General description of FMD outbreaks

FMD outbreaks were defined as the occurrence of new disease in a given areas. The outbreaks were occurred in Tigray (Hintalo Wajirat, Kilte Awilailo, Asgede Tsimbala, Enderta, Saisi Tsaida Imba, Amba Alaje, Raya Alamata and Korem districts), Oromia (Wolmera and Adea Berga districts of West Shewa), SNNPs (Shashogo district) and Oromia regional states of Horo Guduru Wollega zone in Guduru district respectively. In the study areas of the research the disease is locally called Afeme'ar and Anashe in Tigray regional state, Anjicho in Hadiya zone of Shashogo district in SNNPs regional state and Kote bekekisafi gororisisa and Kebenna in Oromia regional state. In outbreak areas, cattle that have exhibited clinical signs of the FMD were identified and samples were collected for confirmatory testing.

3.2. Study areas

The study was conducted from September 2018 to May 2019 in Tigray, Oromia and SNNPs regional states in areas where outbreak of FMD occurred. Totally twelve outbreaks among these eight outbreaks in Tigray, three outbreaks in Oromia and one outbreak in SNNPs regional state were encountered and investigated during the study period as shown in figure (9). In most of the study areas animals were reared under extensive management system although semi-intensive and intensive management system were also practiced in farms found in Adea Berga (Adea Berga dairy farm) and Wolmera (Holleta Agricultural Research Center (HARC) dairy farm). The specific districts, kebeles and farms were purposively selected based on presence of suspected FMD outbreak report.

The state of Oromia sprawls over the largest part of the country and at present consists of 12 administrative zones and 180 woredas. The capital city of the state of Oromia is Finfinnee (Addis

Ababa). The State of Oromia borders Afar, Amhara and the state of Benishangul Gumuz in the north, Kenya in the south, The state of Somali in the east, the state of Benishangul Gumuz in the west, the state of South Sudan, Southern Nations, Nationalities and Peoples' (SNNPs) and the state of Gambella in the south. It covers 366,000 km^2, accounting for 31.17% of the total area of Ethiopia (Menda *et al*., 2014). The region has 24,432,974 cattle, 9,394,430 sheep, 8,591,204 goats, 1,128,901 horses, 134,898 mule, 3,419,932 donkeys, 315,482 camels, 19,014,114 poultry and 3,185,361 bee haves (CSA, 2018). FMD outbreaks were investigated in Horo Guduru Wollega zone of Guduru district, West Shewa zone of Adea Berga district and Oromia special zone of Wolmera district (Figure 9).

Southern Nations, Nationalities, and Peoples' (SNNPs) region is one of the nine ethnically based regional states of Ethiopia. The state of SNNPs' comprises 10% of the total area of the country that is administratively divided in to 9 zones, 72 woredas and 5 special woredas. Its capital city is Hawasa. The SNNPs regional state borders Kenya to the south, south Sudan to the west, the Ethiopian region of Gambela to the northwest, and the Ethiopian region of Oromia to the north and east. The livestock resource of the region consists of 11,883,548 head of cattle (representing 19.68% of Ethiopia's total cattle), 4,639,606 sheep, 4,958,255 goats, 371,298 horses, 91,461 mules, 811,105 donkeys, 10,491,131 poultry, and 1,335,541 beehives (CSA, 2018). The outbreak was investigated in Hadiya zone of Shashogo district (Figure 9). The district is located 360 km South of Addis Ababa and bordered on the south by the Kembata Tembaro zone, on the west by Limo, on the northwest by Ana Lemo, on the northeast by the Silte zone, and on the southeast by the Alaba special zone. It has an altitude of 1800-2300 masl, average annual rainfall of about 900-1250 mm. The minimum and maximum temperature of the area is 11^0c and 37^0c, respectively (Assefa *et al*., 2015).

Tigray regional state is located in Northern Ethiopia. The region has common boundaries with Afar and Amhara regional states at the eastern and southern parts, respectively and international boundaries with Sudan and Eritrea at the western and northern parts, respectively. It covers 54,548.32 km^2 (Menda *et al*., 2014). The livestock resource of the region consists of 4,817,104 cattle, 4,301,221 goats, 2,472,938 sheep, 15,664 horses, 11,308 mule, 886,103 donkeys, 43,332 camel, 6,190,640 poultry and 293,184 bee hives (CSA, 2018). Mekelle is the capital city of Tigray regional state located about 783 km North of Addis Ababa at 38.5° east longitude and 13.5° north latitude at an altitude of 2300 meter above sea level (masl) (Kassaw *et al*., 2013). The climate

conforms to that of the Ethiopian highland. The FMD outbreaks were occurred at Amba Alaje, Asgede Tsimbala, Enderta, Hintalo Wajirat, Kilte Awilailo, Korem, Raya Alamata and Saisi Tsaida Imba districts (Figure 9).

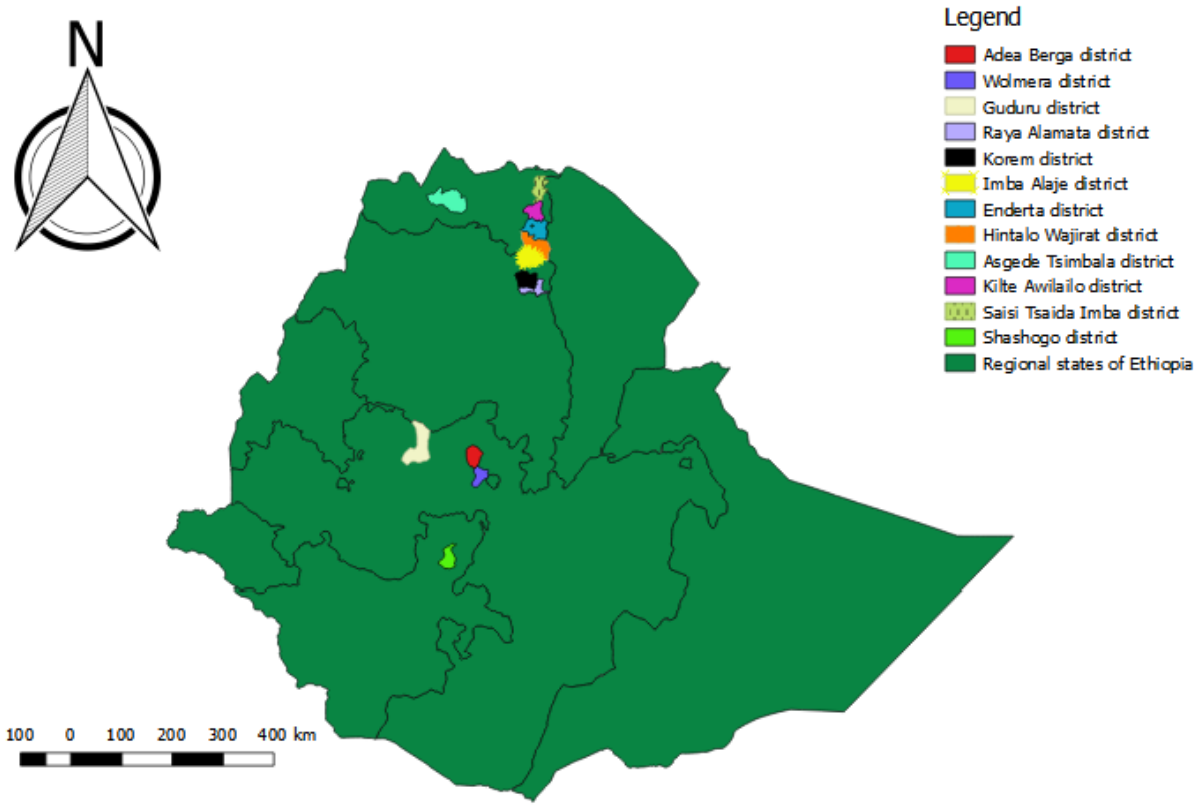


Figure 9: Map of Ethiopia indicating study districts of the research.

Specific sampling districts/areas are indicated in different colors that are explained in the legend

3.3. Study population

The study population consists of cattle that are manifesting the clinical signs of the disease. In each outbreak, cattle manifesting the characteristic signs of FMD like salivation and lameness in each individual animal owner‘s farm and small holding areas were examined from distance. The mouth cavities of salivating animals were examined for evidence of intact and/or ruptured vesicles, erosions and ulcers, and the hooves of lame animals were carefully examined for presence of lesions. Cattle with clinical symptoms irrespective of age groups (Appendix 3), sex, breeds and management systems were recorded during the study period.

3.4. Study design

Proper information channel to National Animal Health Diagnostic and Investigation Center (NAHDIC), regional veterinary diagnostic laboratories, zonal and district veterinary officers were organized and information about the outbreaks were gathered via mobile telephone communication before the beginning of any outbreak investigation. Based on reports of active cases of FMD outbreaks, a cross sectional study design was conducted for outbreak investigation so as to isolate and characterize the serotypes of FMD virus circulating in the study areas. When active outbreak of FMD was reported, field level investigation was conducted purposively at a particular site of outbreaks within the study districts (Figure 9). In each selected kebele, animals having clinical symptoms like vesicles on the dental pad, tongue, muzzle and hooves were purposively sampled as per the extent of the outbreak. The inclusion criteria were cattle with clinical symptoms and the exclusion criteria were cattle in the same farms without any clinical signs when outbreak occurred in the study area. Tissue and oral swab samples were collected from clinically infected animals for diagnostic testing.

3.5. Sampling method

Based on the presence of FMD outbreaks, a field investigation was conducted purposively at the specific sites of the outbreak within the study districts, peasant associations, cattle herds with clear signs, symptoms and suspected to be diseased with FMDV. The information about the disease was first gathered by interviewing livestock owners and animal health professionals of each outbreak site, time, animals affected and death, if any. The collected information were recorded on data collection sheet, and then physical examination was conducted on clinically sick animals to examine for presence of FMD like lesions on the mouth, feet and teat. Accordingly, cattle with clear signs and symptoms of infected with FMDV were selected and sampled.

3.6. Study methodology

3.6.1. Clinical examination

During the FMD outbreak investigation cattle were first physically examined for the presence of typically vesicular lesions in the mouth, lips, tongue, feet and teat. The oral cavity of salivating cattle 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 cattle were also thoroughly washed with clean water and then carefully examined for the presence of lesions particularly on the coronary bands and interdigital spaces of the hooves. Teat of cows were also examined for presence of any vesicular lesion. Sampling was conducted in cattle that showed active clinical signs of the disease (Figure 10).

A **B** **C**

Figure 10: Clinical signs observed on cattle infected with FMDV

(A) Gum lesion, (B) Salivation, (C) Tongue lesion

3.6.2. Sample collection

After the identification of the animal as a suspected case, epithelial tissue samples which were un-ruptured or freshly ruptured vesicle were collected from FMD suspect cattle usually from the tongue, buccal mucosa or feet by using sterile forceps and scissor then placed in universal bottle with viral transport medium (VTM) composed of equal amounts of glycerol and 0.04-M phosphate buffered saline (PBS) solution (pH 7.2-7.6) with antibiotics (OIE, 2004; Mukasa *et al*., 2016).

Oral swab specimens were also obtained by swabbing under the tongue and an area of contact between the lower gum and the inner surface of the lower lip (Callahan *et al*., 2002). In additional oral swab samples were also collected from fresh lesions in mouth by sterile cotton-tipped wood applicators disposable into sterile plastic screw-cap tubes. The oral swabs were preserved using PBS as recommended by OIE (2012). A total of 215 animals were accessed during outbreak study for the presence of FMDV lesion in Tigray, Oromia and SNNPs regional states. Accordingly, 63 (55 epithelial tissue and 8 oral swab) samples were collected from 7 zones of 12 districts (Table 3). Out of which all samples were forwarded for molecular detection, 34 representative samples for cell culture based virus isolation, 26 samples for serotyping and 23 representative culture isolates for sequencing of virus. All samples collected were labeled with identification number, date of collection, sex, breed, age, type of sample, disease suspected and other appropriate information listed under sampling method. Then, the collected samples were transported at 4 ^{0}C by using ice packs box from the collection site to NAHDIC, Sebeta, Ethiopia for sample processing and other laboratory investigation. All the samples were coded by laboratory code at reception room and transferred to laboratory. Upon arrival, the samples were immediately stored at -80ºC until processed.

Table 3: Description of samples collected from infected animals having oral and feet lesions

Region	Zone	District	Kebele	No of animals		samples collected	
				Examined	Affected	ET	OS
Oromia	HGW	Guduru	Kombolcha	10	3	3(30%)	-
			Gutu Abay	9	3	3(33.3%)	-
			Keneni	6	2	2(33.3%)	-
	WS	Adea Berga	Adea Berga dairy farm	36	10	10(27.8%)	-
	OSZ	Wolmera	HARC dairy farm	41	10	10(24.4%)	8
SNNPs	Hadiya	Shashogo	Molelicho	10	2	2(20%)	-
Tigray	SE	Enderta	Messebo	5	1	1(20%)	-
	E	Saisi Tsaida Imba	Senkata	6	1	1(16.7%)	-
	S	Amba Alaje	Teki'a	45	14	14(31.1%)	-
			Ayida	12	3	3(25%)	-
		Raya Alamata	Kidana	13	1	1(7.7%)	-
		Korem	02	7	1	1(14.3%)	-
	NW	Hintalo Wajirat	Dejen	4	1	1(25%)	-
		Kilte Awilailo	Wukro	7	2	2(28.6%)	-
		Asgede Tsimbala	Alogean	4	1	1(25%)	-
Total				**215**	**55**	**55(25.58%)**	**8(3.7%)**

Key: E- East, ET- Epithelial Tissue, HGW- Horo Guduru Wollega, NW- Northwest, OS- Oral Swabs, OSZ- Oromia Special Zone, S- Southern, SE- Southeast, SNNPs- Southern Nations, Nationalists and Peoples, WS- West Shewa

3.6.3. Sample preparation

According to OIE (2012) manual sample preparation was done following the procedure. The tissue 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. Totally 1 gram of epithelial tissue was grinded with sterile sand by a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics (penicillin, streptomycin and Amphotericin B solution). Dulbecco's Modified Eagle Medium (DMEM) was added until a final volume of nine times that of added epithelial sample was reached, giving a 10% suspension (OIE, 2012). The suspension was clarified on a bench centrifuge at 3000 rpm for 10 minutes. The supernatant of the suspension was collected in sterile cryovial tubes and filtered by Millipore filter paper of 0.22 μm pore size, labelled and stored at -80 ^{0}C for until needed for further tests.

3.7. Detection of circulating FMDV

3.7.1. RNA extraction

Total RNA was extracted from supernatants of the homogenized epithelial tissue and oral swab suspensions by Qiagen RNA extraction kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's recommendation. First 140 μl epithelium tissue suspensions and oral swab samples were separately added to with 560 μl of prepared buffer AVL containing carrier RNA into a 1.5 ml micro centrifuge Eppendorf tube, mixed by pulse vortexing for 15second (sec) then incubated at 25 ^{0}C for 10 minute (min) for lysing purpose. The tubes were then briefly centrifuged to remove drops from the inside of the lid. Binding was done by adding 560 μl of ethanol (96%), mixed by pulse-vortexing for 15sec then briefly centrifuged the tube to remove drops from inside the lid. A 630 μl of the solution transferred into QIAamp Mini spin (silica) column, then centrifuged at 8000 rpm for 1min through placing the QIAamp Mini column and collected into a clean 2 ml collection tube. Any left solution was processed again similarly to get more quantity of RNA material. Washing was first conducted by 500 μl of buffer AW1 and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and the column was placed in a fresh 2ml collection tube. Then 500 μl of buffer AW2 were added to the column then centrifuged at 14,000 rpm for 3 min. Then 60 μl of buffer AVE was added to the column and incubated at room temperature for 1 min before centrifuged at 8000 rpm for 1 min. Finally, the RNA of the FMDV was obtained. Extracted RNA was kept at +4°C for

immediate use or stored at -80 ^{o}C until used for real time reverse transcription polymerase chain reaction (rRT-PCR).

3.7.2. Detection of FMDV by real time RT-PCR

The one step real time polymerase chain reaction (rRT-PCR) assay was applied for primary detection of the FMDV. Real time RT-PCR has a sensitivity comparable to that of virus isolation and automated procedures enhanced sample throughput (Reid *et al*., 2003). Reverse transcription of FMDV RNA and PCR amplification of reverse transcribed RNA was conducted using automated one step real time RT-PCR as reported before (Callahan *et al*., 2002) that detects the 3D RNA polymerase encoding gene. The 3D non-structural protein is viral RNA dependent RNA polymerase (RdRp), which is responsible for RNA replication and is highly conserved (94-99% similarity) (Feng *et al*., 2004). The nucleotide sequence of forward primer (FMDV 3DF) 5' - ACT GGG TTT TAC AAA CCT GTG A-3′, the reverse primer (FMDV 3DR) 5' - GCG AGT CCT GCC ACG GA-3′ and the probe (FMDV Probe 3DP) 5'- [6FAM] TCC TTT GCA CGC CGT GGG AC [TAM]-3′ were used (Callahan *et al*., 2002). The probe was labeled with 5'- reporter dye, 6-carboxyfluorescein and 3'- quencher, tetra-methyl-rhodamine in real time RT-PCR reaction to detect the 3D^{pol} gene sequence in all the FMDV serotypes. Real time RT-PCR assay was carried out by using Superscript III/Platinum Taq one-step rRT-PCR kit. The master mix reaction components for one step real time RT-PCR was prepared using, 12.5µl of 2x - reaction mix, 1.5µl of RNAse-free water, 2µl of forward primer, 2µl of reverse primer, 1.5µl of Taqman probe and 0.5µl of superscript ®III RT to make a total of 20µl per sample for each reaction of PCR per well per plate including positive and negative control master mix. Then thoroughly mixed by pulse vortexing (Appendix 4).

A 5µl extracted RNA template was added to each PCR plate and the total volume of PCR plate was 25µl. The PCR plate was sealed by adhesive film then inserted into the thermal cycler machine slots and adjusted according to QIAgen one step RT-PCR kit protocols. The one step rRT-PCR amplification was started with reverse transcription (RT) cDNA synthesis at 50°C for 30 min; followed by initial denaturation at 95°C for 10 min; denaturation at 95°C for 15sec, annealing at 60°C for 1min and extension at 72 ^{o}C for 30 seconds. Totally 50 cycles were taken to finish amplifications (Mukasa *et al*., 2016). Negative control (nuclease free water) and positive control (field isolate) were included in each run.

Interpretation of real time PCR result

The PCR amplification was carried out in the thermal cycler Rotor- Gene Q (Qiagen®, Germany). The successfully amplified target gave an amplification curve and the cycle threshold (C_t) at which the target amplicon was initially detected above the background fluorescent levels as determined by the instrument SDS software. Then FMDV was detected through threshold cycle (C_t) values based on baseline and graphs. The C_t values <32.0 were considered as positive and amplification with C_t values of undetermined were considered as negative. Samples which had C_t values ≥32 and <50 were "inconclusive" and the test was repeated.

3.8. Virus isolation

Virus isolation was established under class II biosafety cabinet. About 1ml of filtered FMDV positive suspensions detected by real time RT-PCR were inoculated to baby hamster kidney (BHK-21) cell line grown in 25cm^2 tissue culture flask and incubated at 37^{o}C for 1 hour (hr) 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% CO_2 in a humidified incubator, while normal non-infected cells served as control. The plates were monitored daily for the presence of cytopathic effect (CPE) for 24-48 hours. CPE was observed after 48 hours even less in some positive cases. CPE was characterized by a fast destruction of the monolayer cell, cell rounding and cell disruption and detached from the flask. If no CPE was observed after 48 hr, the culture was frozen at -80°C and thawed. Freezing and thawing was repeated for three cycles for lysis of cells and release of viruses (OIE, 2012). Then 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 as 'no virus detected' (NVD) as described by Souley *et al*. (2018). The cells were harvested when >85% of monolayer showed CPE. When CPE appeared in the positive cases, supernatants of cultures were used in serotype differentiating antigen detection ELISA for confirmation of serotype of the virus involved in the outbreak. Normal uninfected BHK-21 cell was used for negative control and isolated samples that showed CPE labeled according to a system specified by OIE standards (OIE, 2012). The viral isolates were labeled using the following format: three-letter country code/isolate number/year.

3.9. Typing of detected FMDV by antigen detection ELISA

Isolates that had CPE positive were forwarded for FMDV serotyping. Serotyping was conducted by antigen detection ELISA (IZSLER: Brescia, Italy) with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies. According to the protocol recommended by the Pirbright Institute, UK, the test was applied for detecting and serotyping of FMD viruses. The kit was designed for detection and typing of FMD viruses of type O, A, SAT 1 and SAT 2. A pan FMD test, detecting any isolates of serotypes O, A, C, Asia 1 and some of the SAT serotypes was 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 pre-coated with catching MAbs and with both positive and negative controls already incorporated onto plates from manufacturer. The micro plates were catched with 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 microplate trapped by the respective catched MAbs. The test was conducted as per the manufacturer's instruction. First samples were diluted ½ in diluent buffer and 50µl/well of each sample was distributed to 10 wells of a column (a total of 80 wells of A-H rows). Then, 50µl of diluents per well were added in all wells of 11 and 12 columns (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 tapped 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 (totally three washing cycles). Then all residual fluids were removed by gently 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 room. Finally, the reaction was stopped by adding 50µl/well of stop solution (i.e. sulfuric acid (H_2SO_4)). Immediately after stopping, reading the optical density (OD) of each well was done at 450 nm wavelength using micro plate reader.

Criteria for test validity and interpretation of the results of antigen detection ELISA

The positive inactivated controls were expected to give OD values of ≥1.0 unit higher while the negative control for serotype O, A, C, Asia 1 and Pan-FMDV are expected to give OD values <0.1unit and the negative control for serotype SAT1 and SAT2 are expected to give OD value ≤0.2unit. Results for the samples examined were interpreted as indicated in the table (4) below, after the subtractions of the OD value of each negative control from the OD value measured for samples with the corresponding catching Mab.

Table 4: Interpretation of antigen detection ELISA result

Negative for FMDV	OD <0.1
FMDV +ve for type O	OD ≥ 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 +ve for type A	OD ≥ 0.1 with at least one of the two type A MAbs and with the pan-FMDV MAb
FMDV +ve for Asia 1	OD ≥ 0.1 with the type Asia 1MAb and with the pan-FMDV MAb
FMDV +ve for type C	OD ≥ 0.1 with the type C MAb and with the pan-FMDV MAb
FMDV +ve for SAT1	OD ≥ 0.1 with the type SAT1 catching MAb; some samples could be positive also with the pan-FMDV MAb
FMDV +ve for SAT2	OD ≥ 0.1 with the type SAT2 catching MAb; some samples could be positive also with the pan-FMDV MAb
FMDV +ve (untyped)	OD ≥ 0.1 with the pan-FMDV catching MAb and < 0.1 with the type specific MAbs

Note: OD values ≥ 0.05 and <0.1 should be considered suspect and should be retested; new samples or further investigations by other diagnostic tests may be required.

3.10. FMDV sequencing and phylogenetic analysis

Sequencing and analysis of the VP1 coding region of the FMDV is becoming an increasingly more accessible laboratory tool, providing information that aids our understanding of the spread and global epidemiology of the virus. VP1 sequence data comparisons remain the main method for classification of FMD viruses (Knowles *et al*., 2016). Totally, 23 representative isolates from all outbreak areas (Table 5) were 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. Sequencing and sequence editing were conducted in WRLFMD Pirbright, United Kingdom. The edited sequences were used for molecular characterization of the virus by phylogenetic tree reconstruction. Multiple sequence alignments was conducted by using Clustal W algorithm implemented in BioEdit and MEGA6 software (Thompson *et al*., 1994; Tamura *et al*., 2013) packages to compare with the VP1 sequence of outbreaks isolates retrieved from GenBank. For comparative studies, BLAST search was conducted using MEGA6 software to collect additional FMDV serotype O and A sequences from GenBank (National Centre Biotechnology Information) (Tamura *et al*., 2013). Minimum-evolution methods of analysis as implemented in MEGA6 was used to construct phylogenetic tree of FMDV based on VP1 sequences data for viruses sequenced from Ethiopia, Africa and from other countries. The robustness of tree topology was assessed with 500 bootstrap replicates as implemented in the program and bootstrap values of >70 are shown at the relevant major nodes (Gorna *et al*., 2014).

Table 5: List of representative cell culture isolate samples submitted to WRLFMD

Outbreak site			Country code	Sampling date
Region	**District**	**Kebele**		
Oromia	Wolmera	HARC dairy farm	ETH/2736/19	10/01/2019
			ETH/2737/19	10/01/2019
			ETH/2745/19	10/01/2019
	Guduru	Kombolcha	ETH/8447/19	09/03/2019
			ETH/8452/19	09/03/2019
		Gutu Abay	ETH/8450/19	09/03/2019
		Keneni	ETH/8451/19	09/03/2019
	Adea Berga	Adea Berga dairy farm	ETH/4575/19	06/02/2019
			ETH/4583/19	06/02/2019
			ETH/4580/19	06/02/2019
			ETH/4579/19	06/02/2019
SNNPs	Shashogo	Molelicho	ETH/8442/19	22/02/2019
Tigray	Saisi Tsaida Imba	Senkata	ETH/32297/19	14/12/2018
	Amba Alaje	Teki'a	ETH/32300/19	16/12/2018
			ETH/32309/19	16/12/2018
			ETH/32303/19	16/12/2018

	Ayida	ETH/32313/19	16/12/2018
Korem	02	ETH/4586/19	03/02/2019
Raya Alamata	Kidana	ETH/4585/19	01/01/2019
Hintalo Wajirat	Dejen	ETH/23802/19	06/11/2018
Kilte Awilailo	Wukro	ETH/23803/19	09/11/2018
		ETH/23804/19	09/11/2018
Asgede Tsimbala	Alogian	ETH/23805/19	13/11/2018

HARC- Holleta Agricultural Research Center; SNNPs- Southern Nations, Nationalists and Peoples

3.11. Data management and analysis

Data generated from field and laboratory investigations were recorded and coded by using Microsoft Excel spreadsheet and descriptive statistics were applied to calculate the proportion. Cell culture results, CPE development, serotype identification and molecular characterization results were recorded and tabulated. Multiple sequence alignments was conducted by using Clustal W algorithm implemented in BioEdit and MEGA6 software packages for the new FMDV VP1 sequence data with other reference sequences. Minimum-evolution methods of analysis and maximum likelihood method imbedded in MEGA6 were used to reconstruct the phylogenetic tree of the aligned sequence and confidence levels were assessed by 500 bootstrap replications (Tamura *et al*., 2013). 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. Characteristics of sampled animals

In this study a total of 215 cattle were examined for the presence of typical clinical signs, 55 animals (25.58%) showed clinical signs and lesions suggestive of FMD. Accordingly, it was among these infected cattle that sufficient epithelial tissue and oral swab samples were taken. The cattle were managed under intensive, semi intensive and extensive management system. Most of the sampled animals were adult and the breed composition were 34 (61.82%) local, 10 (18.18%) jersey and 11 (20%) cross (HF). The clinical signs of affected animals were salivation, lameness, interdigital lesion, and oral and tongue lesion.

4.2. Clinical examination

Cattle were carefully examined for the presence of typical clinical signs of FMD. In each outbreak, cattle manifesting the clinical signs of FMD like vesicular lesions (ruptured vesicles) in oral cavity and on the feet and teats, salivation, lameness, and rise in temperature were considered as clinically affected by FMD. Most of the affected animals in the study area showed mouth lesions. No deaths were recorded during the outbreak in both districts of the study areas.

4.3. Molecular characterization of FMDV

4.3.1. Detection of FMDV genome

The presence of FMD viral genetic material was detected by using real time reverse-transcription polymerase chain reaction (rRT-PCR) method. rRT-PCR has been widely adopted by FMD reference laboratories as a principal tool for FMDV detection, offering high sensitivity and rapid sample throughput (Shaw *et al*., 2007; Reid *et al*., 2009). rRT-PCR was used for accurate detection of FMDV in serum, epithelial suspensions and oropharyngeal fluid (OP) (Howson *et al*., 2017). The extracted RNAs from clinical samples were tested by rRT-PCR method targeting the 3D regions of FMDV genome to determine the presence of viral RNA in clinical samples. The successfully amplified target gave an amplification curve and the cycle threshold (C_t), at which the target amplicon was initially detected above the background fluorescent levels as determined by the imbedded software. The C_t value (cycle threshold or crossing point) corresponds to the number of cycles required for a given sample to reach the threshold above is considered as positive. Hence, using a positive C_t value of 32.0 (Shaw *et al*., 2007), FMDV genome was detected by rRT-PCR. The C_t cut-off value is 32.0 and any Ct value <32.0 indicates positive result and Ct values of "Undetermined" are considered negative result. Samples giving Ct values of between ≥32.0 and < 50 are "inconclusive" and the samples were repeatedly tested.

For FMDV detection, real time RT-PCR (using universal primers and probe of FMDV) was performed on all 63 collected samples. Out of 63 samples tested by rRT-PCR, 53 (84.13%) samples were found positive having C_t value ranging from 15.06 to 31.19 and the fluorescence of samples rise above the background fluorescence (Figures 11). Positive reactions were defined as those which gave a detectable C_t (Howson *et al*., 2017). Strong positive FMD samples have a C_t value below 20.0 (Reid *et al*., 2001). A total of 18 (33.96%) samples that were collected from Tigray region (Kilte Awilailo, Amba Alaje, Saisi Tsaida Imba, Hintalo Wajirat and Asgede Tsimbala districts), Oromia region (Guduru and Adea Berga districts) and SNNPs (Shashogo district) were found strong positive having C_t values below 20.0. The lowest C_t value was (15.06) recorded in epithelial tissue sample collected from Amba Alaje district, while the highest C_t value (31.19) was obtained from bovine oral swab sample collected from HARC dairy farm, Wolmera district. The C_t values of oral swab samples were higher than epithelial tissue suspensions, indicating that there were higher levels of viral RNA concentration in the epithelial tissue samples than oral swab samples. Out of 63 samples, 10 (15.87%) samples were negative having C_t values "undetermined" to be no virus

detected (NVD) (Table 6). Two positive controls consisting of viral RNA and two negative controls consisting of nucleus free water (no-template) were included with each rRT-PCR run.

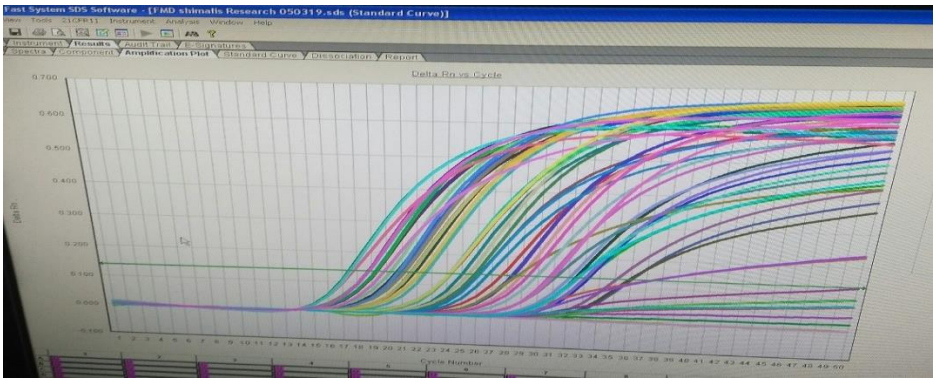


Figure 11: Real time RT-PCR result showing amplification curve.

Green color shows cut-off of point for Ct value positivity and negativity.

Table 6: Sample wise result of rRT-PCR for FMDV

Sample	**Real time PCR**	
	Positive	**Negative**
Tissue	51	4
Swab	2	6
Total	**53**	**10**

4.4. FMD Virus isolation

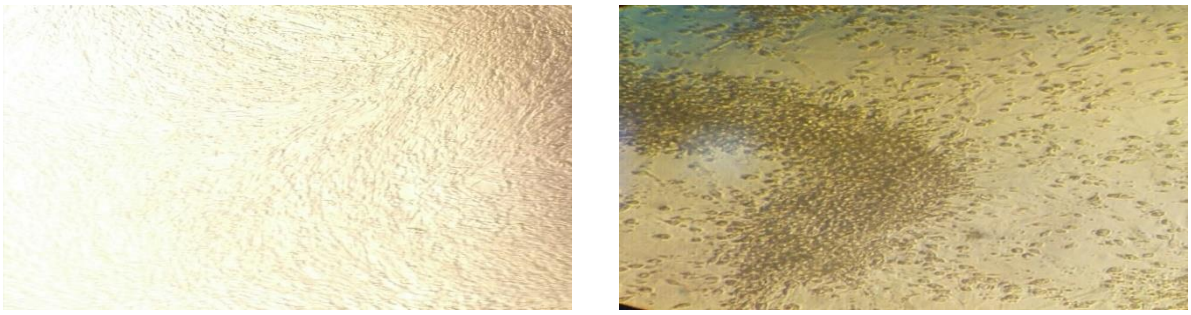
Virus isolation was performed on some samples that were positive in rRT-PCR for which sufficient material was available. Totally 34 representative FMDV samples were cultured for virus isolation. Those samples were from different outbreak areas of the country and inoculated in BHK-21 cell line with three subsequent passages for virus isolation. The results indicated that out of the total 34 bovine epithelial tissue cultured samples, 26 (76.47%) samples were showed morphological alteration (FMDV CPE) on BHK-21 monolayer cell cultures (Table 7). Most of the isolated viruses exhibited CPE characterized by a fast destruction of BHK-21 monolayer cells, and the infected cells were rounded and formed singly (Figure 12). Finally BHK-21 cell deaths which are characteristic findings of FMD virus infected cells.

Table 7: FMDV isolated on BHK-21 cell and the status of viral CPE in different areas

Outbreak sites			**No. of sample**	**CPE status**	
Region	**District**	**Kebele**		**Positive**	**%**
Oromia	Guduru	Kombolcha	2	2	100%
		Gutu Abay	1	1	100%
		Keneni	1	1	100%
	Adea Berga	Adea Berga dairy farm	5	4	80%
	Wolmera	HARC dairy farm	6	4	66.67%
SNNPs	Shashogo	Molelicho	2	1	50%
Tigray	Enderta	Messebo	1	NVD	0%
	Saisi Tsaida Imba	Senkata	1	1	100%
	Amba Alaje	Teki”a	8	5	62.5%
		Ayida	1	1	100%
	Raya Alamata	Kidana	1	1	100%
	Korem	02	1	1	100%
	Hintalo Wajirat	Dejen	1	1	100%
	Kilte Awilailo	Wukro	2	2	100%
	Asgede Tsimbala	Alogean	1	1	100%

Total	**34**	**26**	**76.47%**

HARC- Holleta Agricultural Research center, NVD- No Virus Detected, No.- Number; SNNPs- Southern Nations, Nationalists and peoples



A **B**

Figure 12: BHK-21 cell line infected with FMDV virus (A control, B infected cell) showing typical CPE.

4.5. FMDV serotyping by antigen detection ELISA

The antigen detection ELISA test was conducted for identification and serotyping of FMDV. Out of 26 samples (cell culture supernatants) subjected for serotyping by the antigen detection ELISA, all samples were found positive and two types of FMDV serotypes were detected. Out of 26 positive samples, 14 (53.85 %) were serotype O and 12 (46.15%) were serotype A. No evidence of co-circulation of the two different serotypes within the same animal was found since only one serotype was amplified from each sample. Samples collected from Wolmera district, HARC dairy farm were serotyped into type "A", whereas samples from Amba Alaje, Saisi Tsaida Imba, Korem and Raya Alamata districts were serotyped into type "O" and samples collected from outbreak of Adea Berga district, Adea Berga dairy farm were serotyped into type "A". However, samples collected from Shashogo district were positive for serotype "O" and samples from Guduru districs were serotyped into type "A". The findings also showed that outbreaks from districts of Hintalo Wajirat, Kilte Awilailo and Asgede Tsimbala were due to FMDV serotype "O" as shown in table (8).

Table 8: Summary of FMDV serotypes from samples of cell culture supernatants detected by ELISA

Region	District	Kebele	No. of positive	Identified serotypes
Oromia	Guduru	Kombolcha	2	A
		Gutu Abay	1	A
		Keneni	1	A
	Adea Berga	Adea Berga dairy farm	4	A
	Wolmera	HARC dairy farm	4	A
SNNPs	Shashogo	Molelicho	1	O
Tigray	Saisi Tsaida Imba	Senkata	1	O
	Amba Alaje	Teki'a	5	O
		Ayida	1	O
	Raya Alamata	Kidana	1	O
	Korem	02	1	O
	Hintalo Wajirat	Dejen	1	O
	Kilte Awilailo	Wukro	2	O
	Asgede Tsimbala	Alogean	1	O

Total	**26**	**14 (O) and 12 (A)**

HARC- Holleta Agricultural Research Center; SNNPs- Southern Nations, Nationalists and peoples; No.– Number

4.6. Phylogenetic analysis of FMDV serotype O

Virus protein (VP) 1 coding sequence (639 nucleotides) showing the genetic relatedness between the sequences of FMDV serotype O isolated from the study areas of Ethiopia and previously characterized Ethiopian isolates and other reference viruses from African origin were retrieved from NCBI database. The partial VP1 gene sequence data was used to construct the phylogenetic tree and study the genetic relationships between the current outbreak isolates with the isolates retrieved from the data base. For the analysis 49 nucleotide sequences were included 11 from the current study and 38 nucleotide sequences retrieved from data base. The 11 isolates falls into two different topotypes. The sequenced serotype O isolates of Saisi Tsaida Imba, Amba Alaje, Hintalo Wajirat, Kilte Awilailo, Asgede Tsimbala, Korem and Raya Alamata districts , Ethiopia falls to East Africa-3 (EA-3) but the sequenced serotype isolated from Shashogo district was topotyped to East Africa-4 (EA-4) (Figure 13). All viral isolates from Tigray regional state showed 98.5-100% nucleotide sequence similarity with each other (Table 9). A single isolate from SNNPs regional states of Shashogo district (O/ETH 14/2019) which was topotyped under EA-4 had 80.5-81.3% nucleotide identity with the current strains isolated from Tigray regional state. The viral isolates from Tigray regional state showed 86.4-87.4% nucleotide sequence identity with previous strains of Ethiopia O/ETH/30/94 (AY283386) and O/ETH/3/96 (AY283392) that tends to cluster with them on the tree. The isolates of Shashogo district (O/ETH 14/2019) shared 86.8 and 87% nucleotide identity with previous strains

from Ethiopia (O/ETH/58/2005, accession number FJ798141 and O/ETH/59/2005, accession number FJ798142), respectively both of which are classified under topotype EA-4. This EA-4 topotype virus is clustered with FMD viruses circulating in Uganda and Ethiopia (Figure 13).

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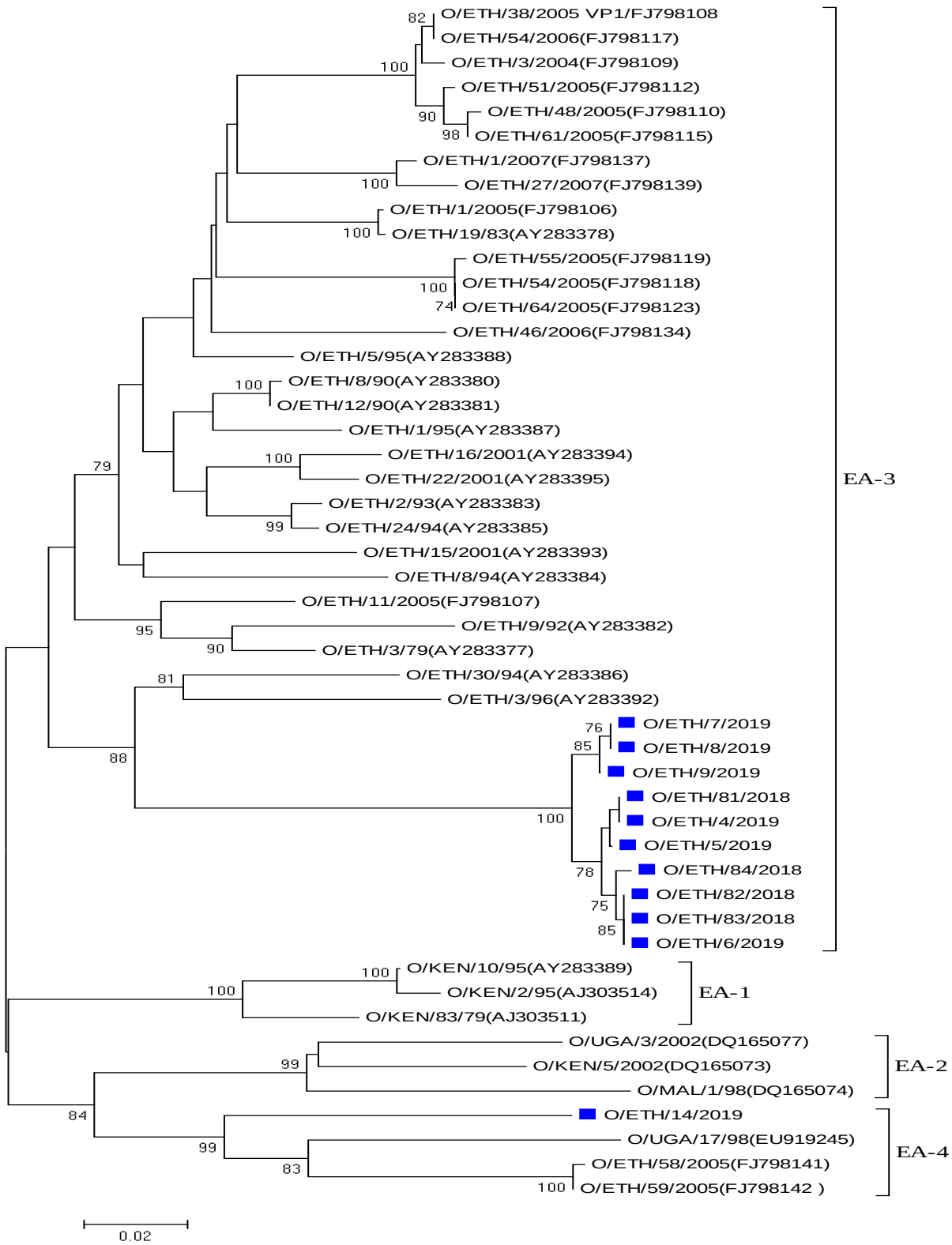


Figure 13: VP1 phylogenetic tree for FMDV serotype O.

The isolates marked with blue color are these sequenced in the current study.

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992) embedded in MEGA software. The optimal tree with the sum of branch length = 1.39524150 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The analysis involved 49 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 459 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

4.7. Phylogenetic analysis of FMDV serotype A

The VP1 gene sequence was used to construct the phylogenetic tree and study the genetic relationships between the current outbreak isolates with the isolates retrieved from the GenBank. The isolated serotype A in the current study were isolated from HARC dairy farm, Adea Berga dairy farm and Guduru district of Oromia regional state. All serotype A isolates sequenced belonged to the Africa topotype of genotype IV (Figure 14). The current isolates from Wolmera districts of HARC dairy farm and Adea Berga districts of Adea Berga dairy farm showed 100% nucleotide sequence similarity. All isolates of serotype A viruses shared 98.9-100% nucleotide similarity as shown in table (10). The current isolates shared 93.2-93.5%, 86-86.3%, 86.3-86.8% and 88.2-88.4% nucleotide similarity with SUD/1/2006 (GU566069) isolate of Sudan, A/ERI/3/98 (EU919238), SUD/2/84 (GU566067) and A/CAR/15/2000 (EF208755), respectively. Molecular characterization of serotype A FMD virus showed that the viruses belonged to African topotype of genotype IV (Figure 14). These virus clustered with FMD viruses circulating in Sudan and Eritrea.

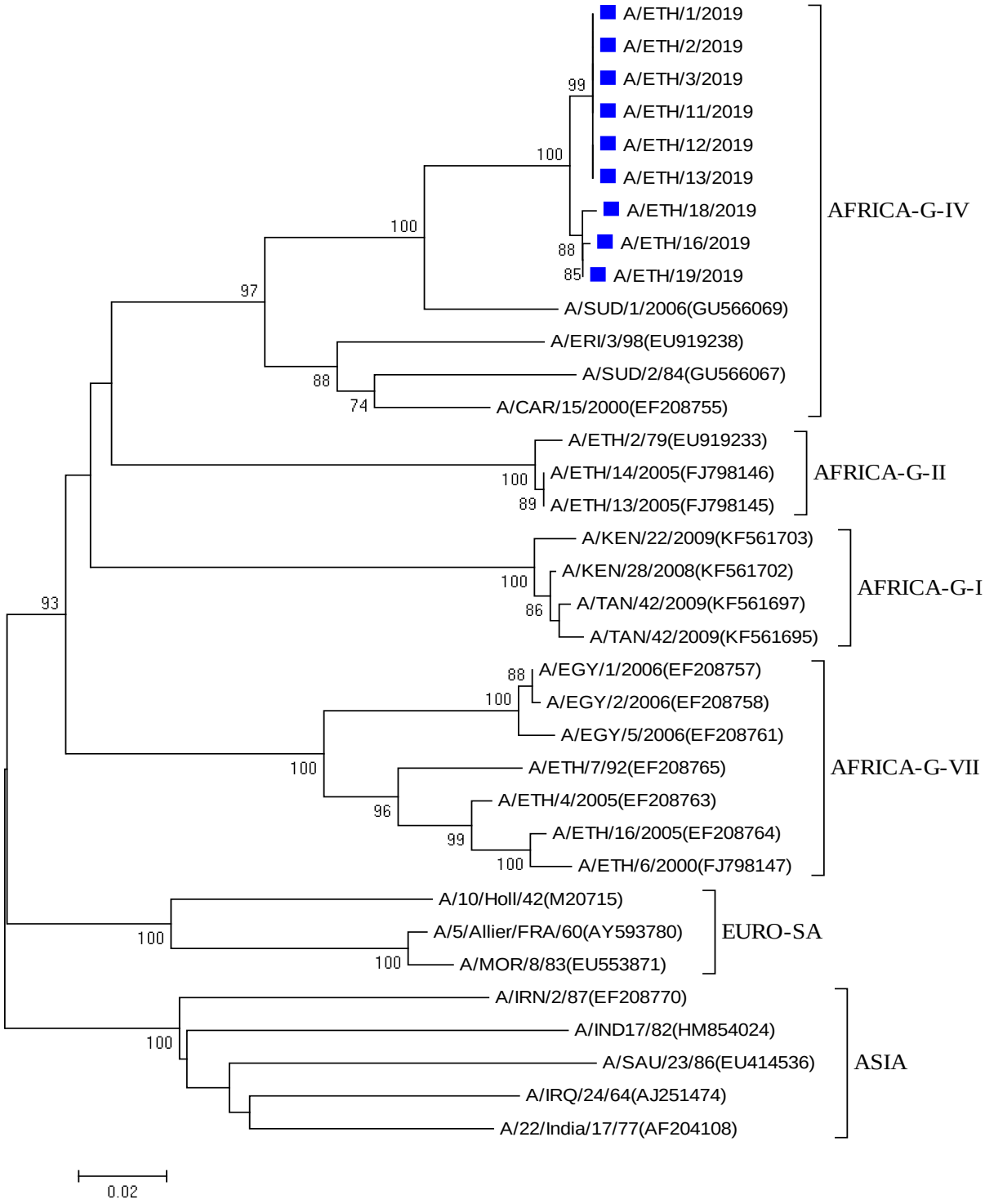


Figure 14: Phylogenetic tree for FMDV serotype A.

The current isolates were marked with Blue color.

The evolutionary history was inferred by using the Minimum Evolution method (Rzhetsky and Nei, 1992) embedded in MEGA software. The optimal tree with the sum of branch length = 1.32892721 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. The analysis involved 35 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 627 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

4.8. Comparison of serotype O with vaccine strain based on VP1 sequence

Analysis on the similarity of the VP1 nucleotide sequences and deduced amino acid changes were performed using BioEdit software. VP1 sequence of serotype O isolates identified in the current study were compared with the VP1 sequence of vaccine strain currently used in National Veterinary Institute (NVI) for vaccine production, Ethiopia to determine their genetic relationship. Accordingly, 84.4 - 85.6% nucleotide similarity were detected between VP1 sequence of the vaccine strain O/ETH/38/2005 (accession number FJ798108) and serotype O isolate of field strain from the current study as shown in table (9). The nucleotide sequence of the vaccine strain was aligned with field isolates of this study to determine the amino acid variations. A total of 27/ 213 (12.68%) amino acid variations were observed in different sites of the partial VP1 gene while the rest 186/213 (87.3%) amino acids (aa) were conserved with reference to the vaccine strain. Amino acid variations were occurred in the antigenic site 3, known to be the immunogenic sites of VP1 (Aggarwal and Barnett, 2002). Substitutions occurred in the critical residues were at positions: 45 (T→Q for all field isolates except O/ETH/14/2019 but strain O/ETH/14/2019 changed T→R) and 48 (Q→L in the O/ETH/14/2019 isolate). Substitutions in non-antigenic sites were also occurred in other positions of the region as shown in figure (15). 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.

Table 9: Percentage of nucleotide identity and divergence among current field isolates and with vaccine strain of FMDV serotype O

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
1		85.6	85.4	85.4	85.4	85.4	85.6	85.4	85.4	85.4	85.6	84.4	**1**
2	14.4		99.3	99.3	99.3	99.7	99.5	99.3	98.5	98.7	98.5	80.7	**2**
3	14.6	0.7		100	99.5	99.1	98.9	100	97.9	98.1	97.9	80.5	**3**
4	14.6	0.7	0.0		99.5	99.1	98.9	100	97.9	98.1	97.9	80.5	**4**
5	14.6	0.7	0.5	0.5		99.1	98.9	99.5	97.9	98.1	97.9	80.1	**5**
6	14.6	0.3	0.9	0.9	0.9		99.3	99.1	98.3	98.5	98.3	80.5	**6**
7	14.4	0.5	1.1	1.1	1.1	0.7		98.9	98.1	98.3	98.1	81.1	**7**
8	14.6	0.7	0.0	0.0	0.5	0.9	1.1		97.9	98.1	97.9	80.5	**8**
9	14.6	1.5	2.1	2.1	2.1	1.7	1.9	2.1		99.7	99.5	81.1	**9**
10	14.6	1.3	1.9	1.9	1.9	1.5	1.7	1.9	0.3		99.7	81.1	**10**
11	14.4	1.5	2.1	2.1	2.1	1.7	1.9	2.1	0.5	0.3		81.3	**11**
12	14.6	19.3	19.5	19.5	19.9	19.5	18.9	19.5	18.9	18.9	18.7		**12**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	

Divergence

1-O/ETH/38/2005_VP1/FJ798108 (Vaccine strain), **2**-O/ETH/81/2018, **3**-O/ETH/82/2018, **4**-O/ETH/83/2018, **5**-O/ETH/84/2018, **6**-O/ETH/4/2019, **7**-O/ETH/5/2019, **8**-O/ETH/6/2019, **9**-O/ETH/7/2019, **10**-O/ETH/8/2019, **11**-O/ETH/9/2019, **12**-O/ETH/14/2019

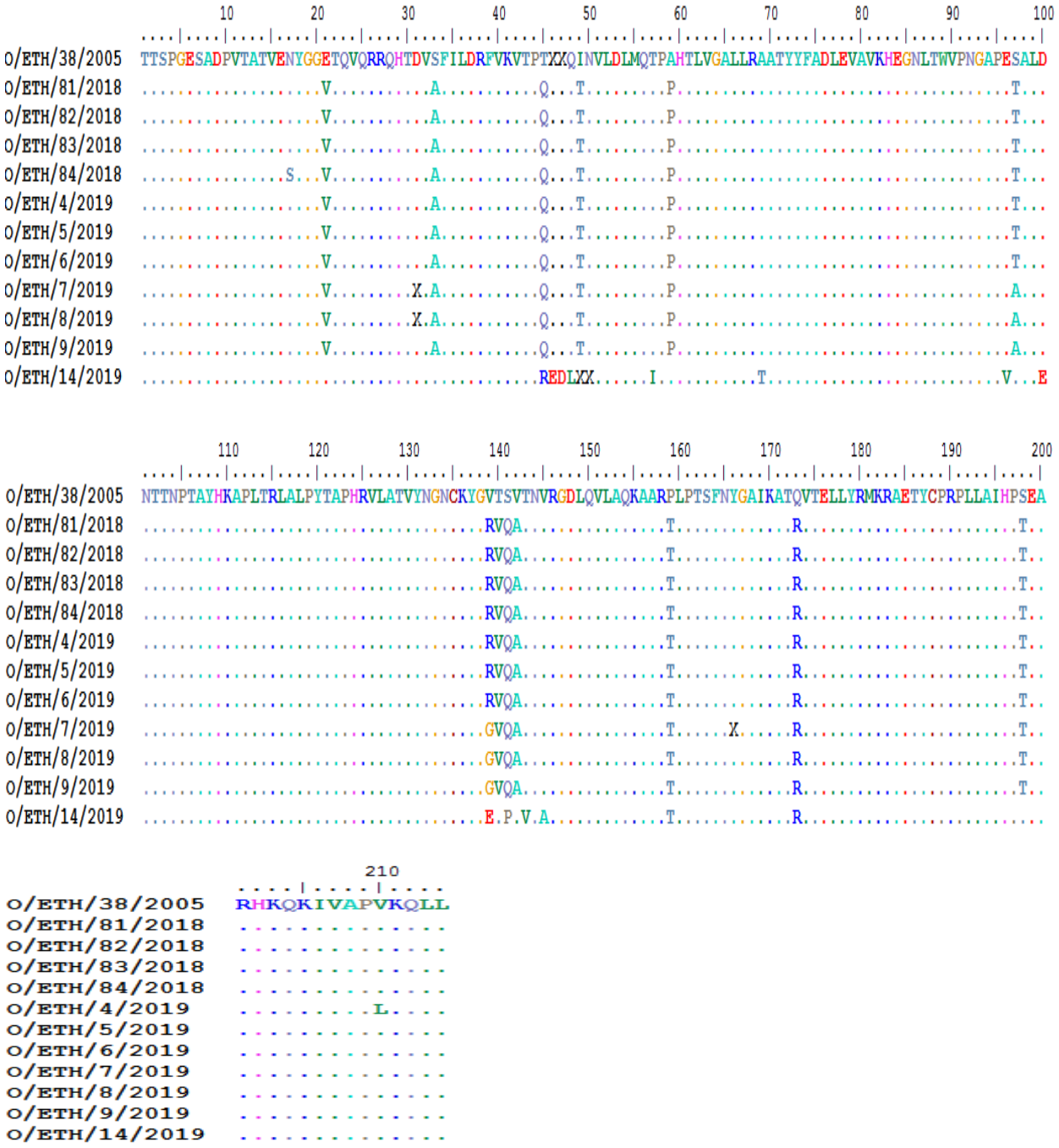


Figure 15: Amino acid sequence alignments of VP1 gene of serotype O FMDV with vaccine strain.

The amino acids sequence of the vaccine strain (O/ETH/38/2005; accession number FJ798108) is written on top row and the conserved amino acids are indicated by dots of different colors to show presence of different amino acids.

4.9. Comparison of serotype A with vaccine strain (A/ETH/6/2000) based on VP1 sequence

Analysis on the similarity of the nucleotide sequences and amino acid variations of FMDV serotype A were performed using BioEdit software. VP1 sequence of serotype A isolates identified in the current study were compared with the VP1 sequence of vaccine strain currently used in National Veterinary Institute (NVI) for vaccine production, Ethiopia to determine their genetic relationship. A total of 78.8-79.4% nucleotide similarity were detected between VP1 sequence of the vaccine strain (A/ETH/6/2000 accession number FJ798147) and serotype A isolate of field strain from this study as shown in table (10). The amino acid sequences of field isolates of this study were aligned with vaccine strain to determine the amino acid variations. Totally 34/213 (15.96%) amino acid variations were observed in different sites of the VP1 gene while the rest 179/213 (84.04%) amino acids (aa) were conserved with reference to the vaccine strain of the country. Amino acid variations identified in the main antigenic sites between the vaccine strain and field isolates of FMDV serotype A at positions, 45 (L→S), 140 (T→G), 141 (T→D and (T→G), 143 (S→P), 149 (G→A) and 157 (A→T). Amino acid substitutions were also occurred in non-antigenic sites in other positions of the region. The RGD cell attachment sites within the G–H loop of the gene at position 145-147 were conserved in all isolates including the vaccine strain as shown in figure (16).

Table 10: Percentage of nucleotide identity and divergence among current field isolates and with vaccine strain of FMDV serotype A

Identity

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		
1		78.8	78.8	78.8	78.8	78.8	78.8	79.0	79.4	79.1	**1**	A/ETH/6/2000
2	21.2		100	100	100	100	100	99.0	98.9	99.2	**2**	A/ETH/1/2019
3	21.2	0.0		100	100	100	100	99.0	98.9	99.2	**3**	A/ETH/2/2019
4	21.2	0.0	0.0		100	100	100	99.0	98.9	99.2	**4**	A/ETH/3/2019
5	21.2	0.0	0.0	0.0		100	100	99.0	98.9	99.2	**5**	A/ETH/11/2019
6	21.2	0.0	0.0	0.0	0.0		100	99.0	98.9	99.2	**6**	A/ETH/12/2019
7	21.2	0.0	0.0	0.0	0.0	0.0		99.0	98.9	99.2	**7**	A/ETH/13/2019
8	21.0	1.0	1.0	1.0	1.0	1.0	1.0		99.5	99.8	**8**	A/ETH/16/2019
9	20.6	1.1	1.1	1.1	1.1	1.1	1.1	0.5		99.6	**9**	A/ETH/18/2019
10	20.9	0.8	0.8	0.8	0.8	0.8	0.8	0.2	0.4		**10**	A/ETH/19/2019
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		

Divergence

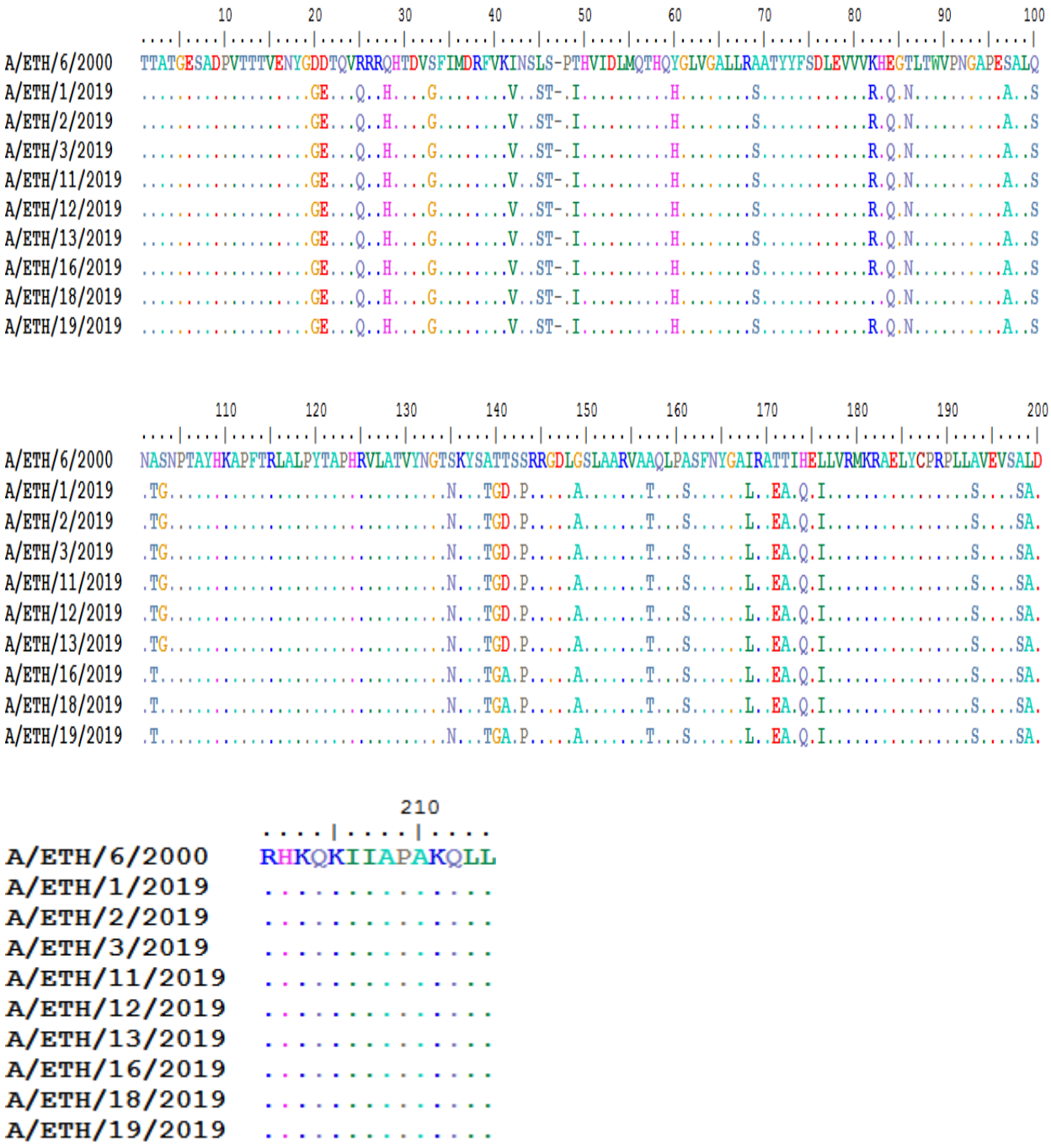


Figure 16: Amino acid sequence alignments of VP1 gene of serotype A FMDV with vaccine strain.

The amino acids sequence of the vaccine strain (A/ETH/6/2000 accession number FJ798147) is written on top row and the conserved amino acids are indicated by dots of different colors to show different amino acids.

5. DISCUSSION

In this study, typical FMD clinical signs were observed in cattle found in three regional states of Ethiopia from which two different serotypes O and A FMD viruses were identified. The identified viruses were characterized using Ag-ELISA and by generation of partial gene sequence data used for molecular evolutionary relationship determination. Of the 215 cattle examined during FMD outbreaks in all districts, 55 (25.58%) animals manifested typical clinical signs and lesions suggestive of FMD 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. The current finding was in agreement with the previous findings by Negusssie *et al*. (2011) and Sulayeman *et al*. (2018) that reported during outbreak investigation of FMD in Ethiopia, 28.2% and 28.8% of animals manifested clinical sign of FMD, respectively.

In the current study, out of 63 samples tested by real time PCR for the presence of genetic material targeting the 3D regions of FMDV in the sample, 84.13% (n = 53) were found positive. Previously, Paixão *et al*. (2008) suggested that real time RT-PCR that targets the 3D region of the viral genome is a powerful technique for reliable detection of FMDV which now becoming key diagnostic test used to confirm FMDV presence in field samples. The negative results for those 10 (15.87%) samples may be attributable to the small quantity of viral RNA in the original samples, which might have resulted from late sample collection after the first appearance of clinical symptoms. From the total of 53 rRT-PCR positive samples, bovine epithelial tissues were accounted for 96.23% (n=51) and bovine oral swab samples were accounted for 3.77% (n=2). The lowest C_t value was 15.06 recorded in bovine epithelial tissue sample collected from Amba Alaje district, Tigray whilst the highest (31.19) C_t value was examined in bovine oral swab sample collected from Wolmera district of HARC dairy farm. Bovine epithelial tissue samples had lower C_t values than oral swab samples this might indicates higher level of viral RNA concentration is found in epithelial samples. In support of this observation, OIE (2012) reported that the preferred sample for virus detection is epithelial tissue that was previously confirmed by Beksisa (2017) who also reported the presence of higher levels of viral RNA in the epithelial tissues. In agreement with this Reid *et al*. (2001) indicated epithelium tissue samples from the vesicular lesions could be used as the sample of choice for FMD virus detection. In this study the detection of genetic material of FMDV from oral swab

samples were in agreement with the findings of Alexandersen *et al*. (2002) who suggested the FMD virus can also be obtained from oral swabs.

BHK-21 cell line was used to isolate the virus from clinical samples. From the total of 34 representative FMDV positive samples detected by rRT-PCR, 26 (76.47%) showed CPE, which appeared as rounding in cell culture followed fast and complete cell destruction and detachment from the surface of the flask. This observation in CPE of FMDV agrees with previous reports (Belay and Muktar, 2015; Ibrahim *et al*., 2015; Beksisa, 2017; Sulayeman *et al*., 2018). Previously, Paixão *et al*. (2008) and Attia *et al*. (2017) reported that FMD virus isolation on BHK- 21 cell is the most reliable diagnostic method. The cell culture negative 8 (23.53%) samples which did not show CPE could be due to death of the virus during transportation (Menda *et al*., 2014) or presence of very small quantity of live virus that could not overcome the challenge of cell culture environment. The failure to isolate all samples on BHK-21 cells may be due to the fact that viral isolation depends on presence of live virus while real time RT-PCR depends on presence of FMDV genome whether dead or live.

FMDV of serotype O and A were isolated in all outbreak sites of Tigray and Oromia regional states respectively. This indicated that the outbreaks with in the two regions could be from the same origin or from closely related viruses. This statement was supported by Samuel *et al*. (1999) who demonstrated that closely related viruses could be either from the same outbreak or from viruses temporally closely related. This similarity might be due to uncontrolled livestock movement among different districts of the region and play an important role in the dissemination of the virus. Similarly Ekboir (1999) also reported that movements of infected animals are the most important dissemination and transmission means for FMD.

Serotyping of the virus revealed that serotype O was the dominant serotype identified from samples collected from different districts of Tigray and Shashogo district of SNNPs, Ethiopia. From the total of 26 representative samples serotyped by antigen detection ELISA, 14 (53.85%) were identified as serotype O while 12 (46.15%) were identified as serotype A (Table 8). Previous studies have also indicated that serotype O was highly prevalent and a dominant serotype causing most of the outbreaks in Ethiopia (Menda *et al*., 2014; Belay and Muktar, 2015; Beksisa, 2017; Sulayeman *et al*., 2018). Gelaye *et al*. (2005) and Ayelet *et al*. (2009) also reported that serotype O was the most

prevalent and dominant FMD virus serotype circulating in Ethiopia, causing most of the outbreaks in the country. Other reports from different countries like Sudan, Kenya and Niger also indicated that, serotype O was the dominant serotype for outbreaks (Habiela *et al*., 2010; Chepkwony, 2011 and Kouato *et al*., 2018, respectively). In the current study, serotype O was isolated from samples collected from Tigray and SNNPs regional states. In line with this, Menda *et al*. (2014) previously reported that the presence of FMDV serotype O from Tigray region (Mekele Universty farm, Enderta) and SNNPs (Alaba and Malga districts). This statement was also supported by the studies of Kassaw *et al*. (2013) who reported that serotype O was the serotype prevailed in Tigray region in Shibta, Ayanalem, Debri, Chelekot and Mekelle city. Additionally, Jemberu *et al*. (2016) also revealed that serotype O was mostly isolated in different parts of the region in Ethiopia. Kitching *et al*. (2007) indicated that serotype O is the most prevalent serotype worldwide. Previously, Rweyemamu *et al*. (2008b) and Klein (2009) reported that serotype O was the most common cause of outbreaks globally followed by serotype A.

In this study, serotype A was isolated from the samples collected from Wolmera district of HARC dairy farm, Adea Berga district of Adea Berga dairy farm (West Shewa) and Guduru district (Horo Guduru Wollega) of Kombolcha, Gutu Abay and Kenneni kebeles from Oromia regional state. Most likely the viruses were responsible for the occurrence of outbreak in these areas. This record has a close agreement with the study of Beksisa (2017) who reported that serotype O, A and SAT 1 of FMDV were circulating in Wolmera and Adea Berga districts of Oromia region. This situation could be associated with the presence of endemic state of the serotype in the districts. Previously serotype A was reported from bovine samples collected from Sinana and Yabello areas (Negussie *et al*., 2013) and from Arsi zone of Guna district (Sulayeman *et al*., 2018), both from Oromia regional state, indicated that serotype A was the circulating FMDVs in cattle.

A phylogenetic tree based on the partial VP1 (1D) region of FMDV is widely used for genetic characterization because of its significance for virus attachment and entry, protective immunity, and serotype specificity (Jackson *et al*., 2003; Burman *et al*., 2006). Phylogenetic analysis conducted to define the genetic relationship between Ethiopian viruses and FMDV isolates that have been collected from other countries. The VP1 sequence of 11 serotype O FMDV isolated in the present study was compared with 38 FMDV O strains and a phylogenetic tree was constructed. All viral isolates from Tigray regional state showed 98.5-100% nucleotide sequence similarity with each

other and assumed to be closely related because FMD viruses with <5% of nucleotide differences in the VP1 sequence are considered as closely related (Knowles and Samuel, 2003). The nucleotide sequence analysis revealed that the nucleotide sequence differences with in Tigray regional state isolates were ≤1.5% and this indicated that outbreaks due to these isolates could be from the same origin as evidenced by their genetic relationship on a phylogenetic tree. This might be due to uncontrolled animal movement from one district to the other which facilitated dissemination of the virus. Those viral isolates from Tigray regional state had 86.4-87.4% nucleotide sequence similarity with previous strains of Ethiopia O/ETH/30/94 (AY283386) and O/ETH/3/96 (AY283392) that clustered with them on the tree. The current isolates from Tigray regional state were homologous, geographically clustered and belongs to East Africa (EA-3) topotype. This is in agreement with previous study on molecular epidemiology of serotype O by Kassaw *et al*. (2013) who demonstrated the existence of EA-3 topotype in Tigray regional state of Ethiopia. This finding agrees with the previously published reports from Ethiopia by Ayelet *et al*. (2009), Negusssie *et al*. (2011), Menda *et al*. (2014) and Deribie *et al*. (2017) who reported the existence of EA-3 topotype. This indicates that the topotype is endemic in the region and in the country.

A single serotype O virus isolated from SNNPs regional state of Shashogo district (O/ETH 14/2019) had 80.5-81.3% nucleotide similarity with the current strains isolated from Tigray regional state. This suggests the two virus groups had nucleotide difference more than 15% and need to belong to different topotypes as recommended by Knowles and Samuel (2003). The FMDV isolates obtained from the two regional states had no any geographic proximity for animal movement for possible disease transmission. The FMDV serotype O nucleotide sequence difference between SNNPs and Tigray region state isolates is between 18.7-19.5% and this indicated that the outbreaks occurred due to two different topotypes of the viruses that is suggestive of originating from different sources. The isolates of Shashogo district (O/ETH 14/2019, SNNPs) shared 86.8% and 87% nucleotide identity with other strains from Ethiopia O/ETH/58/2005 (accession number FJ798141) and O/ETH/59/2005 (accession number FJ798142). The current strain isolated from Shashogo district of SNNPs regional state was geographically clustered and belongs to East Africa topotype 4 (EA-4). The finding was in agreement with the previous studies by Ayelet *et al*. (2009) and Beksisa (2017) who reported the presence of EA-4 topotype in Mizan Teferi area, Wolmera and Adea Berga districts of Ethiopia.

VP1 sequence analysis of Serotype A isolates identified in the current study and other sequences data available in the GenBank database indicated that these sequences of serotype A clustered to genotype IV of African topotype. All the current isolates of serotype A isolated from Wolmera, Adea Berga and Guduru districts of Oromia regional state had ≤1.1% nucleotide differences. This indicated that viruses were closely related and could be genetically related with one another. The identification such closely related isolates could be due to uncontrolled animal movement from one district to the other which facilitated dissemination of the virus from distant places to central part of the country due to cattle marketing. In line with this Knowles and Samuel (2003) reported that serotype A viruses with <5% nucleotide differences in the VP1 sequence are considered as closely related. These isolates also shared 93.2-93.5% nucleotide similarity with SUD/1/2006 (GU566069) isolate of Sudan clustered to genotype IV of African topotype. The virus clustered with FMD viruses circulating in Sudan and Eritrea. This indicated that outbreaks due to these isolates were from the common origin. This might be due to transboundary movement of the cattle between Ethiopia and the neighboring countries. Studies conducted elsewhere Habiela *et al*. (2010) and Di Nardo *et al*. (2011) described further that the spread of FMDV can also be associated by animal movement, especially in the pastoral livestock systems in Africa and Asia. This finding was in agreement with previous study on molecular epidemiology of serotype A by Sulayeman *et al*. (2018) who demonstrated the existence of genotype IV of African topotype in Ethiopia.

The VP1 protein on which the present study was performed comprises of 213 amino acid residues with major antigenic sites of virus capsid. Hence, in this study the phylogenetic tree analysis, nucleotide similarity and amino acid alignments were conducted on VP1 protein to observe the genetic relatedness of FMDV serotype O and A with Ethiopian vaccine strains. From 11 field isolates of serotypes O, a total of 14.4-15.6% nucleotide changes and 12.68% amino acid variations were observed in different sites of VP1 sequence of the vaccine strain (O/ETH/38/2005, an accession number FJ798108). Nucleotide substitutions and amino acid variations were occurred in different parts of VP1 region. Amino acid changes were occurred in antigenic site 3 of critical amino acid residues at positions 45 T (Threonine) was replaced by Q (Glutamine) for all field isolates except O/ETH/14/2019 but in strain O/ETH/14/2019 it was replaced by R (Arginine) and 48 Q (Glutamine) was replaced by L (Leucine) in the O/ETH/14/2019 isolate. Antigenic site 3 involves residues 43, 44, 45, and 48 found on B-C loop of VP1 (Aktas and Samuel, 2000; Sarangi *et al*., 2013). In serotype O the stability of antigenic site 3 has been considered important for the stability

of the VP1 G-H loop, and any destabilization in VP1 residues 43 to 45 may distort the conformation of the flexible VP1 G-H loop (Fry *et al*., 2005). The presence of very limited genetic variation in the antigenic sites can alter the antigenic specificity of FMD viruses isolates (Sahle, 2004). Changes in the amino acid residues have been found to be associated with antigenic variability of the virus because of VP1 has dual function in cell receptor binding and antigenic determination (Kweon *et al*., 2002). In this study, the presence of amino acid variation in antigenic sites 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. In this study amino acid changes were also fixed at positions which have not yet been proven to be antigenically critical in serotype O virus. Amino acid substitutions outside of the known antigenic sites have been shown to play an important role in the antigenic diversification of FMDV (Aggarwal and Barnett, 2002).

A total of 20.6-21.2% nucleotide differences were recorded between VP1 sequence of the vaccine strain A/ETH/6/2000 (accession number FJ798147) and serotype A isolate of field strain from this study. This indicated that the vaccine strain and field isolates had no relations. FMD viruses with >15% of nucleotide sequence differences in the VP1 sequence are considered as unrelated (Knowles and Samuel, 2003). Totally 15.96% amino acid variations were occurred in different parts of VP1 region. Generally, antigenic sites on FMDV has centered on the sequences between amino acids 140 and 160 coupled with minor antigenic sites on VP1 (residue 169) and the C terminus of VP1 (Kitching, 2005). Amino acid residues at VP1-43/44/45 could have potential antigenic significance in serotype A FMDV (Bari *et al*., 2015). Amino acid changes were occurred on the main antigenic sites at positions 45 (L(Leucine) was replaced by S(Serine)), 140 (T(Threonine) was replaced by G(Glycine)), 141 (T(Threonine) was replaced by D(Aspartic Acid) and G(Glycine)), 143 (S(Serine) was replaced by P(Proline)), 149 (G(Glycine) was replaced by A(Alanine)) and 157 (A(Alanine) was replaced by T(Threonine)). Amino acid sequence variations of structural proteins of FMDV are the bases for the antigenic diversity of the virus. These amino acid changes may affect the conformation of the antigenic sites substantially, and thus change the antigenicity and virulence of the virus (Al-Shawkany *et al*., 2012). This finding was supported by results reported by Lin *et al*. (2010), who observed changes in the critical residues of the VP1 viruses isolated in Taiwan in 2009. The presence of amino acid variation in antigenic sites indicates the need for in-vitro vaccine matching studies against the currently circulating viruses and the available vaccine strains so that matching vaccine can be used for control of the disease.

6. CONCLUSION AND RECOMMENDATIONS

FMD is prevalent in the study districts as confirmed by clinically, virologically and molecularly and the disease is endemic in Ethiopia. The presence of this disease in the country is a major obstacle to the development of livestock resource because of its adverse effects on production, live cattle exporting and their product exports. In the current study, serotypes O and A were identified and serotype O was the dominant one. The molecular characterization of those serotypes revealed that the almost all, except one isolate of serotype O was clustered with East African topotype-3 (EA-3) and a single isolate to EA-4. FMDV serotype A was clustered with genotype IV of African topotype. Sequence based analysis of field isolates against vaccine strain revealed that amino acid variations were observed in different sites of the VP1 gene.

Therefore, the following recommendations are forwarded:

- ⇨ Regular FMD outbreak investigation should be conducted to have more detailed information about the serotypes and topotypes circulating in the country.
- ⇨ Livestock movements through regions and international boundaries should be preceded by detailed investigation of animal meant for movement.
- ⇨ Great attention should be given for this economically important disease because its occurrence affects the export earnings of the country thereby threaten the livelihood of farmers and economy of the country at large.
- ⇨ Vaccine matching studies should be conducted for field strains circulating in the country against the vaccine strains.
- ⇨ Regular vaccination program should be started to control the outbreak of the disease.

7. REFFERENCES

Abdela, N. (2017): Sero-prevalence, risk factors and distribution of foot and mouth disease in Ethiopia. *Acta Trop.,* **169**:125–132.

Abubakar, M., Kanwal, S. and Saeed, A. (2012): Persistence, emergence and distribution of foot and mouth disease virus (FMDV); global and pakistan perspectives. *Pak J Biol Sci*., **10**:84-90.

AbuBakar, S., Teoh, B., Sam, S., Chang, L., Johari, J., Hooi, P., Lakhbeer-Singh, H., Italiano, C., Omar, S. and Wong, K. (2013): Outbreak of human infection with Sarcocystis nesbitti, Malaysia, 2012. *Emerg. Infect. Dis.,* **19**:1989.

Admassu, B., Getnet, K., Shite, A. and Mohammed, S. (2015): Review on foot and mouth disease: Distribution and economic significance. *Acad J Anim Dis.,* **4**:160–169.

Aggarwal, N. and Barnett, P. (2002): Antigenic sites of foot and mouth disease virus (FMDV): an analysis of the specificities of anti-FMDV antibodies after vaccination of naturally susceptible host species. *J. Gen. Virol*., **83**:775–782.

Ahmed, H., Salem, S., Habashi, A., Arafa, A., Aggour, M., Salem, G., Gaber, A., Selem, O., Abdelkader, S. and Knowles, N. (2012): Emergence of foot and mouth disease virus SAT 2 in Egypt during 2012. *Transbound. Emerg. Dis.,* **59**:476–481.

Aktas, S. and Samuel, A. (2000): Identification of antigenic epitopes on the foot and mouth disease virus isolate O1/Manisa/Turkey/69 using monoclonal antibodies. *Rev. Sci. Tech. Int. des Epizoot.,* **19**:744–753.

Al-Shawkany, A., Nassiri, M., Zibaei, S., Tahmoorespour, M., Ziaratnia, S., Najafi, M., Haghparast, A., Ghovvati, S., Pourseyed, S. and Rashtibaf, M. (2012): Amino acid diversity of antigenic sites of Iranian type O foot and mouth disease virus. *J. Cell Mol. Res*., **3:**66–74.

Alemayehu, G., Zewde, G. and Admassu, B. (2014): Seroprevalence of foot and mouth disease (FMD) and associated economic impact on Central Ethiopian cattle feedlots. *J. Vet. Med. Anim. Heal*., **6**:154–158.

Alexandersen, S. and Donaldson, A. (2002): Further studies to quantify the dose of natural aerosols of foot and mouth disease virus for pigs. *Epidemiol. Infect.,* **128:**313–323.

Alexandersen, S. and Mowat, N. (2005): Foot and mouth disease: host range and pathogenesis, in: Foot and mouth disease virus. *Springer CTMI*, **288**: 9–42.

Alexandersen, S., Quan, M., Murphy, C., Knight, J. and Zhang, Z. (2003a): Studies of quantitative

parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J. Comp. Pathol.,* **129**:268–282.

Alexandersen, S., Zhang, Z. and Donaldson, A. (2002): Aspects of the persistence of foot and mouth disease virus in animals-the carrier problem. *Microbes Infect.,* **4:**1099–1110.

Alexandersen, S., Zhang, Z., Donaldson, A. and Garland, A. (2003b): The pathogenesis and diagnosis of foot and mouth disease. *J. Comp. Pathol.,* **129**:1–36.

Arzt, J., Baxt, B., Grubman, M., Jackson, T., Juleff, N., Rhyan, J., Rieder, E., Waters, R. and Rodriguez, L. (2011): The Pathogenesis of foot and mouth disease II: Viral pathways in swine, small ruminants, and wildlife; Myotropism, chronic Syndromes, and molecular virus–host Interactions. *Transbound. Emerg. Dis.,* **58:**305–326.

Arzt, J., Pacheco, J. and Rodriguez, L. (2010): The early pathogenesis of foot and mouth disease in cattle after aerosol inoculation: identification of the nasopharynx as the primary site of infection. *Vet. Pathol.,* **47**:1048–1063.

Ashfaq, M., Razzaq, A. and Muhammad, G. (2015): Economic analysis of dairy animal diseases in Punjab: a case study of Faisalabad district. *J. Anim. Plant Sci*., **25:**1482-1495.

Assefa, F., Ano, T., Aba, T. and Ebrahim, Z. (2015): Assessment of improved forage types and their utilization in Shashogo Woreda, Hadiya zone, Southern Ethiopia. *Glob. J. Anim. Sci. Livest. Prod. Anim. Breed.,* **3**:227–230.

Asseged, B. (2005): Review of foot and mouth disease: An in depth discourse of Global, Sub-Saharan

and Ethiopian status; Addis Ababa University Faculty of Veterinary Medicine, Research, and Gradate studies. Debre Zeit, Ethiopia, Pp.3-49.

Attia, M., Elgendy, E., Shahein, M., Kasem, S. and Ibrahim, M. (2017): Co-circulation of three different serotypes of FMD virus in Egypt. *J. of Virol. Sci.,* **2**:102-113.

Awan, F. (2009): Epidemiology of foot and mouth disease in buffaloes of Punjab province. A thesis submitted in the partial fulfillment of the requirements for the degree of doctor of philosophy in Microbiology, University of veterinary and animal sciences, Lahore, Pakistan.

Ayelet, G., Gelaye, E., Negussie, H. and Asmare, K. (2012): Study on the epidemiology of foot and mouth disease in Ethiopia. *Rev. sci. tech. Off. int. Epiz.,* **31**:789–798.

Ayelet, G., Mahapatra, M., Gelaye, E., Egziabher, B., Rufeal, T., Sahle, M., Ferris, N., Wadsworth, J., Hutchings, G. and Knowles, N. (2009): Genetic characterization of foot and mouth disease

viruses, Ethiopia, 1981–2007. *Emerg. Infect. Dis.,* **15:**1409.

Bai, X., Bao, H., Liu, Z., Li, D., Lu, Z., Cao, Y., Shang, Y., Shao, J. and Chang, H. (2011): Evolution and molecular epidemiology of foot and mouth disease virus in China. *Chinese Sci. Bull.,* **56**:2191–2201.

Bari, F., Parida, S., Asfor, A., Haydon, D., Reeve, R., Paton, D. and Mahapatra, M. (2015): Prediction and characterization of novel epitopes of serotype A foot and mouth disease viruses circulating in East Africa using site-directed mutagenesis. *J. Gen. Virol*., **96**:1033.

Bastos, A., Anderson, E., Bengis, R., Keet, D., Winterbach, H. and Thomson, G. (2003): Molecular epidemiology of SAT3-type foot and mouth disease. *Virus Genes,* **27**:283–290.

Bastos, A., Boshoff, C., Keet, D., Bengis, R. and Thomson, G. (2000): Natural transmission of foot and mouth disease virus between African buffalo (*Syncerus caffer*) and impala (A*epyceros melampus*) in the Kruger National Park, South Africa. *Epidemiol. Infect.,* **124:** 591–598.

Bastos, A., Haydon, D., Forsberg, R., Knowles, N., Anderson, E., Bengis, R., Nel, L. and Thomson, G. (2001): Genetic heterogeneity of SAT-1 type foot and mouth disease viruses in southern Africa. *Arch. Virol.*, **146**:1537–1551.

Beksisa U. (2017): Serotyping and molecular characterization of fmd virus isolated from outbreak cases in selected region and Addis Ababa, Ethiopia. MSc thesis Submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia.

Belay, H. and Muktar, Y. (2015): Isolation and identification of foot and mouth disease virus from clinically infected cattle in Ada veterinary clinic. *Am. J. Sci. Res*., **10**:368–374.

Beyi, A. (2012): Costs and benefits of foot and mouth disease vaccination practice in commercial dairy farms in central Ethiopia. MSc thesis submitted to business economics group of Wageningen University, Wageningen, Netherlands.

Biswas, S., Sanyal, A., Hemadri, D., Tosh, C., Mohapatra, J., Kumar, R. and Bandyopadhyay, S. (2006): Sequence analysis of the non-structural 3A and 3C protein-coding regions of foot-and-mouth disease virus serotype Asia1 field isolates from an endemic country. *Vet. Microbiol.,* **116**:187–193.

Bouma, A., Elbers, A.., Bartels, C., De Koeijer, A., Vellema, P., Moll, L., Dekker, A., Van der Wal, P., Pluimers, F. and De Jong, M. (2001): The epidemic of foot and mouth disease in the Netherlands in 2001, in: Proceedings of the 14th Annual Meeting of VEEC, Wageningen, 2001/EAM Graat, K. Frankena.-Wageningen: Wageningen University. Pp. 9–16.

Brito, B., Rodriguez, L., Hammond, J., Pinto, J. and Perez, A. (2017): Review of the global distribution of foot-and-mouth disease virus from 2007 to 2014. *Transbound. Emerg. Dis.,* **64**:316–332.

Bronsvoort, B., Radford, A., Tanya, V., Nfon, C., Kitching, R. and Morgan, K. (2004): Molecular epidemiology of foot and mouth disease viruses in the Adamawa province of Cameroon. *J. Clin. Microbiol*. **42**, 2186–2196.

Brooksby, J. (1958): The virus of foot and mouth disease, in: Advances in virus research. Academic Press. *Elsevier*, **5**:1-37.

Brooksby, J. and Rogers, J. (1957): Methods used in typing the virus of foot-and-mouth disease at Pirbright, 1950-55. Methods typing Cultiv. foot and mouth Dis. virus, Eur. Product. Agency Organ. Eur. Econ. Co-operation, Proj. 31, 34.

Buetre, B., Wicks, S., Kruger, H., Millist, N., Yainshet, A., Garner, G., Duncan, A., Abdalla, A., Trestrail, C. and Hatt, M. (2013): Potential socio-economic impacts of an outbreak of foot and mouth disease in Australia. Abares research report, Canberra. Pp. 40-65.

Burman, A., Clark, S., Abrescia, N., Fry, E., Stuart, D. and Jackson, T. (2006): Specificity of the VP1 GH loop of foot and mouth disease virus for αv integrins. *J. Virol*. **80**, 9798–9810.

Callahan, J., Brown, F., Osorio, F., Sur, J., Kramer, E., Long, G., Lubroth, J., Ellis, S., Shoulars, K. and Gaffney, K. (2002): Use of a portable real-time reverse transcriptase polymerase chain reaction assay for rapid detection of foot and mouth disease virus. *J. Am. Vet. Med. Assoc.,* **220**:1636–1642.

Castro, C., Arnold, J. and Cameron, C.E. (2005): Incorporation fidelity of the viral RNA-dependent RNA polymerase: a kinetic, thermodynamic and structural perspective. *Virus Res.*, **107:**141–149.

Chakraborty, S., Kumar, N., Dhama, K., Verma, A., Tiwari, R., Kumar, A., Kapoor, S. and Singh, S. (2014): Foot and mouth disease, an economically important disease of animals. *Adv. Anim. Vet. Sci.*, 2:1–18.

Chang, H., Du, J., Cong, G., Shao, J., Lin, T. and Xie, Q. (2007): Genome sequencing and analysis of foot and mouth disease virus Asia1/YNBS/58 strain. Bing du xue bao= Chinese *J. Virol*., **23**:407–411.

Chepkwony, E. (2011): An epidemiological survey of the serotypes of foot and mouth disease virus in circulation in the Somali-ecosystem in Kenya. MSc thesis submitted to University of

Nairobi, Kenya.

CSA (2018): Report on livestock and livestock characteristics, volume II, Addis Ababa. Statistical bulletin 587.

DACA (2006): Standard Treatment Guidelines for Veterinary Practice 1st edition. In: Drug Administration and Control Authority of Ethiopia (DACA). Addis Ababa, Ethiopia, Pp. 464-465.

Damena, D., Menkir, D. and Mulatu D. (2017): Serotyping and molecular characterization of foot and mouth disease of cattle in central Ethiopia, MSc thesis, Haramaya University

Deb, R., Chakraborty, S., Veeregowda, B., Verma, A., Tiwari, R. and Dhama, K. (2013): Monoclonal antibody and its use in the diagnosis of livestock diseases. *Adv. Biosci. Biotechnol.,* **4**:50.

Deribie, H., Birhanu, A., Diriba, L., Eyob, E. and Desalegn, D. (2017): Outbreak investigation and epidemiology of foot and mouth disease virus circulating in central areas of Ethiopia. *Academic Journal of Animal Diseases*, **6**:75-82.

Dhanda, M., Gopalakrishnan, V. and Dhillon, H. (1957): Note on the occurrence of atypical strains of foot and mouth diseases virus in India. *Ind J Vet Sci.,* **27**:79–84.

Dhikusooka, M., Tjørnehøj, K., Ayebazibwe, C., Namatovu, A., Ruhweza, S., Siegismund, H., Wekesa, S., Normann, P. and Belsham, G. (2015): Foot and mouth disease virus serotype SAT 3 in long-horned Ankole calf, Uganda. *Emerg. Infect. Dis.*, **21**:111.

Di Nardo, A., Knowles, N. and Paton, D. (2011): Combining livestock trade patterns with phylogenetics to help understand the spread of foot and mouth disease in sub-Saharan Africa, the Middle East and Southeast Asia. *Rev. Sci. Tech.,* **30:**63-85.

Ding, Y., Chen, H., Zhang, J., Zhou, J., Ma, L., Zhang, L., Gu, Y. and Liu, Y. (2013): An overview of control strategy and diagnostic technology for foot and mouth disease in China. *Virol. J.,* **10**:1-6.

Domingo, E., Sheldon, J. and Perales, C. (2012): Viral quasispecies evolution. *Microbiol. Mol. Biol. Rev.,* **76**:159–216.

Dyirakumunda, B., Saidi, B. and Mbanga, J. (2017): Identification of foot and mouth disease virus isolates using Vp1 gene sequencing. *Zimbabwe Journal of Science & Technology,* **12:**15-23.

East, M. (2002): Regional status and approaches to control and eradication of foot and mouth disease in the Middle East and North Africa. *Rev. sci. tech. Off. int. Epiz.,* **21**:451–458.

Ehizibolo, D., Perez, A., Carrillo, C., Pauszek, S., AlKhamis, M., Ajogi, I., Umoh, J., Kazeem, H., Ehizibolo, P. and Fabian, A. (2014): Epidemiological A nalysis, serological prevalence and G enotypic A nalysis of foot and mouth disease in Nigeria 2008–2009. *Transbound. Emerg. Dis.*, **61**:500–510.

Ekboir, J. (1999): Potential impact of foot and mouth disease in California. The role and contribution of animal health surveilance and monitoring services. Agricultural issues Center, Division of Agriculture and Natural Resources, University of California.

El-Khabaz, K. and Al-Hosary, A. (2017): Detection and identification of foot and mouth disease virus serotypes in Assiut governorate, Egypt. *J. Adv. Vet. Anim. Res.,* **4**:32–38.

El Wahed, A., El-Deeb, A., El-Tholoth, M., El Kader, H., Ahmed, A., Hassan, S., Hoffmann, B., Haas, B., Shalaby, M. and Hufert, F. (2013): A portable reverse transcription recombinase polymerase amplification assay for rapid detection of foot and mouth disease virus. *PLoS One*, **8:**71642.

Fandamu, P., Thys, E., Duchateau, L. and Berkvens, D. (2006): Perception of cattle farmers of the efficacy of east coast fever immunization in Southern Zambia. *Trop. Anim. Health Prod.*, **38:**9–16.

Felsenstein, J. (1985): Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**:783-791.

Feng, Q., Chen, X., Ma, O., Liu, Y., Ding, M., Collins, R., Ko, L., Xing, J., Lau, L. and Yu, A. (2003): Serotype and VP1 gene sequence of a foot and mouth disease virus from Hong Kong. *Biochem. Biophys. Res. Commun.*, **302:**715–721.

Feng, Q., Yu, H., Liu, Y., He, C., Hu, J., Sang, H., Ding, N., Ding, M., Fung, Y. and Lau, L. (2004): Genome comparison of a novel foot and mouth disease virus with other FMDV strains. *Biochem. Biophys. Res. Commun.*, **323:**254–263.

Freimanis, G., Di Nardo, A., Bankowska, K., King, D., Wadsworth, J., Knowles, N. and King, D. (2016): Genomics and outbreaks: Foot and mouth disease. *Rev Sci Tech.*, **35:**175–189.

Fry, E., Newman, J., Curry, S., Najjam, S., Jackson, T., Blakemore, W., Lea, S., Miller, L., Burman, A. and King, A. (2005): Structure of Foot and mouth disease virus serotype A1061 alone and complexed with oligosaccharide receptor: receptor conservation in the face of antigenic variation. *J. Gen. Virol.,* **86**:1909–1920.

Gakuya, D., Mulei, C. and Wekesa, S. (2011): Use of ethnoveterinary remedies in the

management of foot and mouth disease lesions in a diary herd. *AJTCAM*, **8:**165-169.

Gao, Y., Sun, S. and Guo, H. (2016): Biological function of foot and mouth disease virus non-structural proteins and non-coding elements. *Virol. J.,* **13**:107.

Garner, M., Fisher, B. and Murray, J. (2002): Economic aspects of foot and mouth disease: perspectives of a free country, Australia. *Rev. Sci. Tech. Int. des épizooties*, **21:**625–632.

Gelaye, E., Beyene, B. and Ayelet, G. (2005): Foot and mouth disease virus serotype identified in Ethiopia. Ethiop. *Vet. J.*, **9**:75–80.

Genchwere, J. and Kasanga, C. (2014): Spatial and temporal distribution of foot and mouth disease virus in the lake zone of Tanzania. *Onderstepoort J. Vet. Res.,* **81**:1–4.

Getachew, M. (2017): Serotyping and molecular characterization of foot and mouth disease of cattle in central Ethiopia. MSc thesis submitted to college of Natural and Computational sciences,

Haramaya University, Ethiopia.

Gladue, D., O'Donnell, V., Baker-Branstetter, R., Holinka, L., Pacheco, J., Sainz, I., Lu, Z., Ambroggio, X., Rodriguez, L. and Borca, M. (2013): Foot and mouth disease virus modulates cellular vimentin for virus survival. *J. Virol.,* **87:**6794–6803.

Gorna, K., Houndjè, E., Romey, A., Relmy, A., Blaise-Boisseau, S., Kpodékon, M., Saegerman, C., Moutou, F., Zientara, S. and Kassimi, L. (2014): First isolation and molecular characterization of foot and mouth disease virus in Benin. *Vet. Microbiol.,* **171:**175–181.

Grazioli, S., Moretti, M., Barbieri, I., Crosatti, M. and Brocchi, E. (2006): Use of monoclonal antibodies to identify and map new antigenic determinants involved in neutralisation on FMD viruses type SAT 1 and SAT 2. Rep. Sess. Res. Gr. Standing Tech. Comm. Control Foot-and-Mouth Dis. Paphos, Cyprus. Pp. 17–20.

Grubman, M. and Baxt, B. (2004): Foot and mouth disease. *Clin Microbiol Rev*., **17:**465–493.

Gulbahar, M., Davis, W., Guvenc, T., Yarim, M., Parlak, U. and Kabak, Y. 2007. Myocarditis associated with foot-and-mouth disease virus type O in lambs. *Vet Pathol*., **44:**589–599.

Habiela, M., Ferris, N., Hutchings, G., Wadsworth, J., Reid, S., Madi, M., Ebert, K., Sumption, K., Knowles, N. and King, D. (2010): Molecular characterization of foot and mouth disease viruses collected from Sudan. *Transbound. Emerg. Dis.*, **57:** 305–314.

Hall, M., Knowles, N., Wadsworth, J., Rambaut, A. and Woolhouse, M. (2013): Reconstructing geographical movements and host species transitions of foot and mouth disease virus serotype

SAT 2. *MBio.,* **4:**5-13.

Hargreaves, S., Foggin, C., Anderson, E., Bastos, A., Thomson, G., Ferris, N. and Knowles, N. (2004): An investigation into the source and spread of foot and mouth disease virus from a wildlife conservancy in Zimbabwe. *Rev Sci Tech.,* **23**:783–790.

Haydon, D., Samuel, A. and Knowles, N. (2001): The generation and persistence of genetic variation in foot and mouth disease virus. *Prev. Vet. Med.,* **51:**111–124.

Howson, E., Armson, B., Madi, M., Kasanga, C., Kandusi, S., Sallu, R., Chepkwony, E., Siddle, A., Martin, P. and Wood, J. (2017): Evaluation of two lyophilized molecular assays to rapidly detect foot and mouth disease virus directly from clinical samples in field settings. Transbound. *Emerg. Dis.,* **64:**861–871.

Ibrahim, M., Ahmed, L., Kasem, S. and Hodhod, A. (2015): Molecular study on foot and mouth disease virus in Beheira governorate, Egypt during 2014. *Alexandria J. Vet. Sci.,* **45**: 151-160.

Jackson, A., O'neill, H., Maree, F., Blignaut, B., Carrillo, C., Rodriguez, L. and Haydon, D. (2007): Mosaic structure of foot and mouth disease virus genomes. *J. Gen. Virol.,* **88**:487–492.

Jackson, T., King, A., Stuart, D. and Fry, E. (2003): Structure and receptor binding. *Virus Res.* **91:**33-46.

Jamal, S. and Belsham, G. (2013): Foot and mouth disease: past, present and future. *Vet. Res.,* **44:**116.

Jamal, S., Ferrari, G., Ahmed, S., Normann, P. and Belsham, G. (2011): Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; emergence of a new genetic Group and evidence for a novel recombinant virus. *Infect. Genet. Evol.,* **11:**2049–2062.

James, A. and Rushton, J. (2002): The economics of foot and mouth disease. *Rev. Sci. Tech. Int. des Epizoot.,* **21**:637–641.

Jemberu, W., Mourits, M., Sahle, M., Siraw, B., Vernooij, J. and Hogeveen, H. (2016): Epidemiology of foot and mouth disease in E thiopia: A retrospective analysis of district level outbreaks, 2007–2012. *Transbound. Emerg. Dis.,* **63:**246–259.

Jori, F., Vosloo, W., Du Plessis, B., Bengis, R., Brahmbhatt, D., Gummow, B. and Thomson, G. (2009): A qualitative risk assessment of factors contributing to foot and mouth disease outbreaks in cattle along the western boundary of the Kruger National Park. *Rev. Sci. Tech. Int. des épizooties,* **28:**917–931.

Kasambula, L., Belsham, G., Siegismund, H., Muwanika, V., Ademun-Okurut, A. and Masembe, C. (2012): Serotype identification and VP1 coding sequence analysis of foot and mouth disease viruses from outbreaks in eastern and northern Uganda in 2008/9. *Transbound. Emerg. Dis.*, **59:**323–330.

Kasanga, C., Sallu, R., Kivaria, F., Mkama, M., Masambu, J., Yongolo, M., Das, S., Mpelumbe-Ngeleja, C., Wambura, P. and King, D. (2012): Foot and mouth disease virus serotypes detected in Tanzania from 2003 to 2010: Conjectured status and future prospects. *Onderstepoort J. Vet. Res.*, **79**:80–83.

Kasanga, C., Wadsworth, J., Mpelumbe-Ngeleja, C., Sallu, R., Kivaria, F., Wambura, P., Yongolo, M., Rweyemamu, M., Knowles, N. and King, D. (2015): Molecular characterization of foot and mouth disease viruses collected in Tanzania between 1967 and 2009. *Transbound. Emerg. Dis.*, **62:**19–29.

Kassaw, K., Afera, B., Amasalu, K. and Hussien, D. (2013): Serotype identification and molecular characterization of foot and mouth disease in and around Mekelle, Tigray region. *Glob. Vet.*, **11:**390–394.

Kitching, P., Hammond, J., Jeggo, M., Charleston, B., Paton, D., Rodriguez, L. and Heckert, R. (2007): Global FMD control—Is it an option? *Vaccine*, **25**:5660–5664.

Kitching, R. (2005): Global epidemiology and prospects for control of foot and mouth disease, in: Foot and mouth disease Virus. *Springer CTMI*, **288**: 133–148.

Kitching, R. (2002): Clinical variation in foot and mouth disease: cattle. Scientific and Technical *Rev. Sci. Tech. Int. des Epizoot*., **21:**499–502.

Kitching, R. and Hughes, G. (2002): Clinical variation in foot and mouth disease: sheep and goats. *Rev. Sci. Tech. Int. des Epizoot*., **21:**505–510.

Klein, J. (2009): Understanding the molecular epidemiology of foot and mouth disease virus. *Infect. Genet. Evol*., **9:**153–161.

Klein, J., Parlak, Ü., Özyörük, F. and Christensen, L. (2006): The molecular epidemiology of foot and mouth disease virus serotypes A and O from 1998 to 2004 in Turkey. *BMC Vet. Res*., **2**:35.

Knight-Jones, T. and Rushton, J. (2013): The economic impacts of foot and mouth disease–What are they, how big are they and where do they occur? *Prev. Vet. Med*., **112:**161–173.

Knowles, N., He, J., Shang, Y., Wadsworth, J., Valdazo-González, B., Onosato, H., Fukai, K., Morioka, K., Yoshida, K. and Cho, I. (2012): Southeast Asian foot-and-mouth disease viruses

in Eastern Asia. *Emerg. Infect. Dis*., **18**:499-501.

Knowles, N., Hovi, T., Hyypiä, T., King, A., Lindberg, A., Pallansch, M., Palmenberg, A., Simmonds, P., Skern, T. and Stanway, G. (2011): Virus taxonomy: classification and nomenclature of viruses. Ninth Rep. Int. Comm. Taxon. Viruses. Pp. 855–880.

Knowles, N. and Samuel, A. (2003): Molecular epidemiology of foot and mouth disease virus. *Virus Res*., **91**: 65–80.

Knowles, N., Samuel, A., Davies, P., Kitching, R. and Donaldson, A. (2001): Outbreak of foot and mouth disease virus serotype O in the UK caused by a pandemic strain. *Vet. Rec*., **148:**258–259.

Knowles, N., Wadsworth, J., Bachanek-Bankowska, K. and King, D. (2016): VP1 sequencing protocol for foot and mouth disease virus molecular epidemiology. *Rev Sci Tech*., **35**:741–755.

Knowles, N., Wadsworth, J., Reid, S., Swabey, K., El-Kholy, A., El-Rahman, A., Soliman, H., Ebert, K., Ferris, N. and Hutchings, G. (2007): Foot and mouth disease virus serotype A in Egypt. *Emerg. Infect. Dis*., **13:**1593-1596.

König, G., Palma, E., Maradei, E. and Piccone, M. (2007): Molecular epidemiology of foot and mouth disease virus types A and O isolated in Argentina during the 2000-2002 epizootic. *Vet. Microbiol*., **124:**1–15.

Korboukh, V., Lee, C., Acevedo, A., Vignuzzi, M., Xiao, Y., Arnold, J., Hemperly, S., Graci, J., August, A. and Andino, R. (2014): RNA virus population diversity, an optimum for maximal fitness and virulence. *J. Biol. Chem.*, **289:**29531–29544.

Kweon, C., Ko, Y., Kim, W., Kwon, B., Hyun, B., Sohn, H., Choi, K. and Shin, J. (2002): Molecular characterization of foot and mouth disease virus O/SKR/2000. *Virus Res*., **90:**15–22.

Lee, S., Lee, Y., Kim, R., Park, J., Park, M., Ko, M., Choi, J., Chu, J., Lee, K. and Kim, S. (2017): Rapid engineering of foot and mouth disease vaccine and challenge viruses. *J. Virol.*, **91**:15-17.

Lefebvre, D., De Vleeschauwer, A., Goris, N., Kollanur, D., Billiet, A., Murao, L., Neyts, J. and De Clercq, K. (2014): Proof of concept for the inhibition of foot and mouth disease virus replication by the anti-viral drug 2′-c-methylcytidine in severe combined immunodeficient mice. *Transbound. Emerg. Dis*., **61**:89–91.

Lefevre, P. (2010): Infectious and parasitic diseases of livestock: Bacterial diseases, fungal diseases, parasitic diseases. Éd. Tec. & doc.

Leforban, Y. (2005): Report of a mission on foot and mouth disease in Ethiopia. Propos. a Strateg.

plan a Control Progr. oriented to Export 1022, 1242.

Legesse, Y., Asfaw, Y., Sahle, M., Ayelet, G., Jenberie, S. and Negussie, H. (2013): First confirmation of foot and mouth disease virus serotype SAT-1 in cattle and small ruminants in Ethiopia in 2007/08. *Trop. Anim. Health Prod*., **45**:1265–1267.

Lin, Y., Jong, M., Huang, C., Shieh, H. and Chang, P. (2010): Genetic and antigenic characterization of foot and mouth disease viruses isolated in Taiwan between 1998 and 2009. *Vet. Microbiol*., **145:**34–40.

Lloyd-Jones, K., Mahapatra, M., Upadhyaya, S., Paton, D., Babu, A., Hutchings, G. and Parida, S. (2017): Genetic and antigenic characterization of serotype O FMD viruses from east Africa for the selection of suitable vaccine strain. *Vaccine*, **35:**6842–6849.

Lomakina, N., Fallacara, F., Pacciarini, M., Amadori, M., Lomakin, A., Timina, A., Shcherbakova, L. and Drygin, V. (2004): Application of universal primers for identification of foot and mouth disease virus and swine vesicular disease virus by PCR and PCR ELISA. *Archives of virology*, **149**(6), 1155-1170.

Longjam, N., Deb, R., Sarmah, A., Tayo, T., Awachat, V. and Saxena, V. (2011): A brief review on diagnosis of foot and mouth disease of livestock: conventional to molecular tools. *Vet. Med. Int*., **2011**: 1-17.

Lordwin, K. (2011): Inter-epidemic molecular characterization of foot and mouth disease Viruses in eastern and northern Uganda between 2008 and 2009 (doctoral dissertation, Makeree University, Uganda).

Ludi, A., Mioulet, V., Knowles, N. and King, D. (2017): Laboratory diagnostic methods to support the surveillance and control of foot and mouth disease. Foot and mouth dis. virus, Pp. 275.

Lyytikäinen, T., Niemi, J., Sahlström, L., Virtanen, T. and Lehtonen, H. (2011): The spread of Foot-and-mouth disease (FMD) within Finland and emergency vaccination in case of an epidemic outbreak, Evira Research Reports 1. Pp. 1-147.

Ma, L., Zhang, J., Chen, H., Zhou, J., Ding, Y. and Liu, Y. (2011): An overview on ELISA techniques for FMD. *Virol. J.,* **8:**419.

Mahapatra, M., Upadhyaya, S., Aviso, S., Babu, A., Hutchings, G. and Parida, S. (2017): Selection of vaccine strains for serotype O foot and mouth disease viruses (2007–2012) circulating in Southeast Asia, east Asia and far east. *Vaccine*, **35:**7147–7153.

Mahy, B. (2005): Introduction and history of foot and mouth disease virus, in: Foot and mouth

disease virus. *Springer CTMI*, **288**: 1–8.

Mansley, L., Donaldson, A., Thrusfield, M. and Honhold, N. (2011): Destructive tension: mathematics versus experience-the progress and control of the 2001 foot and mouth disease epidemic in Great Britain. *Rev. Sci. Tech.*, **30:**483.

Maree, F., Blignaut, B., de Beer, T. and Rieder, E. (2013): Analysis of SAT type foot and mouth disease virus capsid proteins and the identification of putative amino acid residues affecting virus stability. PLoS One, **8:**61612.

Maree, F., Blignaut, B., Esterhuysen, J., De Beer, T., Theron, J., O'Neill, H. and Rieder, E. (2011): Predicting antigenic sites on the foot and mouth disease virus capsid of the South African Territories types using virus neutralization data. *J. Gen. Virol.,* **92:**2297–2309.

Maree, F., Kasanga, C., Scott, K., Opperman, P., Chitray, M., Sangula, A., Sallu, R., Sinkala, Y., Wambura, P. and King, D.P. (2014): Challenges and prospects for the control of foot and mouth disease: an African perspective. *J Vet Med Res Rep.,* **5:**119–138.

Martel, J. (1975): Comparative serological study of the principal strains of the foot and mouth disease virus isolated in Ethiopia 1969-1974. *Rev. Elev. Med. Vet. Pays Trop*., **28:**287.

Martel, J. (1974): Foot and mouth disease in Ethiopia. Distribution of serotypes of foot and mouth disease virus. *Rev. Elev. Med. Vet. Pays Trop*., **27:**169.

Martinez-Salas, E., Pacheco, A., Serrano, P. and Fernandez, N. (2008): New insights into internal ribosome entry site elements relevant for viral gene expression. *J. Gen. Virol*., **89:**611–626.

Mason, P., Bezborodova, S. and Henry, T. (2002): Identification and characterization of a cis-acting replication element (cre) adjacent to the internal ribosome entry site of foot and mouth disease virus. *J. Virol.,* **76:**9686–9694.

Mason, P., Grubman, M. and Baxt, B. (2003): Molecular basis of pathogenesis of FMDV. *Virus Res*., **91:**9–32.

Mateo, R., Luna, E., Rincón, V. and Mateu, M. (2008): Engineering viable foot and mouth disease viruses with increased thermostability as a step in the development of improved vaccines. *J. Virol*., **82:**12232–12240.

Menda, S., Jenberie, S., Negussie, H., Ayelet, G. and Amasalu, K. (2014): Molecular epidemiology of foot and mouth disease Virus Outbreaks in Ethiopia in 2011/2012. *Academic Journal of Animal diseases*, **3:**8-16.

Metages Y. (2018): Molecular characterization of foot and mouth disease viruses in cattle from

outbreaks occurred in different parts of Ethiopia from October, 2017 to May, 2018. MSc thesis submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu,

Ethiopia.

Michel, A. and Bengis, R. (2012): The African buffalo: a villain for inter-species spread of infectious diseases in southern Africa. *Onderstepoort J. Vet. Res*., **79:**26–30.

Motuma, D. (2017): Molecular characterization and vaccine matching test of foot and mouth disease viruses isolated from outbreak cases in cattle in selected sites of central part of Oromia,

Ethiopia. MSc thesis submitted to College of Veterinary Medicine and Agriculture, Addis Ababa

University, Bishoftu, Ethiopia.

Mukasa, H., Mwiine, F., Atuhaire, D., Ochwo, S. and Nanteza, A. (2016): Comparative detection of foot and mouth disease virus by reverse transcription loop-mediated isothermal amplification assay and real time polymerase chain reaction in Uganda. *International Journal of Biotechnology and Food Science*, **4:**22-33.

Musser, J. (2004): A practitioner's primer on foot and mouth disease. *J. Am. Vet. Med. Assoc.*, **224:**1261–1268.

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A., Khan, G., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X. and Modarresi-Ghazani, F. (2018): Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed. Pharmacother*., **97:**67–74.

Negussie, H., Ayelet, G., Jenberie, S., Minda, S. and Tesfaw, L. (2013): Molecular epidemiology and vaccine matching study on foot and mouth disease virus circulating in Ethiopia. *African J. Microbiol. Res.*, **7:**5101–5106.

Negusssie, H., Kyule, M., Yami, M. and Ayelet, G. (2011): Outbreak investigations and genetic characterization of foot and mouth disease virus in Ethiopia in 2008/2009. *Trop. Anim. Health Prod*., **43:**235–243.

Nei, M. and Kumar, S. (2000): Molecular evolution and phylogenetics. Oxford university press.

OIE (2004): Foot and mouth disease.In: Manual of Standards for Diagnostic Tests and Vaccines. 5th ed, Office international des Epizooties (OIE), Paris, France. Pp: 111-128.

OIE (2008): Manual of diagnostic tests and vaccines for terrestrial animals. Revue Scientifique Et Technique-Office International Des Epizooties, Paris, France. Pp. 1092-1106.

OIE (2009): Foot and mouth disease, manual of standard for diagnostic tests and vaccine for terrestrial animals. 6th ed. Paris. Pp. 156-212.

OIE (2012): Foot and mouth disease, manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees) 7th ed, vol 1, Paris. Pp 145-173.

Oleksiewicz, M., Donaldson, A. and Alexandersen, S. (2001): Development of a novel real-time RT-PCR assay for quantitation of foot and mouth disease virus in diverse porcine tissues. *J. Virol. Methods,* **92:**23–35.

Paarlberg, P., Lee, J. and Seitzinger, A. (2002): Potential revenue impact of an outbreak of foot and mouth disease in the United States. *J. Am. Vet. Med. Assoc.,* **220:**988–992.

Paixão, T., Neta, A., Paiva, N., Reis, J., Barbosa, M., Serra, C., Silva, R., Beckham, T., Martin, B. and Clarke, N. (2008): Diagnosis of foot and mouth disease by real time reverse transcription polymerase chain reaction under field conditions in Brazil. *BMC Vet. Res*., **4:**53.

Paton, D., Sumption, K. and Charleston, B. (2009): Options for control of foot and mouth disease: knowledge, capability and policy. *Philos. Trans. R. Soc. London B Biol. Sci*., **364:**2657–2667.

Paul, A. and Wimmer, E. (2015): Initiation of protein-primed picornavirus RNA synthesis. *Virus Res*., **206:**12–26.

Prempeh, H., Smith, R. and Müller, B. (2001): Foot and mouth disease: the human consequences: The health consequences are slight, the economic ones huge.

Radostits, O., Gay, C., Hinchcliff, K. and Constable, P. (2007): A textbook of the diseases of cattle, horses, sheep, pigs and goats. *Vet. Med*., **10:**2045–2050.

Reeve, R., Borley, D., Maree, F., Upadhyaya, S., Lukhwareni, A., Esterhuysen, J., Harvey, W., Blignaut, B., Fry, E. and Parida, S. (2016): Tracking the antigenic evolution of foot-and-mouth disease virus. *PLoS One*, **11:**159360.

Reid, S., Ebert, K., Bachanek-Bankowska, K., Batten, C., Sanders, A., Wright, C., Shaw, A., Ryan, E., Hutchings, G. and Ferris, N. (2009): Performance of real-time reverse transcription polymerase chain reaction for the detection of foot and mouth disease virus during field outbreaks in the UK in 2007. *J. Vet. Diagnostic Investig.,* **21:**321–330.

Reid, S., Ferris, N., Brüning, A., Hutchings, G., Kowalska, Z. and Åkerblom, L. (2001): Development of a rapid chromatographic strip test for the pen-side detection of foot and mouth disease virus antigen. *J. Virol. Methods*, **96:**189–202.

Reid, S., Ferris, N., Hutchings, G., Zhang, Z., Belsham, G. and Alexandersen, S. (2001): Diagnosis

of foot and mouth disease by real-time fluorogenic PCR assay. *Veterinary Record*, **149:**621-623.

Reid, S., Grierson, S., Ferris, N., Hutchings, G. and Alexandersen, S. (2003): Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot and mouth disease virus. *J. Virol. Methods*, **107:**129–139.

Reid, S., Mioulet, V., Knowles, N., Shirazi, N., Belsham, G. and King, D. (2014): Development of tailored real-time RT-PCR assays for the detection and differentiation of serotype O, A and Asia-1 foot-and-mouth disease virus lineages circulating in the Middle East. *J. Virol. Methods*, **207:**146–153.

Reller, L., Weinstein, M., Jorgensen, J. and Ferraro, M. (2009): Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin. Infect. Dis*., **49:**1749–1755.

Rodriguez, L. and Gay, C. (2011): Development of vaccines toward the global control and eradication of foot and mouth disease. Expert Rev. *Vaccines*, **10:**377–387.

Roeder, P., Abraham, G., Mebratu, G. and Kitching, R. (1994): Foot and mouth disease in Ethiopia from 1988 to 1991. *Trop. Anim. Health Prod*., **26:**163–167.

Roeder, P. and Knowles, N. (2008): Foot and mouth disease virus type C situation: the first target for eradication. Open Sess. Eur. Comm. Control Foot and Mouth Dis. Standing Tech. Comm. 14–17.

Roeder, P. and Taylor, W. (2007): Mass vaccination and herd immunity: cattle and buffalo. *Rev. Sci. Tech. Int. des Epizoot*., **26:**253.

Rufael, T., Catley, A., Bogale, A., Sahle, M. and Shiferaw, Y. (2008): Foot and mouth disease in the Borana pastoral system, southern Ethiopia and implications for livelihoods and international trade. *Trop. Anim. Health Prod*., **40:**29–38.

Ruiz-Sáenz, J., Goez, Y., Tabares, W. and López-Herrera, A. (2009): Cellular receptors for foot and mouth disease virus. *Intervirology*, **52:**201–212.

Rushton, J. (2009): The economics of animal health and production. CABI, Nosworthy Way, Wallingford, Oxfordshire, UK. Pp. 193-200.

Rweyemamu, M., Roeder, P., MacKay, D., Sumption, K., Brownlie, J. and Leforban, Y. (2008a): Planning for the progressive control of foot and mouth disease worldwide. *Transbound. Emerg. Dis*., **55:**73–87.

Rweyemamu, M., Roeder, P., Mackay, D., Sumption, K., Brownlie, J., Leforban, Y., Valarcher, J.,

Knowles, N. and Saraiva, V. (2008b): Epidemiological patterns of foot and mouth disease worldwide. *Transbound. Emerg. Dis*., **55:**57–72.

Rzhetsky, A. and Nei, M. (1992): A simple method for estimating and testing minimum-evolution trees. Pp. 945-967.

Sahle, M. (2004): An epidemiological study on the genetic relationship on FMDV in east Africa (Doctoral dissertation, University of Pretoria, South Africa).

Saitou, N. and Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4:**406–425.

Samuel, A. and Knowles, N. (2001): Foot and mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.,* **82:**609–621.

Samuel, A., Knowles, N. and Mackay, D. (1999): Genetic analysis of type O viruses responsible for epidemics of foot and mouth disease in North Africa. *Epidemiol. Infect*., **122:**529–538.

Sangare, O. (2002): Molecular epidemiology of foot and mouth disease virus in west Africa (Doctoral dissertation, University of Pretoria, South Africa).

Sangare, O., Bastos, A., Marquardt, O., Venter, E., Vosloo, W. and Thomson, G. (2001): Molecular epidemiology of serotype O foot and mouth disease virus with emphasis on west and south Africa. *Virus Genes*, **22**:345–351.

Sangare, O., Bastos, A., Venter, E. and Vosloo, W. (2003): Retrospective genetic analysis of SAT-1 type foot and mouth disease outbreaks in West Africa (1975–1981). *Vet. Microbiol*., **93:**279–289.

Sangula, A., Belsham, G., Muwanika, V., Heller, R., Balinda, S. and Siegismund, H. (2010): Co-circulation of two extremely divergent serotype SAT 2 lineages in Kenya highlights challenges to foot and mouth disease control. *Arch. Virol*., **155:**1625–1630.

Sangula, A., Siegismund, H., Belsham, G., Balinda, S., Masembe, C. and Muwanika, V. (2011): Low diversity of foot and mouth disease serotype C virus in Kenya: evidence for probable vaccine strain re-introductions in the field. *Epidemiol. Infect*., **139:**189–196.

Sarangi, L., Mohapatra, J., Subramaniam, S., Sanyal, A. and Pattnaik, B. (2013): Antigenic site variation in foot and mouth disease virus serotype O grown under vaccinal serum antibodies in vitro. *Virus Res*., **176:**273–279.

Sebhatu, T. (2014): Foot and mouth disease sero-surveillance in Africa and vaccine matching (Doctoral dissertation, Utrecht University, Netherlands).

Seyoum, B. and Teshome, E. (2018): Major Transboundary Disease of Ruminants and their Economic Effect in Ethiopia. *Glob. J. Med. Res.*, **17:**27-36

Shaw, A., Reid, S., Ebert, K., Hutchings, G., Ferris, N. and King, D. (2007): Implementation of a one-step real-time RT-PCR protocol for diagnosis of foot and mouth disease. *J. Virol. Methods*, **143:**81–85.

Simmonds, P. (2006): Recombination and selection in the evolution of picornaviruses and other mammalian positive-stranded RNA viruses. *J. Virol.*, **80:**11124–11140.

Singh, C., Verma, A.K. and Pal, B. (2008): Prevalence of protected animals against foot and mouth disease in Uttar Pradesh. *Haryana Vet*., **47:**107–109.

Sørensen, J., Jensen, C., Mikkelsen, T., Mackay, D. and Donaldson, A. (2001): Modelling the atmospheric dispersion of foot and mouth disease virus for emergency preparedness. *Phys. Chem. Earth, Part B Hydrol. Ocean. Atmos*., **26:**93–97.

Sørensen, J., Mackay, D., Jensen, C. and Donaldson, A. (2000): An integrated model to predict the atmospheric spread of foot and mouth disease virus. *Epidemiol. Infect*., **124:**577–590.

Souley K., Elliot, F., King, D., Hyera, J., Knowles, N., Ludi, A., Mioulet, V., Matlho, G., De Clercq, K. and Thys, E. (2018): Outbreak investigations and molecular characterization of foot and mouth disease viruses circulating in south-west Niger. *Transbound. Emerg. Dis*., **65:**146–157.

Sulayeman, M., Dawo, F., Mammo, B., Gizaw, D. and Shegu, D. (2018): Isolation, molecular characterization and sero-prevalence study of foot and mouth disease virus circulating in central Ethiopia. *BMC Vet. Res*., **14:**110.

Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci*., **101:**11030–11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013): MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., **30**:2725–2729.

Teifke, J., Breithaupt, A. and Haas, B. (2012): Foot and mouth disease and its differential diagnoses. *Tierarztl. Prax. Ed. G. Grosstiere. livestock*, **40**:225–237.

Tesfaye, Y. (2014): Isolation, Molecular characterization and vaccine matching of foot and mouth disease virus circulating in Ethiopia. MSc thesis submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia.

Thompson, J., Higgins, D. and Gibson, T. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap

penalties and weight matrix choice. *Nucleic Acids Res.*, **22**:4673–4680.

Thomson, G., Vosloo, W. and Bastos, A. (2003): Foot and mouth disease in wildlife. *Virus Res*., **91:**145–161.

Timoney, J., Gillespie, J., Scott, F. and Barlough, J. (1988): Hagan and Bruner's microbiology and infectious diseases of domestic animals. Cornell University Press, Itacha, USA.

Tully, D. and Fares, M. (2008): The tale of a modern animal plague: tracing the evolutionary history and determining the time-scale for foot and mouth disease virus. *Virology*, **382**:250–256.

Upadhayay, U. and Ewam, P. (2012): Epidemiology and diagnosis of foot and mouth disease: A review. *Indian J. Anim. Sci.*, **82:**543–551.

Valarcher, J., Knowles, N., Fernandez, R., Statham, B., Davies, P., Midgley, R., Hutchings, G., Newman, B., Ferris, N. and Paton, D. (2004): Global foot and mouth disease situation 2003-2004. Rep. Sess. Res. Gr. Standing Tech. Comm. EUFMD, Chania, Crete, Greece 12, 15.

Valarcher, J., Knowles, N., Ferris, N., Paton, D., Zakharov, V., Sherbakov, A., You-jun, S., Zai-xin, L., Xiang-tao, L. and Sanyal, A. (2005): Recent spread of FMD virus serotype Asia 1. *Vet. Rec*., **157:**30.

Valdazo-González, B., Knowles, N., Hammond, J. and King, D. (2012): Genome sequences of SAT 2 foot-and-mouth disease viruses from Egypt and Palestinian Autonomous Territories (Gaza Strip). *Journal of Virology*, **86:** 8901-8902.

Vallée, H. and Carré, H. (1922): On the plurality of the foot and mouth virus. *Accounts of the Academy of Sciences. Series III, Life Sciences,* **174:**1498–1500.

Verma, A., Sehzad, S., Kumar, A. and Yadav, S. (2009): LPB elisa based pre-and post-vaccination seroprevalence of foot and mouth disease virus. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.*, **30:**130–132.

Vosloo, W., Bastos, A. and Boshoff, C. (2006): Retrospective genetic analysis of SAT-1 type foot and mouth disease outbreaks in southern Africa. *Arch. Virol*., **151**:285–298.

Vosloo, W., Bastos, A.., Sangare, O., Hargreaves, S. and Thomson, G. (2002): Review of the status and control of foot and mouth disease in sub-Saharan Africa. *Rev. Sci. Tech. Int. des épizooties*, **21**:437–445.

Vosloo, W., Dwarka, R., Bastos, A., Esterhuysen, J., Sahle, M. and Sangare, O. (2004): Molecular epidemiological studies of foot and mouth disease virus in sub-Saharan Africa indicate the presence of large numbers of topotypes: implications for local and international control. Proc.

Open Sess. EU-FMD Res. Gr. Pp. 149–158.

Vosloo, W. and Thomson, G. (2017): Natural habitats in which foot-and-mouth disease viruses are maintained. Foot and mouth dis. virus curr. res. emerg. trends. Madrid, Spain CSIC-UAM. Pp. 179–210.

Wagari, A. (2016): Seroprevalence of foot and mouth disease in bulls of Borana origin quarantined in Adama. *Inernational J. Biochem. Biophys. Mol. Biol.,* **1:**1–10.

Waldmann, O. and Trautwein, K. (1926): Experimental studies on the plurality of foot and mouth disease virus. *Berl. Tierarztl. Wschr.,* **42:**569–571.

Wondwossen. T. (2017): Isolation, molecular characterization and vaccine matching of foot and mouth disease virus circulating in central Ethiopia. MSc thesis submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia.

Xiao, C., Rajput, Z., Liu, D. and Hu, S. (2007): Enhancement of serological immune responses to foot and mouth disease vaccine by a supplement made of extract of cochinchina momordica seeds. Clin. *Vaccine Immunol.,* **14:**1634–1639.

Xu, L., Hurtle, W., Rowland, J., Casteran, K., Bucko, S., Grau, F., Valdazo-González, B., Knowles, N., King, D. and Beckham, T. (2013): Development of a universal RT-PCR for amplifying and sequencing the leader and capsid-coding region of foot and mouth disease virus. *J. Virol. Methods,* **189:**70–76.

Yang, M., Xu, W., Goolia, M. and Zhang, Z. (2014): Characterization of monoclonal antibodies against foot-and-mouth disease virus serotype O and application in identification of antigenic variation in relation to vaccine strain selection. *Virol. J.,* **11:**136.

Yoon, H., Yoon, S., Wee, S., Kim, Y. and Kim, B. (2012): Clinical manifestations of foot-and-mouth disease during the 2010/2011 epidemic in the Republic of Korea. *Transbound. Emerg. Dis.,* **59:**517–525.

8. APPENDIXES

Appendix 1: List of regions, zones, districts and PAs/kebeles selected during the study period

S/No.	Region	Zone	District	Kebele
	Tigray	South east	Enderta	Messebo
		Eastern	Saisi Tsaida Imba	Senkata
		Southern	Amba Alaje	Teki"a
				Ayida
			Raya Alamata	Kidana
			Korem	02
		North Western	Hintalo Wajirat	Dejen
			Kilte Awilailo	Wukro
			Asgede Tsimbala	Alogean
	Oromia	Horo Guduru Wollega	Guduru	Kombolcha
				Gutu Abay
				Keneni
		West Shewa	Adea Berga	Adea Berga dairy farm
		Oromia special zone	Wolmera	HARC dairy farm
	SNNPs	Hadiya	Shashogo	Molelicho

Appendix 2: FMD field outbreak investigation and data collection recording sheet

A. FMD Outbreak sample collection sheet

Region____ Zone ___ District ___PA ____Village ____Date ___/ ____/ ____

Code	Species	Breed	Age	Sex	Management	Sample type	Remark
01							
02							
03							
04							
05							
06							
07							
08							
09							
10							
11							
12							

B. Number of animals examined, clinically affected and died animals during FMD Outbreak

Region_____ Zone _____ District ____PA _____Date ___/ ____/ ____

Owners name	Species	Breed	Age	No. of animal examined	No. of clinically affected animal	No. of died	Total	Remark

C. Any other animals included (affected) during the outbreak……..

D. Major Clinical signs observed in sick animals (Mark x)

- ✓ Profuse salivation _____
- ✓ Lameness _____
- ✓ Interdigital lesion _____
- ✓ Udder lesion _____

- ✓ Oral and tongue lesion _____
- ✓ Other signs _____

Appendix 3: Animals age determination based on teeth eruption

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

Source: (Merck Veterinary Manual, 1998).

Appendix 4: Master Mix components of real rime RT-PCR

- ❖ 2x - reaction mix
- ❖ RNAse-free water
- ❖ Forward primer
- ❖ Reverse primer
- ❖ Taqman probe
- ❖ superscript ®III RT
- ❖ Extracted template

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 37oc.
- ✓ 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 37oc.
- ✓ 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 25cm2 tissue culture flasks and rock the flask gently to distribute inoculum evenly over the monolayer cell.
- ✓ Incubate inoculated culture in incubator at 37oc 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 lay out for FMDV antigen detection and serotyping ELISA

NATIONAL ANIMAL HEALTH DIAGNOSTIC AND INVESTIGATION CENTER (NAHDIC)

QUALITY ANAGEMENT SYSTEM

PLATE LAY OUT FOR FMDV ANTIGEN DETECTIONN AND SEROTYPING ELISA

Type of test: FMDV antigen detection Test date:……..

Kit batch No: 01-2016 161201a Plate No:

Expiry date: August 2021

Purpose: Research

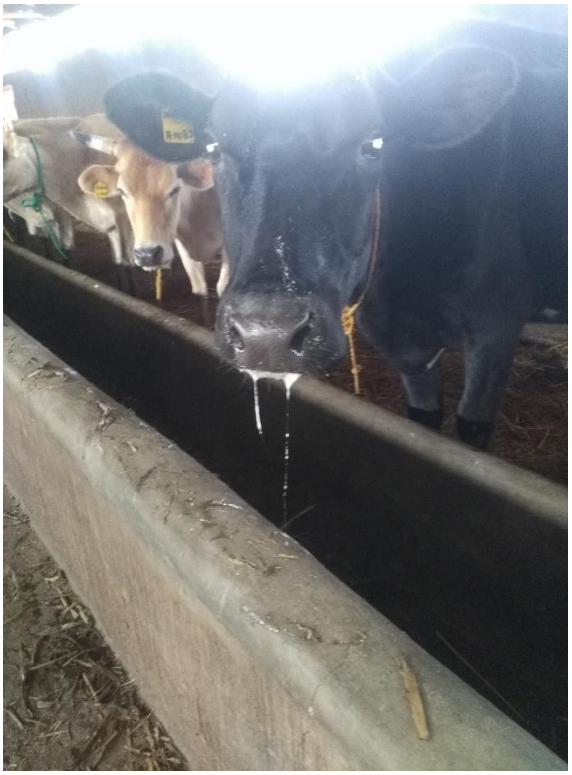
Catching MAbs		Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Positive control	Negative control	
		1	2	3	4	5	6	7	8	9	10	11	12	
Type O	A													Conjugate A (Pan-O-A—Asia 1 + someSATs)
Type A(1st mab)	B													
Type A (2nd mab)	C													
Type Asia 1	D													
Type C	E													
Pan-O-A-C-Asia1	F													
Type SAT 2	G													Conjugate B (SAT1-
Type SAT 1	H													

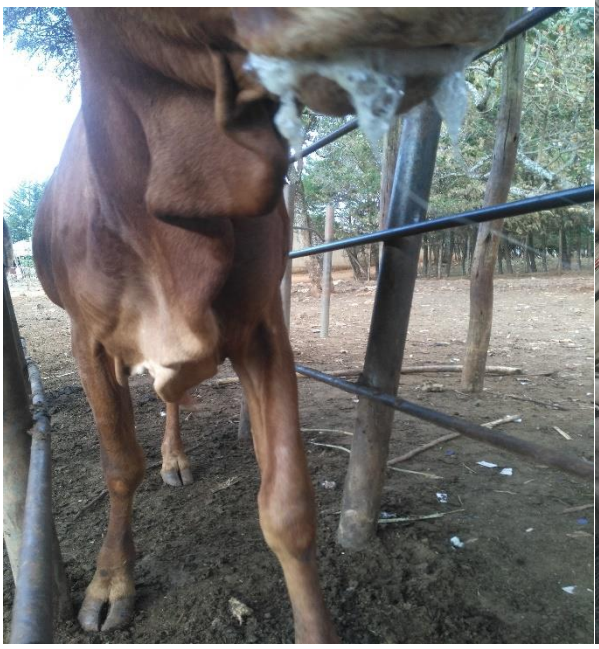
														2)

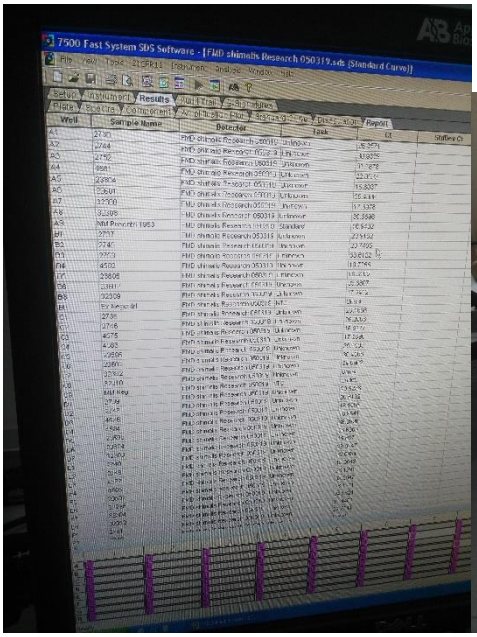
Appendix 8: Nucleotide sequence of primer used for sequencing VP1 protein of FMDV in WRLFMD

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

Appendix 9: Miscellaneous pictures



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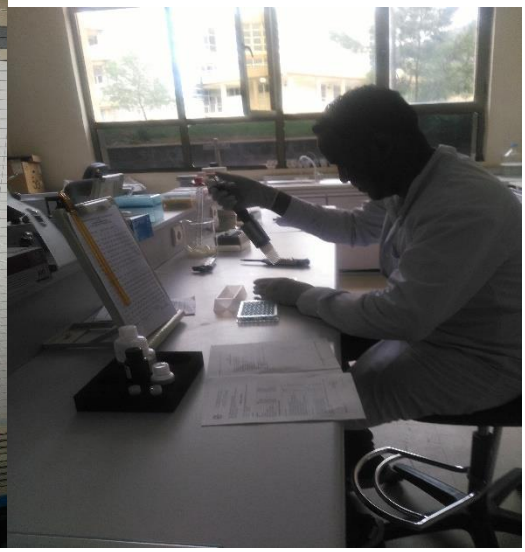

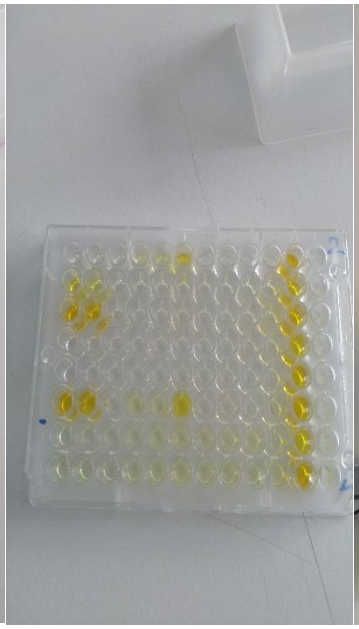

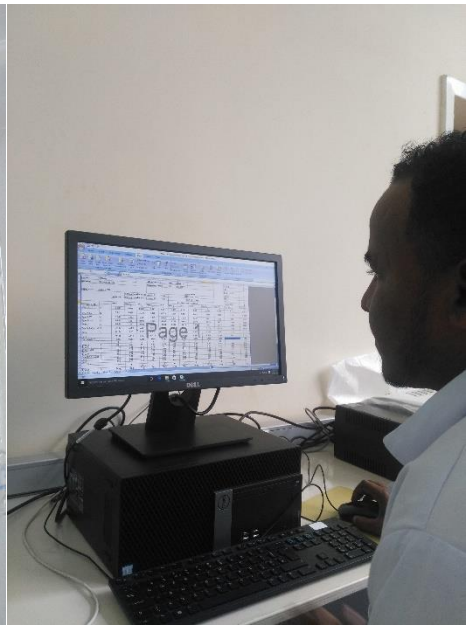
Page 1