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SEROTYPING AND MOLECULAR CHARACTERIZATION OF FMD VIRUS ISOLATED FROM OUTBREAK CASES IN SELECTED AREAS OF OROMIA REGION AND ADDIS ABABA, ETHIOPIA

MVSc. Thesis

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

BY

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JUNE, 2017

BISHOFTU, ETHIOPIASEROTYPING AND MOLECULAR CHARACTERIZATION OF FMD VIRUS ISOLATED FROM OUTBREAK CASES IN SELECTED AREAS OF OROMIA REGION AND ADDIS ABABA, ETHIOPIA

MVSc. Thesis

A Thesis Submitted to College of Veterinary Medicine and Agriculture, Addis Ababa University, in partial fulfillment of the requirements for the degree of Masters of Veterinary Science in Veterinary Mirobiology.

By

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As MVSc research advisors, we here by certify that we have read and evaluated this Thesis prepared under our guidance by Beksisa Urge Hurrisa entitled: "Serotyping **and Molecular Characterization of Foot and Mouth disease virus isolated from outbreak cases in selected areas of Oromia region and Addis Ababa Ethiopia;** we recommend that it can be submitted as fulfilling the MVSc thesis requirement.

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DEDICATION

This thesis manuscript is dedicated and memorial to my beloved Father ***Urge Hurrisa Gudeta*** *whom I could not forget him since he has passed away because of emergency case before a year!! The grief I felt has hurt me so much.*

May your soul rest in peace

STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

ASS	Agricultural Sample Survey
BCS	Body Condition Score
BHK21	Baby Hamster kidney 21 Cell
CPE	Cytopathic Effect
CT	Cycle Threshold
CME	Clathrin mediated Endocytosis
ELISA	Enzyme Linked Immunosorbant Assay
FAO	Food and Agriculture Organization
LMP	Livestock Master Plan
KB	kilo Base Pair
MEM	Minimum Essential Media
MABS	Monoclonal Antibodies
NAHDIC	National Animal Health Diagnostic and Investigation Centre
NCR	Non-Coding Region
NVI	National Veterinary Institute
OD	Optical Density
OIE	World Animal Health Organization
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAT	Southern African Territories
SsRNA	Single Stranded Ribonucleic Acid
VP1	Viral Capsid Protein
VPG	Viral genomic Protein
WRL	World reference laboratory
RGD	arginine-glycine-aspartic acid

ABSTRACT

Foot and mouth disease (FMD) is an epitheliotropic and trans-boundary viral disease affecting cattle, sheep, goats and wild animals around the globe including Ethiopia. Outbreak investigation and cross-sectional study design was conducted from September 2016 to April 2017 with the objectives of isolation, serotyping, molecular characterization and to determine risk factors for the occurrence of the disease in selected outbreak areas of Oromia region and Addis Ababa, Ethiopia. Purposive sampling was done in the respective districts and kebeles where the outbreaks occurred. A total of 586 accessible cattle were examined in six districts and 226 (38.6%) showed clinical signs of the disease. Out of this, 352 animals were examined at Adea Berga dairy farm. Hence, outbreaks were investigated by collecting 123 samples from sick animals out of which 37 samples for virus isolation, 41 samples for identification of serotypes and 35 samples for molecular assays. Moreover, 312 bovine sera were collected from Welmera district during outbreak. The risk factor outbreak analysis showed that the overall morbidity and mortality at Adea Berga dairy farm was 36.9% and 3.12%, respectively. The risk of FMD infection in this farm was increased by 4.2 times (OR = 4.2, 95% CI (1.925-8.810) in calves than adult cattle. Among 312 sera samples, 30.8% (n=96) were FMD seropositive. The occurrence was (38.5%) in females and (15.8%) in males and it was statistically significant ($\chi 2$= 16.93, p = 0.001). Cross bred cattle were three times more likely to be infected (OR = 3.47, 95% CI: 2.10 -5.74) than local breeds. Furthermore, out of 37 cultured samples, 56.8% (n=21) exhibited FMDV cytopathic effect (CPE) and the viruses were isolated. A total of 41 samples were processed for antigen typing and 39.02% (n=16) were positive. The identified serotypes were; serotype O (34.2%), serotype A (17.1%), serotype SAT1 (4.9%) and serotype SAT2 (2.4%). Out of 50 samples tested by rRT- PCR, 18.1% (n=9) were positive for FMDV genome with Ct values ranging from 16.03-26.98 that were recorded in epithelial tissues and probang samples. Of the four serotypes identified, only serotype O virus isolates were characterized at molecular level by phylogenetic tree reconstruction. The isolates belonged to East Africa topotype-4 (EA-4). In conclusion, the study showed four serotypes were prevalent in the study areas; FMD control therefore depends to be instituted based on the availability of matching and potent vaccine against the serotypes per area.

Key words: *Addis Ababa, Cattle, FMDV, Risk factor, Serotypes, Seroprevalence*

1. INTRODUCTION

Ethiopia has the largest livestock inventories among African countries. Recent report showed that, 56.71 millions of cattle, 29.33 million of sheep, 29.11 millions of goats and 54.5 million chickens are found in Ethiopia (ASS, 2015/16). Even though the country is endowed with huge livestock population, it happens to be a passageway of trans-boundary and internationally important livestock disease like foot-and-mouth diseases (FMD) (Livestock Master Plan, 2015). FMD is one of the major endemic diseases in Ethiopia and other developing countries with abundant socioeconomic importance as a result of loss of production; huge cost of veterinary services and restrictions on the trade of animals both locally and internationally (James and Rushton, 2002). Moreover, livestock and livestock product exports to the Middle East and African country has been hampered because of the presence of FMD recently (Bayissa *et al*., 2011). The Egyptian ban of 2003 on Ethiopia's livestock market alone resulted in market loss of 14.36 million USD (MoARD, 2007) and it is a threat to Ethiopia's live animal export and export of animal products. The disease is also characterized by low mortality in adult animals and sometimes high mortality in among young animals and attributed to acute myocarditis (Kandeil *et al.*, 2013).

FMD is an endemic disease in most of the African countries where serotypes O, A, SAT 1 and SAT 2 predominate (Vosloo *et al*., 2002). FMD outbreaks have been reported in Ethiopia with a sero-prevalence of 9-26% at animal and 28% at herd level (Mohamoud *et al*., 2011). Other studies conducted by (Gulima *et al.*, 2011) also showed that the herd and individual prevalence of the disease was 57.6 % and 11.9% respectively. The real epidemiological data of the disease in the country is definitely underestimated due to factors such as low trans-boundary animal disease reporting rate (35%) and limited awareness of farmers. Recently, out of the six serotypes established in the Sub-Saharan Africa, four serotypes (A, O, C and SAT 2) have been identified and reported (Sahle, 2004).

Besides this, studies conducted by (Ayelet *et al*., 2009 and Negussie *et al*., 2011) also indicated that four out of seven serotypes (O, A, SAT 2, and SAT 1) have been reported. SAT2 serotype with topotype VII, XIII and XIV reappeared after an apparent gap of sixteen years. Beside their difference in serotypes, there is genetic heterogenecity because of error-prone replication which improves viral fitness by changing antigenically important sites of the virus that enabled them to escape from protection by the developed vaccine (Grazioli *et al.*, 2013).

FMDV recovered from outbreaks during 1981-2007 supported that serotype O (73.30%) was most prevalent followed by type A (19.50%), SAT-2 (4.10%), SAT-1 (1.80%) and C (1.40%) (Ayelet *et al.,* 2009). Moreover, (Klein, 2009) also indicated that serotype O among other serotypes is prevalent worldwide and causing huge losses. Report of genetic characterization of FMDV from 1981-2007 disclosed additional serotypes of the virus like SAT-1, and SAT-2 circulate in host species (cattle, sheep, goats, and pig), and high numbers of wildlife which has uncontrolled cross borders of neighboring countries were involved (Ayelet *et al*., 2009). Uncontrolled animal movements are some of the risk factors of FMD outbreak in Ethiopia (Bewket *et al*., 2012).

FMD occurs in the form of outbreak that rapidly spread from herd to herd before it is controlled (Radostits *et al.*, 2000). However, a study on subtypes of virus that causes outbreak is limited. The origin of the serotype is also difficult to determine due to lack of available sequences from the region. The viruses do not appear to be closely related to any of the other sequences**.** With globalization of trade even areas where FMD is endemic, there is introduction of virus strains that are exotic to the region and hence continuous research is required (Bruckner *et al*., 2002).

The molecular epidemiology of FMDV has been extensively studied using the VP1 coding region of the virus genome. Genetic analysis of viral protein 1 (VP1) region of FMD virus has been used to study the molecular epidemiology of the disease worldwide

and plays a role in the phylogenetic characterization of FMD virus. The establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks and transmission of the disease over time in a given area is required (Knowles and Samuel, 2003). Topotypes are defined as geographically clustered viruses that form a single genetic lineages sharing >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3) nucleotide identity in the VP1-coding region (Ayelet *et al.,* 2009). Rapid identification of FMDV serotypes during outbreak is very important to determine the origin of infection and to use appropriate emergency vaccine (Yang *et al*., 2013).

Molecular epidemiological studies based on phylogenetic analysis is currently missing in these areas. 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 because the existence of other serotypes and new virus subtype circulating in the field might be changed and causes severe outbreaks. Hence, the availability of updated information on genetic characteristics of FMD field isolates and strains helps to know circulating strains in the field. This is also an important element in understanding the new strains that could aid in instituting proper control and prevention measures against the occurrence of the disease in the area. In majorities of the current FMD outbreak areas, it was not known the serotypes circulating in these areas except Addis Ababa were source due to common source, new introduction of the virus from a single or from the same serotypes and the genetic relationships among the viruses responsible for the outbreak is not known. Therefore, the objectives of the current study were:

- ✓ To isolate and characterize FMD virus from outbreak cases
- ✓ To determine the serotypes of FMD virus strains responsible for outbreak cases
- ✓ To characterize the FMD viruses at molecular level by phylogenetic tree reconstruction
- ✓ To determine seroprevalence and risk factors associated with the occurrence of FMD outbreas

2. LITERATURE REVIEW

2.1. Definition of disease

Foot and mouth disease (FMD) also known as Aphthous fever or infectious aphthous stomatitis is a highly contagious viral trans-boundary disease of both domestic and wild cloven hoofed animals characterized by the formation of vesicles in the buccal cavity (Dinardo *et al.*, 2011). The disease is extremely contagious acute viral disease of animals, resulted in fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats and sudden death of young stock (Quinn *et al.*, 2005). It is one of the most globally important notifiable diseases of livestock due to its high infectious and trans-boundary distribution nature (Knight *et al*., 2013).

2.2. Etiology of FMD Virus

2.2.1. Nomenclature of FMD virus

FMD virus was defined in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as the virus belongs to the genus *Aphthoviru in the* family Picornaviridae. The name, picornaviride is derived from the Latin word “Pico” meaning small and “rna “ meaning RNA, which refers to the size and genome type, of the virus while the genus name Aphthovirus refers to the vesicular lesions Produced in cloven-hoofed animals (OIE, 2009). The seven serotypes of FMD virus (O, A, C, SAT1, SAT2, SAT3 and Asia1) were named after the geographical area where they caused FMD outbreak for the first time (kasanga *et al.,* 2014). O and A serotypes were discovered first in 1922 by Valle and Carre who demonstrated that from France that recovered from FMD infection, were re-infected with FMD after being mixed with another herd of cattle from German, infected with another serotype of FMD. This phenomenon explained the distinct serotypes with no antibody cross protection were responsible for FMD outbreaks in

France and Germany For this reason, FMD which caused disease outbreak in France was named serotype O (O for department of Oise in France) and the FMDV which caused an outbreak in Germany was named serotype A (A for Allemagne which literally means Germany in French Language) (Jamal and Belshman, 2013).

2.2.2. Physicochemical properties of FMD virus

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 (EWCA , 2009). The virus is pH sensitive to both acidic and alkaline conditions, It is more stable between pH 7 and 9 at 4°C and -20°C and but inactivated when exposed to PH below 4 or above 11 (Domingo *et al.*, 2002) . However, in milk and milk products, the virion is protected, and can survive at 70°C for 15 seconds and pH 4.6. In meat, the virus can survive for long periods in chilled or frozen bone marrow and lymph nodes. Two percent solutions of NaOH or KOH and 4% Na2Co3 are effective disinfectants for FMD contaminated objects, but the virus is resistant to alcohol, phenolic and quaternary ammonium disinfectants and the virus is also sensitive to other chemicals like trypsin, which causes cleavage and denaturation of the virtual capsid protein (VP1) (Sahle, 2004).

At temperature below freezing point, the virus is also stable and exposed to 56°C for 30 minutes is sufficient to destroy most strains, although there is some variation between strains in resistance to temperature and pH stress. Sunlight has little or no direct effect on infectivity, any loss is due to secondary drying and temperature. The survival of airborne virus is mainly influenced by relative humidity with good survival above 60% relative humidity and rapid inactivation below 60% relative humidity. The size of droplet aerosol also plays a role in the survival or drying out of the virus, where a droplet aerosol size of 0.5-0.7μm is optimal for longer survival of the virus in the air while smaller aerosols dry out. In dry conditions the virus also survives longer in proteins and epithelial fragments (Donaldson, 2000).

2.2.3. Virus morphology

Picorna viruses virions are icosahederal with no envelop and contain one molecule of infectious, positive sense, single stranded RNA (ssRNA), ranging from 7-8.5 kb in length. A viral genomic Protein (Vpg), which is enclosed by the 3B genome region, is covalently linked to the 5' end of the genome and a poly (A) tract of variable length is located at the 3' terminus. The diameter of 22 - 25 nm capsids is composed of 60 capsomers each consisting of four capsid proteins (VP1-4). VP1-3 is exposed on the surface of the virion and contributes to the antigenic properties of the virus, whilst VP4 is located internally within the virus particle at the pentametric apex of the icosahedrons and contains a myristic acid molecule attached to the amino terminal glycine. The Vp1 contains two important immunogenic sites, the G-H loop (at amino acid positions 141–160) and the C-terminus (residues 200–213). One copy of each capsid protein assembles to produce a protomer, five protomers form a pentamer, and 12 pentamers make the complete capsid (Domingo *et al*., 2002).

2.2.4. Genomic organization of FMD virus

FMDV has single stranded, positive sense RNA that is approximately 8500 bases long and consists of a 5' non-coding region (NCR), a single open reading frame, and a short 3' polyadenylated NCR. It has small virus encoded protein, VPg, covalently attached to the 5' terminus. Four distinct regions are distinguished for the poly protein namely the L, P1, P2, and P3. Another characteristic, unique to FMDV, is that there are three species of Vpg encoded by protein 3B, termed 3B1, 3B2, and 3B3. It is roughly spherical in shape and about 25–30 nm in diameter, consists of the RNA genome surrounded by a protein shell or capsid. The capsid is composed of 60 copies of the capsomers. Each capsomer consists of four structural polypeptides, VP1, VP2, VP3 and VP4. The L protein represents the leader protein, where 2 initiation sites (AUG codons) have been identified in FMD virus, namely Lab and Lb (Sangar *et al.*, 2005).

The P1 gene product is the precursor of the capsid proteins 1D, 1B, 1C, and 1A. P1 precursor is processed with the help of viral protease 3Cpro to produce VP0, VP1, and VP3 where the products combine to form empty capsid particles (Figure 1). The mature virion is produced after the encapsidation of the virion RNA by the cleavage of VP0 to VP2 and VP4. The P2 (2A, 2B, 2C) and P3 (3A, 3B, 3C, 3D) regions encode for non-structural proteins that are involved in viral RNA replication and protein processing (Belsham, 1993).

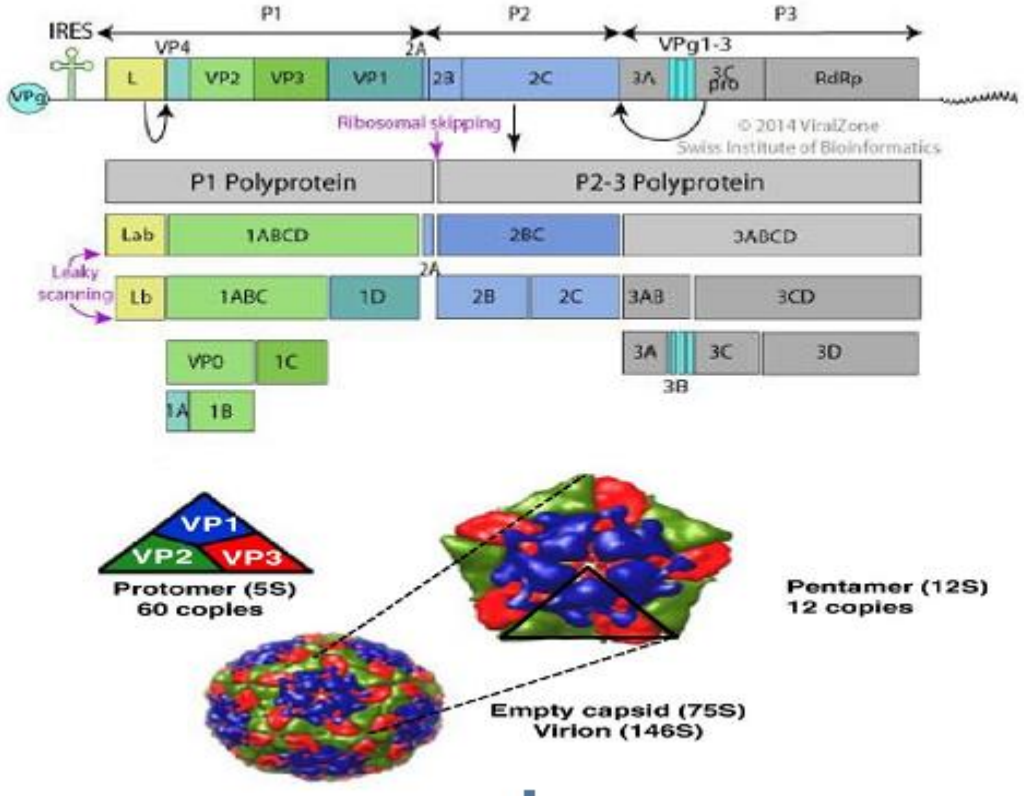

Figure 1: Foot-and-mouth disease virus genome. Source: Taken from Viral Zone (2014)

The virus capsid consists of 60 copies of each of the four structural polypeptides (VP1 to VP4), which are self-assembled into an icosahedral structure with a diameter of 30 nm (Fry *et al.*, 2005). Studies on structural information and protein interaction have shown that the structural protein or the precursor products VP0 (VP2/4 or 1AB), VP1 (1D), and VP3 (1C), which are encoded by P1 region, form immature protomers through weak

chemical bond interaction. Pentamers are assembled by five protomers. After self-assembly of pentamers to generate an empty capsid, the viral genomic RNA covalently linked to VPg at the 5′ end enters the capsid to produce provirion. The provirion is then processed into a mature virion following the RNA-triggered auto-cleavage of VP0 (Han *et al.,* 2015). Finally, the virion particles with complete assembly are released from the infected host cells (Figure 2).

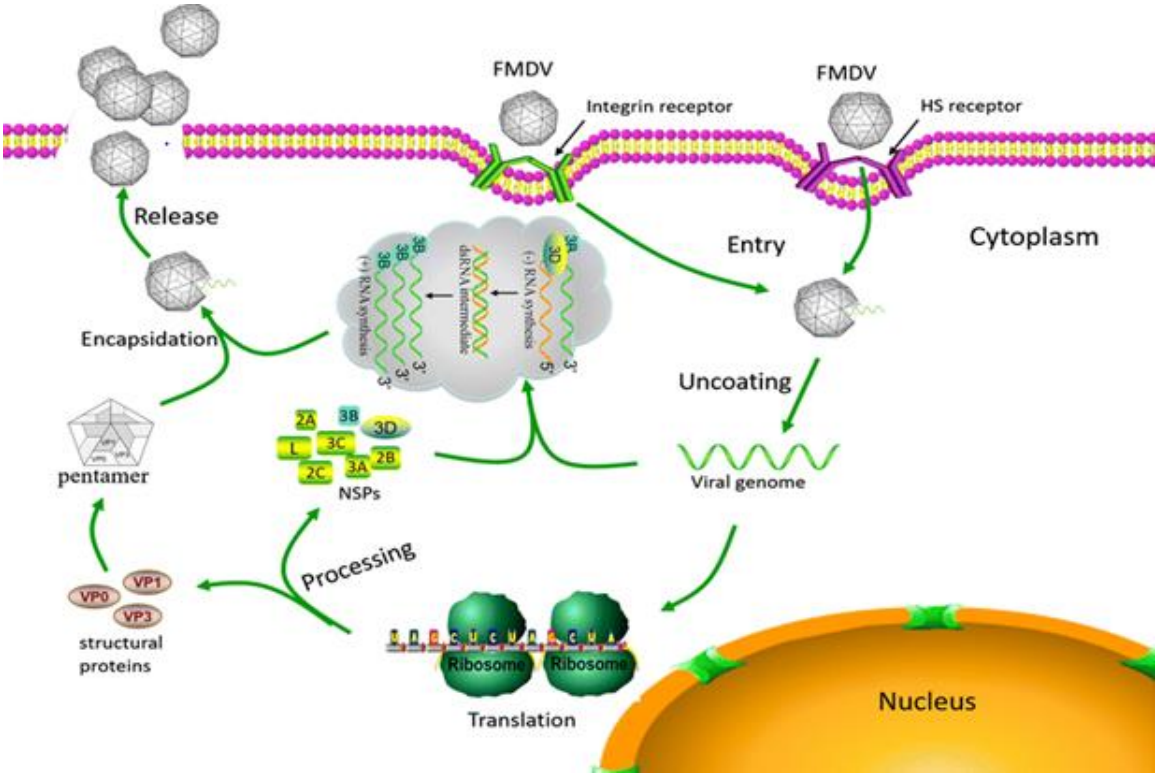

Figure 2: Life cycle of FMDV in host cells, Source: (Gao e*t al.*, 2016)

FMD virus infection is initiated by the attachment of the RGD loop of viral capsid protein (VP1) to host virus immune response and vaccines insufficient FMD virus immunity can be attributed to the epitope between amino acids 140 to 160 having affinity for B-lymphocytes. The three dimensional structure of FMDV includes G-H loop in VP1 (Rweyemamu *et al.*, 2008). This G-H loop is highly conserved arginine-glycine-aspartic acid sequence that invloves in binding to cell receptors. Viruses containing a single point

mutation in the RGD segment of VP1 regain virulence upon restoration of the RGD sequence (Sobrino *et al*., 2001).

2.2.5. Genetic variation

The observed genetic variation in FMD viral genome is the result of a two-step process. Firstly, the replication of viral RNA is error-prone due to absence of proofreading in the 3D-encoded RNA dependent RNA polymerase. Secondly, competitive selection is acting on the genome. Those mutants with a selective advantage in the prevailing environment will be better represented than those with a selective disadvantage (Sahle, 2004).

In RNA viruses, variation is favored by high mutation rates during replication of the virus and emerging viruses are due to mutation and recombination. Recombination is another important process driving viral biology and evolution. It involves the exchange of genetic material between two non-segmented RNA genomes resulting from polymerase jumping during RNA synthesis. It has been shown that genetic recombination occurs between viruses of the same serotype Intratypic recombination occurs more frequently than intertypic recombination and it appears that recombination events in FMD occur more readily in the 3' half of the genome, than in the capsid genome of the FMDV. Mutations through recombination could result in the exchange of genetic material that could lead to the generation of new antigenic variants that may escape immune pressure and may lead to modifications of cell tropism and host range (Drake and Holland, 1999).

One or a few amino acid substitutions at the surface of virus particles may result in alteration of receptor recognition and use, the same virus may acquire the capacity to enter cells via alternative receptors (Baranowski *et al*., 2003).

2.2.6. Immune selection pressure

The evolutionary mechanisms used by RNA virus is the profile mutant production. In addition, the immune system of an infected animal, which provides a power full selective

force, is another driving force in viral evolution for the reason that the viruses are exposed to immune sera (Domingo *et al.*, 1990).

2.2.7. Antigenic variation

Antigenic variation arises due to amino acid mutations that alter the recognition of virus proteins by immune effector systems. Surface exposed structures of the virus are particularly prone to immune attack. Changes to the genes encoding capsid proteins through mutation can result in antigenic variation and evolvement of new subtypes (Haydon *et al.*, 2001).

This gives rise to immunological distinct variants that can re-infect individuals that have been previously infected by related viruses. The concept of antigenic variation came from the observation of Vallee and Carre in 1922, animal that has recovered from FMD virus infection can be re-infected and develops clinical signs. Subsequently, cross challenge experiments in cattle have allowed the classification of FMD virus into seven immunologically distinguishable serotypes such as O and A which exist in Asia, South America and Africa, and Asia-1 and the SAT types (South African Territories SAT-1, SAT-2 and SAT-3) which occur in Asia and sub-Saharan Africa, respectively. The degree of virulence of the virus in recovered animals has shown differences between strains within each serotype or subtypes exsits. The study on field isolates of type C using a panel of monoclonal antibodies identified several antigenic groups showing variation in FMD virus epitopes (Mateu *et al.*, 1996).

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 (Sangare, 2005; Bari *et al.*, 2014). 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).

2.3. FMD Viral Proteins

2.3.1. Features of structural proteins

The P1 gene product is the precursor of structural capsid proteins 1D, 1B, 1C and 1A and named viral proteins VP1, VP2, VP3, and VP4 (Belsham, 1993). The intermediate P1 precursor is initially processed with the help of viral protease 3Cpro to produce the four major structural capsid proteins (VP1-VP4) and the mature virion is produced after the encapsidation of the virion RNA. VP1 is an important protein for epidemiological studies of virus as it is the most antigenic protein involved in cell attachment and carries immunologically important G-H loop. VP3 is the most conserved surface exposed structural protein among different FMD viruses (Acharya *et al.,* 1990). FMD viruses have a high concentration of histidine residues lining the pentamer interfaces that also plays a role in virus instability at acidic pH (Stanway, 1990). Heat or acid treatment of the virus disrupts the interactions between VP2 and VP3 as the pentameric interfaces resulting in pentamer dissociation with release of the internal capsid protein VP4 and RNA genome. The VP4 is entirely internal within the virus particle whereas VP1, VP2 and VP3 are surface exposed and contribute to the antigenic properties of the virus. The VP1 contains important immunogenic sites, the G-H loop (at amino acid positions 141–160) and the C-terminus (residues 200–213). The G-H loop includes an arginine-glycine-aspartic acid (RGD) motif, which is required for attachment of the virus to the host cell via an integrin receptor (Jackson *et al.,* 1997).

2.3.2. Features of nonstructural proteins

The genome segments P2 and P3 precursors are processed into non-structural proteins which are involved in virus RNA replication and protein processing.The genome segment P2 encodes the non-structural proteins 2A, 2B, and 2C. While P3 region is a precursor of 3A, 3B, 3C and 3D, respectively. The protein 2A is a protease and cleaves itself liberating the precursor of the capsid proteins whilst 3C is the major protease and carry out the majority of the processing of the poly protein (Stanway, 1990). The protein 3D is

the RNA-dependent RNA polymerase and is required for the replicative intermediate stage. Animals that have recently recovered from infection will have antibodies to the NSPs, because as the virus replicates in the tissues of the animals, these proteins will be expressed and stimulate the production of specific antibodies by the host. The role of the proteins 2B, 2C, and 3A still remains unclear (Sangare, 2005).

2.4. FMDV capsid epitopes and paratopes

An epitope, also known as antigenic determinant is a part of antigen recognized by the immune system. Epitopes can be B-cell and T-cell epitopes. In FMD virus, B-cell epitopes are well studied and there are four or five known antigenic sites that contain at least one B-cell epitope. An antigenic site is, an area on the protein that induces an immune response (immunogenic) when detected by the body and contains at least one epitope. These areas are important in vaccine preparation as they induce protective immune responses in target hosts. In the different serotypes of FMD virus, these antigenic sites, including the VP1 GH loop contains receptor binding sites and linear epitopes **(**Bari, 2015). Antibodies are essential components of the immune system that are able to identify and neutralize foreign immuno-stimulating objects such as viruses, by binding to specific parts on their surface that are called antigens (Janeway *et al.*,2001). However, the degree of success of any epitope depends on its ability to induce the most specific and detectable rapid immune response while it lies on its capability to confer a neutralizing safe B-cell dependent response. Paratope is specific part of an antibody, binds to a particular region on the antigen that is called the epitope or the antigenic determinant (Ponomarenko and van regenmortel, 2009).

Unlike T-cell epitopes,the majority of functional B-cell epitopes are discontinuous nonlinear epitopes having 3D conformational structures. The paratope epitope interaction offers benefits to the fields of research in immune response, vaccines and diagnostics design, passive immunization, allergens, and auto-immunity (Kringelum *e t al*., 2013).

2.4. Model of the FMDV internalization pathway

FMDV entered host cells by macro-pinocytosis. For the macropinocytic entry, the binding of FMD virus to surface receptors may activate cellular actin modulators and other factors, such as sodium hydogen and dynamin, which trigger actin rearrangement and plasma membrane ruffling. The virions are internalized into macropinosomes, and the membrane fussion events that separate the macropinosomes from the extracellular space occur in a myosin II dependent manner. After closure, the early macropinosomes containing FMD virus acquire Rab5 and EEA1,which facilitate intracellular trafficking. The acidic pH of macropinosomes may trigger viral uncoating. As an alternative entry route of FMD virus, virion binding to integrin receptors induces viral internalization via clathrin mediated endocytosis (CME). The internalized vesicle is then delivered to early endosomes, and the endosomal acidic pH triggers viral uncoating (Figure 3). (https://www.google.com.eab and Model of the FMDV internalizationpathway).

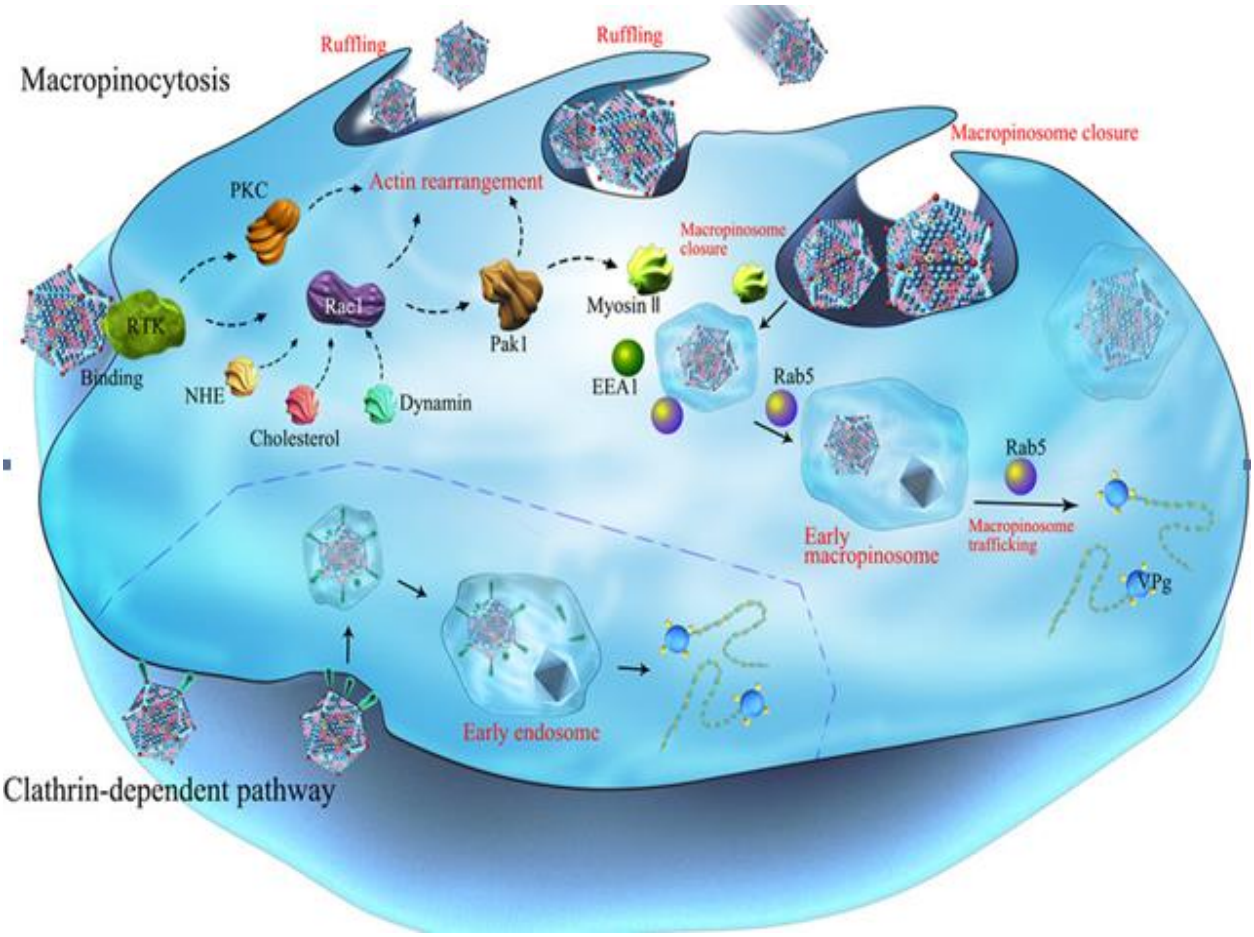

Figure 3: Productive Entry of FMD via Macropinocytosis Independent of Phosphatidylinositol 3-kinase, Source: (shichong *et al.*, 2016).

2.5. Integrin-Mediated FMD infection

Receptors are the major determinant factors for the tropism and pathogenesis of viruses. Viruses may utilize different cell surface receptors during pathogenesis (Zhao *et al*., 2003). Integrins are extensively distributed cell surface primary receptors for FMD virus. Members of this family are heterodimeric transmembrane glycoproteins containing α and β subunits formed by non-covalent interactions. The spherical region formed by the N-terminal α and β chains is the extracellular ligand-binding domain. Integrin mediates cell adhesion and signal transduction in physiological functions, such as cell growth, development, differentiation, and apoptosis. Integrin-mediated FMDV infection occurs through clathrin-dependent endocytosis (CME) followed by acidified endocytic vesicles that cause rapid cleavage of the viral capsid protein structure, causing acid pH-dependent RNA release and translocation of RNA across the endosomal membrane. Apart from the arginine-glycine-aspartic acid (RGD) integrin mechanism, there are isolates of FMDV such as cell culture-adapted viruses which use heparan sulphate (HS) as the predominant cell surface ligand (Fry.*et al* , 1999) and FMDV can also cause infection via the antibody-dependent enhancement pathway, in which virus bound to virus-specific antibodies could enter cells via the Fc receptor, thus by passing the RGD mechanism others can establish RGD. HS independent infections (Baranowski *e t al.*, 2000) (Table 1).

Table 1: Summary of Integrin-mediated FMDV infection.

Integrin	Features of cell surface receptor integrin	References
αvβ1	αvβ1-mediated virus adsorption could be blocked by monoclonal antibodies(Mabs) and its ability to utilize as virus receptor depend on a cellular regulatory mechanism underlying the interaction between integrin protein and ligands	Berryman *et al.*, 2005
αvβ3	Cellular receptor for virus infection	Berinstein *et al*., 1995
αvβ6	Expressed on the surface of epithelial cells targeted by virus, as major receptor determining virus tissue tropism, associated with the endocytosis function of integrins in the porous structure which is mediated by clathrin and plays a role in the cell adsorption process,virus un-coating and replication	Berryman *et al* ., 2005

2.6. Cell surface heparan sulfate-mediated FMDV infection

Cell surface heparan sulfate (HS) can substitute for FMDV integrin receptor and virus variants with improved affinity for heparin are selected after propagation in cell culture. Interaction with heparin often involves the acquisition of positively charged residues on the viral capsid. Under physiological conditions, the N-sulfate group or the O-sulfate group in the HS carbon chain provides negative charges to the sugar chain. This sulfated polysaccharide sequence structure provides HSPGs with not only their anionic feature and high density negative charges but also the ability to interact with other extracellular substances, including viruses (Bernfield *et al.,* 1999). Heparin could specifically block FMDV infection in cultured cells and heparin-treated cells had significantly reduced plaque formation after FMDV infection. In addition, FMDV could not infect HS-

deficient cells. HS is considered a co-receptor for the O type FMDV strain to enter cells and other serotypes (such as A, C, Asia1, and SAT1) of FMD virus could also bind to HS (Baranowski *et al*., 2000). These results indicated that HS could have a direct electrostatic adsorption function with the positively charged arginine residue at position 56 in the VP3 protein. The C-terminal amino acids at positions 201–211 of VP1 might participate in the adsorption process between viruses and cells. HS receptor mediated FMDV infection through Caveola-dependent endocytosis pathway (Ruiz-Saenz, *et al.,* 2009). However, because different strains of FMDV can use different types of receptors, the functional relation between HS and integrin remains lacking. In FMDVs, the integrin-binding site overlaps antigenic sites 1 and 5 as the HS-binding site overlaps antigenic site 4 (Figure 4).

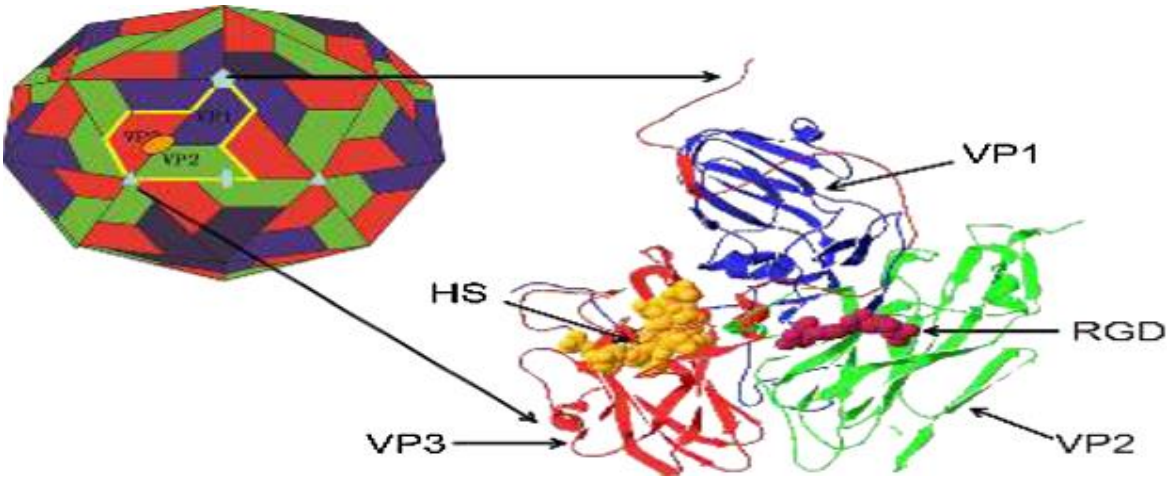

Figure 4: Model of the interaction of FMD virus with heparan sulfate receptor binding site and RGD integrin-binding motif, Source: (Han *et al.,* 2015).

2.7. FMD virus replication

In the initial event of the replication process, FMD virus uses highly conserved triplet sequence (Arg-Oly-Asp) motif on the G-H loop to attach to specific receptors on the cell membrane (Mateu *et al.,* 1996).. These receptors mediate the release of the viral genome from the protein shell into the cytoplasm. The incoming RNA uses the host cell protein-

synthesizing machinery causing shut down of host cell replication. Complementary negative (-) RNA strand synthesis of the positive (+) RNA strand is initiated by a virus-encoded RNA polymerase. The synthesis of (+) RNA strands leads to the formation of multi-stranded replicative intermediates (RI) with a 3' poly (A) which are transcribed from the poly (U) tract in the RI. The RI generates a poo1 of (+) RNA for translation and some for synthesis of additional (-) RNA. Proteolytic cleavages occur during shell assembly in a polyprotein precursor of structural and non-structural proteins. Complete virus particles are released by cell lysis (Belsham, 1993).

2.8. Serotypes and sub types of FMD virus

Currently there are seven serotypes of Foot and Mouth disease virus (FMDV), namely O, A, C, Southern African Territories (SAT) 1, 2 and 3, and Asia 1, which infect cloven-hoofed animals (Quinn *et al*., 2005). Within these serotypes, over 60 subtypes have been described using biochemical and immunological tests as well as new subtypes arise spontaneously. At specific time, there are only a few subtypes causing disease throughout FMD endemic areas. The importance of subtypes is that a vaccine may have to be tailored to the subtype present in the area in which the vaccine is being used (OIE, 2009).

Foot-and-Mouth disease in Ethiopia has evidenced by laboratory diagnosis for multiple serotypes of the virus O, A, C, SAT 1, and SAT 2 (Gelagay *et al*., 2009). Recovery from Infection with any one serotype does not confer immunity against another (OIE, 2009). viruses are assigned to different serotypes on the basis of lack of cross protection following infection (convalescent animals) or vaccination. Viruses showing partial cross protection were categorized to the same serotype but to different subtype. Antigenic variation within a type occurs as continuous process of antigenic drift without clear-cut demarcation between sub types (Quinn *et al.,* 2005) and there are also biotypical strains (strains which become adapted to particular animal species) and topotypes (antigenic entity specific to a given topography) (Samuel and Knowles, 2001).

2.8. 1. Characteristics of serotype "O" FMD virus

FMDV serotype O is the most prevalent globally and has been studied most extensively. Five neutralizing antigenic sites (1–5), involving three of the capsid proteins (VP1–3), have been identified on the surface of serotype O FMDV (Aktas and Samuel, 2000).

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. However, problem with this approach is that antibody bound to one site may interfere with a competitive ligand targeted elsewhere on the virus surface, due to small size of the virion and close vicinity of different antigenic sites (Aktas and Samuel, 2000).

2.8.2. Characteristics of serotype "SAT 2" FMD virus

Two antigenic sites exist. The antigenic site located in the G-H loop of VP1, downstream of the RGD motif, at residues 147, 148, 156, and 158 and residue 154. The SAT serotypes of FMDV are not well characterized as there have been very limited studies (Grazioli *et al*., 2006). These studies reported the presence of 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 154 or 146 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. 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) and (Bari *et al*., 2015).

2.*8.3. Characteristics of Serotype "A" FMD Virus*

The structural alignment and differences between the tertiary structures of these subtypes were found. The integrin-binding site Arg-Gly-Asp (RGD) was in serotype A isolates. Amino Acid alignment of the GH-loop is at residues 140–160. 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. Serotype A displays a great antigenic diversity of subtypes and there is no cross-protection between them (Kitching, 2005).

2.9. Epidemiology of Foot and Mouth Disease

2.9.1. Global distribution

FMD has occurred in most parts of the world. Europe has experienced a number of sporadic outbreaks since the cessation of vaccination on the continent during 1990 - 1991 (Rweyemamu and Astudillo, 2002). Currently almost all European countries are recognized by the World Animal Health Organization (OIE) as free of FMD. The disease is common in most developing nations of South America, Asia, Middle East and it is highly endemic in most sub-Saharan African countries (FAO, 2006 (Table 2).

Table 2: Geographical distribution of Foot and Mouth disease virus serotypes

FMD virus serotypes	Global distribution
O, A, C	Europe
O, A, C, Asia 1	Asia
O, A, C, SAT1, SAT2, SAT3	Africa
O, A, C	South America

Source: Adapted from (Quinn *et al*., 2005)

2.9.2. Distribution of disease by serotypes

FMD serotypes are not uniformly distributed in the regions of the world where the disease still occurs. Africa has the greatest diversity of FMD serotypes. Six of the seven serotypes of FMD virus except Asia 1 are prevalent in Africa. Three of the South African Territories (SAT) serotypes are unique to Africa. Asia contends with four serotypes (O, A, C, Asia 1), and South America with only three (O, A, C). Serotype Asia1 is restricted only to Asia subcontinent and the capacity to invade free areas is common to all serotypes (Rweyemamu *et al.,* 2008).

Type O is the most widely prevalent serotype in the world followed by serotype A. South America has had a genetically stable type O virus for nearly the past 50 years under study. Serotype C appears extremely rare and disappearing from the world as a whole with the exception of Kenya and some foci of Brazil in South America probably because of the circulation of this particular serotype extinct in wildlife (FAO, 2006). The last confirmed case was in the Amazon region of Brazil in 2004 and Kenya in 2005. In history it is the rarest of the FMD types to have occurred in Africa, having been recorded only in three countries, namely Ethiopia, Kenya and Angola (Rweyemamu *et al.,* 2008).

The SAT serotypes have an intimate and probably ancient association with African buffalo that is instrumental in their maintenance (Vosloo *et al.*, 2002; FAO, 2006). Of the

three SAT types, SAT-3 has the most restricted distribution to South African countries, while serotypes SAT-1 and SAT-2 are widespread and occur throughout sub-Saharan Africa and have also made recent incursions into the Middle East. Due to globalization, FMD epidemics were changed from local and regional spread to wide international spread (Knowles *et al.,* 2005). The globalization of trade even areas where FMD is endemic can suffer from introduction of virus strains that are exotic to the region (Bruckner *et al.,* 2002). The risk of FMD entry into free areas is low through legal trade of animal and animal products from zones or countries officially recognized as FMD free by the OIE. However, smuggling of animal product is a significant issue and the main route of virus introduction into FMD free areas (WRL, 2007).

2.9.3. The Disease situation in sub-Saharan African countries

The epidemiology of FMD in sub-Saharan Africa is probably more complicated than in any other regions of the world. Not only six of the seven serotypes prevalent in Africa (only Asia1 has never been recorded), but marked regional differences in the distribution and prevalence of serotypes and intratypic variants occur. Wildlife plays a unique and important role in the epidemiology of the disease in Africa although this aspect has been investigated only in Southern Africa (FAO, 2006). Regardless of the disease endemicity in nearly countries of sub-Saharan Africa, the majority of outbreaks remains unrecorded and is not notified timely due to trade restrictions and pastoral systems where inadequate in disease surveillance; the transport of sampling material is difficult and expensive, few African laboratories are able to confirm the diagnosis of FMD (FAO, 2006; Rweyemamu *et al.,* 2008).

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

East African countries are not only having large livestock populations but also own the highest concentration of wildlife in the world. Livestock farming is dominated by agro-pastoral and pastoral communities and it is characterized by communal grazing and migrations. This cluster probably contains several major FMD endemic foci and the most complicated FMD situation in the world. There are also wide genetic variations in the FMD virus strains and the role of African buffalo in the maintenance and transmission of FMD serotypes and the role of other potential wildlife reservoirs of the disease that occur in this epidemiological unit have not been systematically studied (Rweyemamu *et al.,* 2008).

The situation of FMD in infected areas indicates that FMD types continually spread within endemic regions give rise to virus types that break immunity and cause regional epidemics. In this cluster as in other parts of Africa, the use of vaccines is sub-optimal in relation to the size of population and most of the FMD susceptible animal populations are found at risk. Countries in southern Africa, contrary to the general trend in Africa, have been largely successful in controlling FMD to ensure access to international markets (Sutmoller *et al.,* 2000).

2.9.4. Susceptible hosts

FMD is highly contagious and affects over 70 domestic and wild life species of animals. However, not all FMD viruses have the same host range. Sensitive species belong to the mammalian order of *Artiodactyls and* of the domesticated species, cattle, pigs, sheep, goats and buffalo are susceptible to FMD. Similarly, many species of wild life, such as deer, antelope, wild pigs, warthogs, elephants, giraffes, camels and llamas, may become infected with FMD viruses. Except African buffalo, the importance of other wild life involvement in the epidemiology of FMD is not well studied (OIE, 2008).

The susceptibility of cloven-hoofed species varies with breed of animal and strain of the virus. The disease is considerably less obvious or sub-clinical in breeds of cattle, sheep and goats indigenous to Africa and Asia, where FMD is endemic; and these animals are

believed to have been the source of infection for countries previously considered disease free (Kitching, 2002).

2.9.5. Incubation period

The incubation period of an infectious disease is defined as the time interval between exposure to infective dose and first appearance of clinical signs. The incubation period for FMD is highly variable, and depends on the strain and dose of virus, the route of transmission, the animal species and the husbandry conditions (Alexanderson e*t al.,* 2003).

The incubation period of the virus in natural infections is usually between 2 and 3 days and could be as long as 14 days in cattle and lasts for 4 to 8 days in pigs. Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact: the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion, it may last up to 3 years. In African buffalo, individual animals have been shown to harbor the virus for at least 5 years, but it is probably not a lifelong phenomenon. Sheep and goats do not usually carry FMD viruses for more than a few months, whilst there is little information on the duration of the carrier state in Asian buffalo species and subspecies (OIE, 2012).

2.9.6. The role of carrier animals

Carrier, in FMD, is defined as an animal from which FMD virus can be isolated from the oesophageal pharyngeal (OP) area, 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), the mechanisms underlying persistence and the Immunological pathway that eventually leads to viral clearance are not well

understood. However, the carrier state can develop either after the acute stage or without any clinical disease, in vaccinated animals exposed to live virus. Nevertheless, in both situations, the main part of the virus replication is controlled, either by vaccine-induced immunity or after a few days by antibody being produced (Alexanderson *et al*., 2002). But, host response is not efficient in clearing the infection in the pharynx and may harbor virus even in animals with antibodies. This may provide a mechanism for the maintenance of the virus in nature and the cause of acute episodes of disease that may contribute to the emergence of new variant viruses (Kitching, 1998; Sahle, 2004).

2.10. Molecular Epidemiology

Phylogenetic analysis of the VP1 region of FMD viruses has been used extensively to investigate the molecular epidemiology of the disease worldwide. The techniques have assisted in studies of the genetic relationships between different FMDV isolates, geographical distribution of lineages and genotypes, and the establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks (Knowles and Samuel, 2003; Sangare, 2005). Topotypes are defined as geographically clustered viruses that form a single genetic lineage generally sharing >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3) nucleotide identity in the VP1 coding region and named by the geographic region in which they occurred (Ayele *et al.*, 2009).

Currently, sequencing and phylogenetic trees are widely used to illustrate the genetic relationships between viruses. Sequence differences of 30% to 55% of the VP1 gene were obtained between the seven serotypes of FMD virus while 15% to 20% sequencing difference within each sub-group were observed (Knowles and Samuel, 2003). Analysis of the genetic distance and phylogenetic resolution of the sequence of VP1 encoding gene have provided crucial epidemiological information covering different degree of genetic relationships between isolates (Samuel *et al.*, 1999).

2.10. Source of Infection and Mode of Transmission

The disease is contagious because a small dose of the virus is infectious and several routes of infection and excretion have been reported. The primary mode of transmission is via respiratory aerosols since the virus can replicate mainly in the respiratory tract of animals and large amount of the virus particles are excreted from this area although the virus may occur in all the secretions and excretions of infected animals during the acute phase of infection (Kitching *et al.,* 2007).

Other important means of spread is by direct contact between infected and susceptible animals. In densely populated areas the disease may spread rapidly because of the high level of challenges from infected animals. The virus also readily spreads indirectly by inanimate objects including animal food staff, beddings, farm equipment, livestock holding areas and transport vehicles that have been contaminated with acutely infected animals excretions and secretions such as saliva, milk, faces and urine. Outbreaks can occur because of viruses escaping from research and vaccine production centers and the semen of infected bull can be a source of infection by artificial insemination (Radostits *et al.,* 2000).

Personnel handling infected animals can be contaminated on hands, clothes or in nasal passages with live FMD virus and mechanically carry virus to susceptible animals by direct contact. A person in contact with infected animals can serve as a source of infection for 24 hours post infection (Kitching *et al.,* 2007). Dogs, cats, horses and birds can transmit the disease mechanically (Quinn *et al.,* 2005).

The spread of FMD virus by wind over 250 km across the sea and 60 km across the land has been reported in temperate climates during an outbreak of the disease. Early recognition of disease, followed by slaughter of infected livestock and the introduction of movement controls reduces the risk of mechanical spread. However airborne spread of the virus cannot be controlled by these means (Gloster and Alexanderson, 2004).

Airborne spread of the virus is determined by the numbers and species of animals affected by the virus strain and the environmental conditions. Infected pigs emit high levels of airborne virus which can be as high as 4 x 106 infectious units per day, but are relatively resistant to infection by the airborne route (Kitching *et al.*, 2007). In contrast cattle, infected with FMD virus excrete less airborne virus between 103 to 108 infectious virus units per day as an aerosol, but are highly susceptible to airborne infection of respiratory route and a dose of 20 tissue culture infective dose of virus is sufficient to establish infection. The virus is stable in aerosols at a relative humidity above 60% and at temperatures below 33°C. Sunlight and the pollution complex termed the outside air factor have minimal direct effects on virus survival (Gloster and Alexanderson, 2004).

2.11. Immune response to FMD virus

Serotype specific immunity is based on the presence of neutralizing antibodies to one of the viral capsid protein, VP1, develops 7 to 21 days after exposure to the virus. The immunoglobulin M is most prevalent in the early convalescent serum and is less specific to the different serotypes than Immunoglobulin G. Immunoglobulin G is produced in the later stage during the FMD infection and the reaction between the serotype and the homologous antibodies is highly specific. It has been reported that healing of lesions and clinical recovery in infected animals would not occur until a few days after the IgG1 antibodies have developed. The localized antibody response, specific to anti-FMD IgM and IgA antibodies in the pharyngeal fluid of cattle develops 7 days after exposure to the virus, while IgG activity reaches pick in serum only 14-21 days after infection. Although serum antibody levels play an important role in host protection against FMD virus infection, the cellular responses mediated by T-helper and T- cytotoxic cells also play a role in the immune response to FMD virus infection. Protection of a susceptible host against FMD virus correlates with the neutralizing antibody level. Infection with one-serotype produces complete protection against homologous virus, but no protection against heterologous viruses (Samina *et al.*, 1998).

2.12. Clinical signs

The severity of clinical signs of the disease varies with the strain of the virus, the exposure dose, the age and breed of animal, the host species and their status of immunity. Viremia in cattle lasts for 3-5 days and the lesions develop 2-14 days post infection (Kitching and Hughes 2002). In acutely infected cattle, the initial signs are fever or a rise in temperature (40-40.6^{o}C), dullness, anorexia and marked drops of milk production. Within 24 hours, there appears excessive salivation, the saliva hanging in long, ropy strings and leads to nasal discharge (Quinn *et al.,* 2005).

These signs are accompanied by vesicle formation on the dorsum of the tongue, soft palate, dental pads, lips and gums, which result in opening and closing of their mouth with characteristic smacking sound. Vesicles may also be found in areas where there is high friction, such as inter-digital space and coronary band of feet with consequent lameness, on nostrils, muzzle and teats. Pregnant cows may abort, presumably as a consequence of fever (Murphy *et al.,* 1999). Young calves up to 6 months of age may die suddenly before the appearance of any clinical signs of the disease due to virus infection of the developing lesion in heart muscle (Radostits *et al.,* 2000).

Most infected animals recover within 2 to 3 weeks although infection may delay recovery of mouth, feet and teat lesions, resulting in hoof deformation, mastitis, low milk production, failure to gain weight, and breeding problems. A lactating animal may not recover to pre infection production status because of damage to the secretory tissue. In lame animals, there may be vesicles or erosion on the coronary band or in the inter-digital space. Infected animals may abort and nursing lambs may die without showing any clinical sign (Hughes *et al.,* 2002).

2.12.1. Morbidity and mortality status

The morbidity rate in outbreaks of FMD in susceptible animals can rapidly approach 100% but some strains are limited in their infectivity to particular species. However, the

case fatality is low, about 2% in adults and 20% in young stock (Radostits *et al.*, 2007). Mortality in adult animals is usually low to negligible; up to 50% of calves may die due to cardiac involvement and complications such as secondary infection, exposure or malnutrition and mortality decrease rapidly as animals get older (exceed 4weeks). During outbreaks in endemic and developed countries, most deaths are due to a slaughter policy that usually involves all susceptible animals and herds in contact with or within a certain radius of infected herds also persist (Hirsh and Zee, 1999).

2.12.2. Pathogenesis of FMD virus in cattle

The respiratory system is the most important portal of infection. 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 (Lefevere *et al.,* 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 (Hirsh *et al.,* 2004).

Gross lesions develop only in areas subjected to mechanical trauma or unusual physiological conditions such as the epithelium of the mouth, feet to a less extent, the teats. Bacterial complication aggravates the lesions, particularly those of the feet and the teat, leading to severe lameness and mastitis, respectively. In young animals, especially neonates, the virus frequently causes necrotizing myocarditis and this lesion may also be seen in adult infected with some strains of the virus particularly type O (Radostits *et al.,* 2007). In fatal cases, death is caused either by dehydration or by ventricular fibrillation during cardiac attacks or as a result of bacterial complication (Lefevere *et al.,* 2010).

2.13. Diagnosis

In cattle, FMD should be considered whenever salivation and lameness occur as well as vesicular lesion is seen or suspected, the laboratory diagnosis and serotype identification of the virus should be done in a virus secure laboratory (OIE, 2009). Diagnostic samples include vesicular fluid epithelium, blood in anticoagulant, serum and esophageal or pharyngeal fluids collected with a Pro-probing (Quinn *et al.,* 2005).

When epithelium tissue is not available from ruminant animals in advanced or convalescent cases and infection is suspected in the absence of clinical sign, samples of oesophageal-pharyngeal fluids is collected by means of a probang and used for virus isolation. For virus isolation specimens suspected to contain FMDV are inoculated into cell culture (primary pig, calf, and lamb kidney), BHK-21 incubated at 37ºC and examined for Cytopathic effect (CPE) after 48 hours. The antibody detection by 3 ABC ELISA can be used on a herd basis to detect FMDV infection in vaccinated and unvaccinated population (OIE, 2010). The polymerase chain reaction can be used to amplify the genome fragments of FMDV in diagnostic material (Quinn *et al.*, 2005).

2.13.1. Prevention and Control

Foot and mouth disease is subjected 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. Countries free of FMD impose strict import regulation on animals, animal products and potentially contaminated materials from FMD countries. Quarantine and vaccination programs are also used to control outbreaks and to prevent spread of the disease (Hirsh *et al.,* 2004).

In countries where the disease is endemic, efforts are directed at protecting high yielding dairy cattle by a combination of vaccination and control of animal movement. Preventive measures in the absence of disease should be implemented through Control of national

borders to prevent movement of animals and livestock products from non-free neighbors. For officially free countries, prohibition of imports of animals and their products from no free countries in accordance with the OIE standards, prohibition to distribute untreated catering waste to pigs. Emergency measures in the event of outbreaks is through Rapid slaughter of infected animals, in contact animals and herds have received infection, to reduce the quantity of virus released policy of stamping-out (Lefevere *et al.,* 2010), 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. Intensive investigations to determine if infection is likely to have spread to additional locations within or outside of the protection and surveillance zones and control measures for such herds or villages, depending on the risk identified and emergency vaccination is important (Radostits *et al.,* 2007). Active disease surveillance must be effective which needs a strong field service as well as proper laboratory facilities with efficient methods of detection and characterization of the virus (OIE, 2009).

In the situation of Ethiopia the control of FMD is practiced by involvement of quarantine, restriction of animal movement, isolation of infected animals, vaccination programs, and proper disposal of infected carcass. Currently there is no country-wide vaccination program aimed to control FMD and a ring vaccination is carried out around infected area. Considering the wide prevalence of serotypes O, A and SAT 2 the National Veterinary Institute is producing an inactivated trivalent vaccine (Tesfaye, 2014).

2.13.2. Economic impact of FMD

Foot and mouth disease remains one of the most important livestock diseases of the world, its highly infectious nature, its broad economic impacts on animal wellbeing and productivity, its implications access to domestic and export markets for livestock and products. The impacts of the disease vary between developed and developing countries, and also within many developing countries depending on the species involved, the genotype, the level of productivity, the significance of livestock to livelihoods, and the

effectiveness of indigenous coping mechanisms for controlling the effects of FMD (Catley *et al.,* 2004).

The direct effects of an outbreak of FMD in terms of morbidity, mortality, expenditure to treat the disease, and measures taken to avoid further spread can lessen incomes, particularly in susceptible highly productive animals (James and Rushton, 2002). Clinical disease affects animal performance and causes reduction of milk yield in dairy cattle by 20%, growth rate of beef animal by 10-20%, pig meat production up to 20% per annum and inflict fertility impairment due to increased abortion rate up to 10% (Knight *et al*., 2013). Moreover, death of young animals, culling of unproductive and chronically infected animals, and loss of valuable breeding stock and disruption of livestock improvement programs are also attributed to the direct effect of the disease. The impact on the livelihoods can be devastating and similarly pastoralists severely suffer from the direct impact of the disease since their livelihood depends on livestock production (Rufael *et al.,* 2008).

From a current global perspective, the indirect effect and the risk of the disease have a much greater impact. Burden of containing outbreaks, as well as maintaining the ongoing capacity and measures intended to reduce the risk of FMD such as vaccination, surveillance and movement control can incur high additional economic cost (Kitching *et al.*, 2006). In developed nations containment measures especially stamping out policies are seen to be associated with animal welfare and environmental impacts that increasingly draw attention. The highest value markets for livestock products are in FMD free countries, and these countries are allowed to restrict or ban imports of livestock products and other products as well as a potential risk of introducing FMD. The presence of FMD in a country is considered as binding constraint to developing an export sub-sector within the livestock industry. Unfortunately, FMD is widely distributed in the developing world, in particular Africa, South America, south East Asia, regions of the world that support 75 per cent of the world's poor and thus hampering utilization of their

huge livestock resources for economic development and poverty alleviation (Rich and Winter, 2007).

2.14. Foot and Mouth Diease situation in Ethiopia

2.14.1. Status of disease

In Ethiopia, foot and mouth disease is endemic and a notifiable disease; the national animal health regulatory directorate sends monthly and annually official reports to OIE (MoLF, 2016). 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 (Tefera, 2010).

2.14.2. Spatial distribution

FMD is widely distributed in all areas of Ethiopia, although the level of the disease prevalence shows significant variations across the different farming systems and agro-ecological zones of the country. The national picture of FMD status outbreak data were reported to Ministry of Livestock and Fishery (MoARD) from 2009-2015.

2.14.3. FMD prevalence

Despite the widespread of disease, clinical, serological and virological studies to characterize the disease have never been exhaustive. The prevalence of the disease is varying from place to place and studies conducted so far did not cover all corners of the country. The lack of well-equipped regional animal laboratories, inaccessibility of certain areas and suboptimal routine surveillance and reporting could hinder to have the overall estimate of the disease magnitude at a national view contrary to its endemicity (Sahel, 2004).

Table 3: The overall seroprevalence of FMD in different parts of the country

Location	Prevalence (%)	References
Borana	23	Bayissa *et al.,* 2009
South omo	8.2	*Molla et al.,* 2009
Somali regional state	14.05	*Mohamoud et al.,* 2011
Kellem wellega zone	21.4	*Desissa et al.,* 2014
Central ethiopia	14.5	*Alemayehu et al.,* 2014
Western Ethiopia	9	*Beyene et al.,* 2015
Adama	26.84	*Mishamo et al.,* 2016

2.15. Current scenario of FMD virus serotypes circulating in Ethiopia

Research findings and records from National Animal Health Diagnostic and Investigation Centre (NAHDIC) and National Veterinary Institute (NVI) of Ethiopia indicated that five of the seven FMDV serotypes (O, A, C, Southern African Territories SAT-1, and SAT-2) were identified in in the country and the isolated serotypes were responsible for FMD outbreaks during 1974-2007. In terms of species, these serotypes were identified from bovine, swine, ovine, and caprine samples collected from outbreak areas. Cattle were found to be infected with all circulating serotypes of FMDV, whereas swine had only serotype O (Sahle, 2004; Gelaye *et al.*, 2005; Legesse, 2008; Nigussie, 2010; Ayelet *et al.,* 2009). Serotypes O, SAT-2, A and C viruses were considered as the major causative agents of FMD. However, recent report of serotype C specific antibodies in cattle in Ethiopia indicated that circulation of serotype C viruses in the country may have gone unnoticed (Rufael *et al.,* 2008).

Recent reports indicated that serotype SAT2 was once more recorded in 2007 from a bovine of Bambasie, Benshangul-Gumuz, western Ethiopia (Ayelet *et al.,* 2009). Presence of serotype SAT-2 specific antibodies in cattle in Ethiopia has been reported (Rufael *et al.,* 2008). SAT-2 may have been introduced to Ethiopia by free animal movement across the border with Sudan because SAT-2 is endemic in Sudan (Vosloo *et*

al., 2002). SAT 2 was also recently isolated from bovine and small ruminants of Itang, Gambella, Abobo and Lare Districts, Gambella region (Tefera, 2010) and serotype O was also isolated from the outbreaks occurred in Addis Ababa, Debre Berhan and Bishoftu (Tesfaye, 2014). The presence of FMDV serotype SAT-1 in Ethiopia was isolated and reported in 2008 (Legesse, 2008), from cattle, sheep and goats. Although SAT-1 has not been previously reported in Ethiopia, it might be circulating in wildlife and transmitted to domestic animals (OIE, 2012). FMDV recovered from the outbreaks in Ethiopia during 1981-2007 witnessed that serotype O (73.30%) was most prevalent followed by types A (19.5%), SAT-2 (4.10%), SAT-1 (1.80%) and C (1.40%), respectively (Gelaye *et al.,* 2007; Ayelet *et al.,* 2009).

Table 4: FMD virus circulating in the country

Region	**Outbreak sites**	**Circulating serotypes**	**References**
Oromiya	Borena	O, A, SAT2	Rufael *et al*., 2006
	-	O, A, SAT2, C, SAT1	Ayelet *et al*., 2009
	Bishoftu	O	Sentayehu *et al*.,
	Adama		2014
	Adami Tulu		
Addis Abeba	Akaki kaliti		
SNNP	Sidama zone		
Amhara	Debreberhan		
Tigray	Mekele,Ayanalem,	O	Kiros *et al*., 2013
	shibta and debra		
Addis Abeba	-	O	Tesfaye *et al*., 2014
Oromiya	Arsi	O, A, SAT2	Mishamo *et al*.,
	East shoa		2016
Addis Abeba	-		

2. 15.1. Risk factors

Risk factors for FMD includes factors that may change the level of risk (new serotypes or biotypes, or changing epidemiological or livestock husbandry patterns) and factors that may interrupt on the national veterinary service to respond effectively to the disease threats (Wondwossen and Tariku, 2000). The occurrence of new topotype and uncontrolled animal movement are some of the risk factors. Hence, FMD outbreaks occurred in different regional state of country showed the virus serotypes and topotypes were similar, that is O serotypes, EA-3 except the Mekele outbreak topotype, which was identical with Sudan topotype and its phylogenetic analysis indicated that the isolate was much related to the Sudan 1999, 2004 and 2008 isolates (Bewket *et al.,* 2012) and SAT2 serotype, VII, XIII and XIV topotype reappeared after a gap of sixteen years (Ayelet *et al.,* 2009).

2.15. 2. FMD control program

FMD is considered as one of the most important livestock diseases demanding urgent control intervention to minimize the impact of disease because of international trade barrier. But the complex nature of the disease, its distribution and absence of vaccination program within the country demanded that control strategies be implemented progressively on term basis. Actions such as disease free zone establishment and mass vaccination of cattle herds may have important contributions to minimize the impact of this disease. However, the short term FMD vaccination program give emphasis to the control of outbreaks occurring in the country through ring vaccination and vaccination of export cattle before entering the quarantine stations. To protect export animals from contracting the disease while being kept in quarantine sites cattle found within 10 km radius of these sites could be vaccinated. All dairy animals should also be vaccinated (MoARD, 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 first FMD outbreaks investigated in this study have been occurred by mid-september 2016, in the dairy farm of Adea Berga district of West Shewa zone and there have been FMD clinical cases and death cases in the farm. The same kebeles within the above mentioned district experienced the second outbreak of the disease. The outbreaks were then continued and occurred in Welmera district of Special Oromia zone. By October, 2016 the third outbreaks were occurred at the Addis Ababa Kolfe, Keranyo in dairy farms. In November 2016, the fourth outbreaks were occurred in North Shewa zone of Oromiya region (Mulo, Wuchale and Kimbibit districts) (Figure 5). In this FMD affected Areas, cattle that have exhibited clinical signs of the disease were identified in the village and samples were collected for confirmatory testing.

3.2. Study areas of FMD outbreaks

The study was carried out in areas where FMDs outbreaks occurred from mid-September, 2016 to March 2017 in selected district of Oromiya region and Addis Ababa, Ethiopia. A total of six outbreaks were encountered and investigated during the entire period. These outbreaks were reported in west Shewa zone (Adea Berga and Welemra district), North Shewa zone (Kimbibit, Wuchale and Mulo districts) and Addis Ababa (Kolfe Keranyo 01) in 2016. Adea Berga district is located in Oromia regional State, West shewa zone which is about 70 km from the capital city of the country; Addis Ababa. The total area of the woreda is 798.35 square kilometers. It is located at 9^{o} 12’ to 9^{o} 37’ latitude and 38^{o}17’ to 38^{o} 36’ longitude. The altitude of the area ranges from 1400 to 3500 meters above sea level. It is characterized by agro ecologies like low land, middle and high lands which covers 37%, 34% and 29% of the area respectively. An annual average of rainfall ranges from 918mm to 1368mm while the minimum and maximum temperature reaches

10^{o}C and 25^{o}C respectively. The primary wet season extends from June through October. July and August are the wettest months. The farming system of the area is mixed type where crop production and livestock rearing are done side by side (ABLHO, 2016).

FMD outbreak was also investigated in Addis Ababa city Kolfe Keranyo. The city is located on a well-watered plateau surrounded by hills and mountains, in the geographic center of the country. It has an altitude of 2300 meter above sea level with a subtropical high land climate. The average annual rainfall, maximum and minimum temperature for the area are 1180 mm and 22.8ºC and 10.6^{o}C, respectively. It is found between 9°1′48″N latitude and 38°44′24″E longitude. It has a humid subtropical mild summer climate that is mild with dry winters, mild rainy summers, and moderate seasonality. The city is completely surrounded by Oromia Regional Government in all directions. In Addis Ababa, there are about 5,200 dairy farms with some 58,500 cattle, and almost 50% are cross breed (CSA, 2009).

The other outbreak site was Welmera district which is a part of the Oromiya special zone Surrounding Finfinne/Addis Ababa. The district is 40 km away from the capital city, Addis Ababa and the area is situated at 9^{o}04'- 9^{o}13' N latitude and 38^{o}29'-38^{o}39' E longitude. It is bordered on the south by the Sebeta Hawas, on the west by West shewa zone, on the North by Mulo district, on the Northeast by the Sululta. Menagesha Kolobo and Holeta Genet are the towns located in the district. The average altitude of the area ranges from 2200-2500 meter above sea level. The rainfall pattern of the district is bimodal, with a short rainy period from February to April and a long rainy season from mid-June to September. The annual temperature and rainfall ranges from 18°C to 24°C and 1000 to 1100 mm, respectively (CSA, 2009).

FMD Outbreak was also occurred in Kimbibit district which is located in North Shewa zone of Oromiya Region at distance of 78km from Addis Ababa. There are 29 rural kebele administrations and two town dwellers Association in the district. Agriculture is the main source of income of the population. The district has total area of about 752 square kilometers and the population density was 123.3 per square kilometer. The area is

characterized by bimodal rainfall pattern with the main rainy season extending from June to September and short rainy season that stretches from February to March. The area is known by production of sheno butter due to huge livestock number. Sheno is the name of district town and butter produced in this area was named within the name of town. The area is situated at an altitude of 2630-3020 meters above sea level and has an annual rainfall of 913 mm and an average maximum and minimum temperature of 24^0c and 8.5^oC (CSA, 2012).

Mulo is one of the districts where FMD outbreaks were occurred. The 2007 National census reported a total population for this woreda of 35,138, of whom 17,708 were men and 17,430 were women; 2,296 or 6.53% of its population were urban dwellers. Wuchale is district is a part of North shewa zone which is bordered on the south by Aleltu, on the west by Mulo-Suluta, on the Southeast by kembibti.Muka Turi is a town located in Wuchale district. Based on figures published by the Central statistical Agency in 2005, wuchale had an estimated total population of 142,131 and 4.46% of its population was urban dwellers (CSA, 2009).

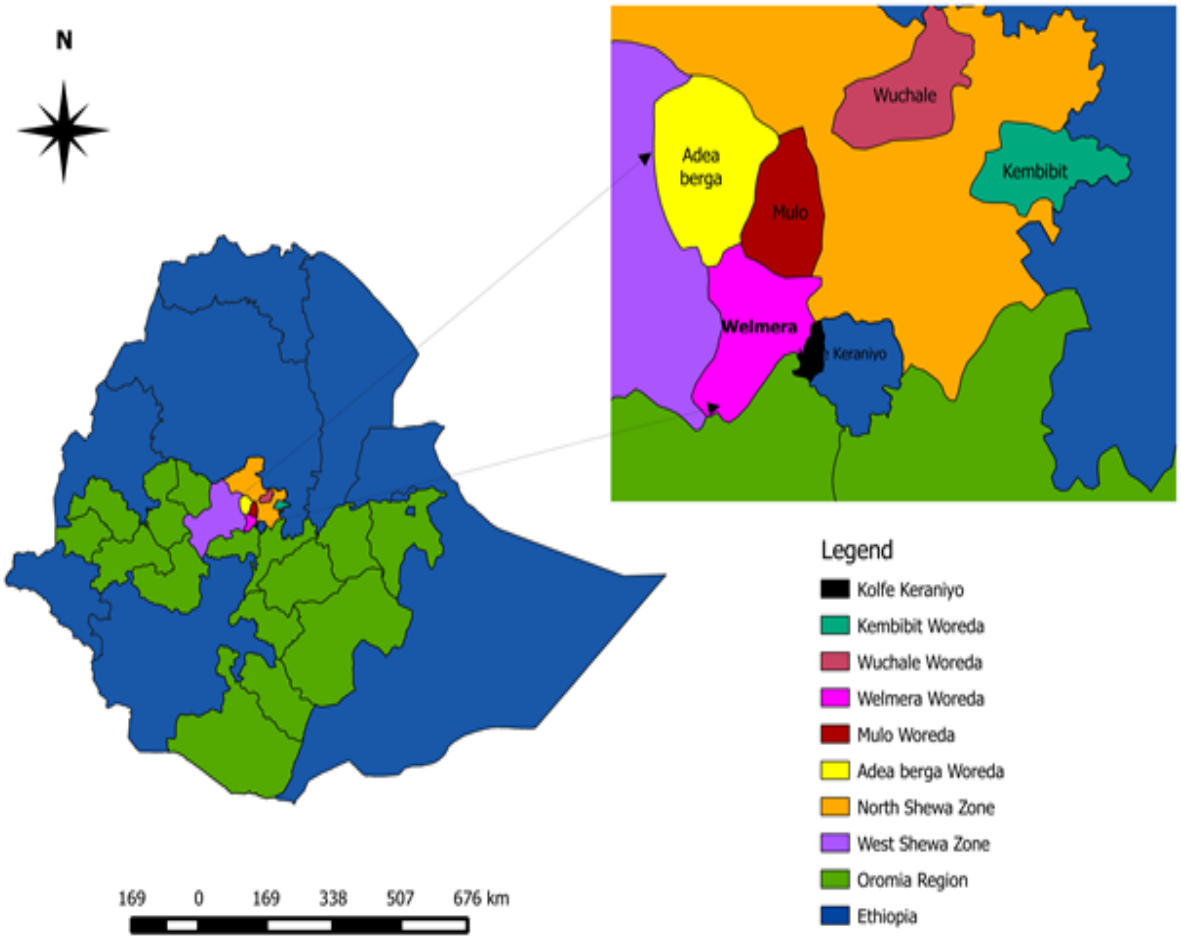

Figure 5: Map showing the study areas where FMD outbreaks occurred in 2016

3.3. Study Population

The study population was cattle that had experienced outbreaks of disease and manifested clinical signs of FMD in the outbreaks and those in close contact with outbreaks. Cattle with clinical symptoms and asymptomatic ones in the same farm in respective of age groups(Appendix 2), sex, breeds of animals that were kept in intensive and semi intensive management systems and body condition scores (Appendix 3) were included and considered as risk factors during the entire period of investigation.

3.4. Study Design

On the basis of FMD outbreaks and active cases, a cross-sectional study design was used during outbreaks to collect samples. When active outbreak of FMD was encountered or reported, field level investigation was conducted purposively at a particular site of outbreaks within the study districts (Table 6). In each selected villages, animals having clinical symptoms like oral lesion, history of infection but having healing lesion and any other asymptomatic cattle (with no evident clinical signs for FMD) in the same farm or grazing with the symptomatic cattle (with evident clinical signs of FMD) were purposively sampled as per the extent of the outbreak. The inclusion criteria were cattle with clinical symptoms and asymptomatic ones in the same farm while the exclusion criteria were cattle in the same farms without any clinical signs or not experienced the disease before or when cases occurred in a defined area and history of clinical signs. Besides this, cross-sectional study design was also conducted to determine the seropositivity of disease in Welmera district based on the abundance of dairy animals.

3.5. Sampling Techniques and Sample Size Determination

Based on the presence of FMD outbreaks, field observation was conducted at the specific sites of the outbreaks. Purposive sampling technique was applied at certain sites of FMD affected areas to select district outbreaks, peasant associations, cattle herds and sampling animals. In the outbreak areas, physical examination was conducted on clinically sick animals to record clinical signs and disease conditions in the farm and herd. Accordingly, animals with clear signs, symptoms and suspected of infected with FMDV were selected and sampled. In addition, prevalence calculation is possible as recommended by Buderer (1996) from outbreak cases.

A total of 586 accessible animals were used during outbreak study. Accordingly, 113 samples were collected out of which 41 samples for serotyping of virus, 37 samples for cell culture based virus isolation and 50 samples for molecular assays. In addition, systematic random sampling was applied for determination of FMD seropositivity in

Welmera district. The district was selected based on its accessibility, geographical location, proximity to livestock market, population density and recent outbreak. The required sample size in this district was 96 by using 6.7% previous report by (Shanko *et al*., 2015) at precision of 5% within 95% Confidence interval consideration. (Thrusfield , 1995). However, to increase the representativeness and improve precision, the sample size was increased by three folds and, a total of 312 bovine sera were collected for serological test using Chekit FMDV NSP ELISA to determine the seropositivity of FMD in the district.

3.6. Ethical Consideration

Ethical clearance for the study was given by Animal research ethical review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture. A nine page requests for explanation of the purpose of conducting the study and possible precautions to minimize suffering of animals during sampling was given to the committee. The significance of this research was evaluated from ethical perspectives, applicability, and originality technical competence point of view. Finally, the approval was given with minute's number of (VM/ERC/06/017) and Reference number of (VM/ERC/14/06/09/2017). Sampling was done following the care of animals as per the willingness of animal owners for this study.

3.7. FMD Outbreaks Investigation

3.7.1. Investigation through field observation

In order to come across with non-biased findings during outbreak investigation, frequent field assessment of the target areas was done to point out the existence of recent outbreaks within the each specific area in line with the onset dates and times of outbreaks. During field assessment, at least one case suggestive of FMD in an area was considered as an outbreak. Using this report base wise, information was collected from such particular disease affected areas and purposive sampling strategy was performed.

Therefore, field observations and exploration have been considered in the representative sampled areas of outbreaks.

3.7.2. Field level clinical examination

As soon as particular outbreak areas were identified during investigation, cattle included in this study were carefully examined for the presence and appearance of clinical signs of FMD. In each outbreak, animals that have manifested the signs of disease such as visible typical lesions on the tongue or ruptured vesicle in the oral cavity, on the feet and teat as well as excessive salivation, lameness, anorexia and rise in temperature were considered as clinically sick animals (OIE, 2012). The mouth cavities of salivating animals were opened and examined for evidence of intact or ruptured vesicles, erosions, and ulcers on the tongue, dental pad, and mucosa of the oral cavity. The hooves of lame animals were exhaustively washed with water and then wisely examined for similar lesions on the coronary bands and inter digital spaces of the hooves. Other animals in the herd without these signs were similarly examined, but sampling was done only when active lesions suggestive of FMD cases were noticed. So, a study combined active disease investigation and clinical examination in response to time of sampling that is purposive sampling (Figure 6).

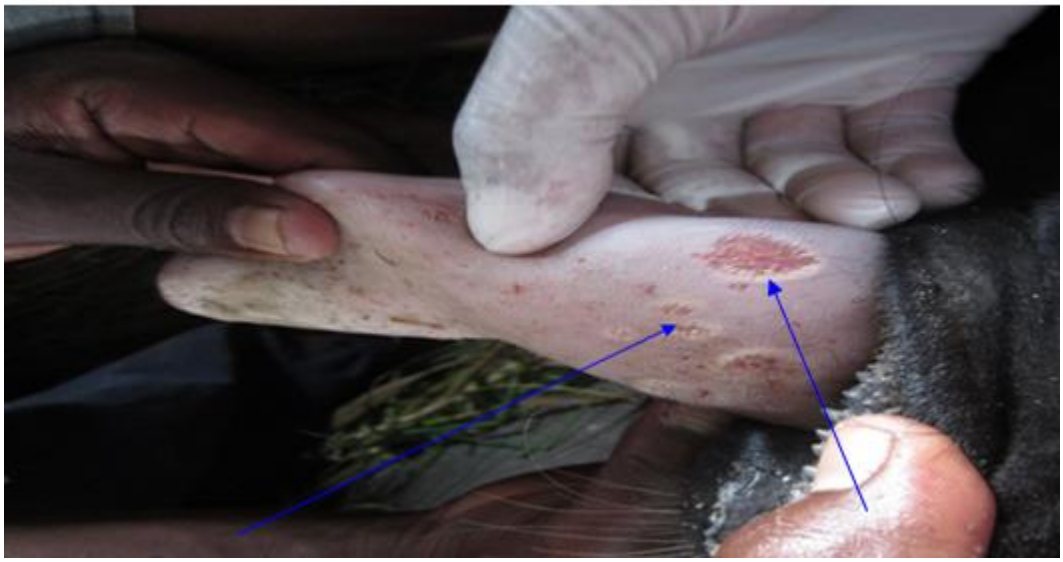

Figure 6: Tongue lesion observed on cattle infected with Foot and Mouth disease

3.8. Collection of Samples

Samples were collected from clinically sick cattle and from those which had healing lesion in the mouth, dental pad or on the feet and the asymptomatic animals in the same farm from the same farmer (Kafeero *et al.,* 2016). Different types of samples such as bovine epithelial tissues, bovine oral swab, probang sample and sera were collected.

3.8.1. Collection of bovine epithelial tissues

Representative active bovine epithelial tissues were aseptically taken from gum and tongue of clinically affected animals during the course of field outbreak to isolate the circulating viruses responsible for the occurrence of disease. Epithelial tissues were also collected from un-ruptured or freshly ruptured vesicles and lesions of the tongue, buccal mucosa were placed in a bottle with transport medium composed of equal amount of glycerol and 0.04M phosphate buffer saline (PBS) at pH 7.2–7.6 with some antibiotics and antifungal (OIE, 2004). Bovine oral swabs were also obtained from animals with clinical signs sharing the same farm with those having clinical signs. The exclusion criterion involved cattle from farms with no any animals having clinical signs and these were taken as non-cases**.** After identification of sick animals, bovine epithelial tissues and swabs were collected in the crayon vials containing virus transport medium, given a field identification number, species, sex, age, village, and type of tissue were labeled. Samples were immediately placed in a cooler containing ice and transported to National Animal Health Diagnostic and Investigation Center (NAHDHIC) Sebeta. Once the samples arrived at NAHDHIC, It was then stored at -80°C until processed and placed at −20°C until laboratory analysis**.**

3.8.2. Bovine probang samples

Probang samples were collected from previously suspected of FMD infected cows and asymptomatic cattle that were found in close proximity to the affected herd. Probang sample was collected in advance or convalescent cases by a probang cup and poured into

a 20 ml bottle. Equal amount of transport media (0.08 m phosphate buffer with pH7.2-7.4) with antibiotics and glycerol were added. The fluid was then examined and about 2 ml of it containing cellular material was added to 5 ml tube containing about 2ml of transport medium (OIE, 2012). Then, the samples were labeled and transported to NAHDIC, Sebeta and kept at -80^{o}C until ready for laboratory investigation.

3.8.3. Serum sample

About 10 ml of blood sample was collected from the jugular veins of each animal using plain vacutainer tube, and the tube containing the blood sample was kept and protected from direct sunlight and put in slant position at room temperature until the blood clotted for serum separation. The separated serum was transferred into sterile cryovials bearing the names of the owner and herd code, species, village, age, sex, date of collection and transported in ice box to NADHIC, Sebeta cell culture laboratory and then stored at -80°C for analysis (OIE, 2004).

3.9. FMD Virus Isolation and Characterization

The samples collected were processed and cultured on BHK-21 cell monolayer with three subsequent passages as follows. About 1 gram of each tissue was taken and washed three times using sterile phosphate buffered saline containing antibiotics and antifungal (PBS) on petridish. The washed tissues were transferred to sterile mortar, cut into pieces using scissor and minced by scalpel blade. The minced tissues were then grounded and homogenized in sterile sand with a sterile pestle and mortal. Nine ml of PBS was added to the homogenized tissues and well mixed as well as small volume tissue culture made and small amount of five percent antibiotics (penicillin, streptomycin and Amphotericin B solution) containing medium were added so that the final volume was ten times that of the epithelial tissue, producing of ten percent suspension (OIE, 2012).

Then, epithelial tissue suspension was transferred to test tube and then clarified by centrifugation at 3500 rpm for ten minutes. The supernatant was collected and filtered by

Millipore filter paper of 0.22 µm pore size and about 0.5ml of the supernatant or filtered tissue suspension was inoculated to baby hamster kidney (BHK-21) cells grown in25 cm^2 tissue culture flask, incubated at 37^oc with 5% CO2. The cells were observed daily for the appearance of FMD virus induced cythopathic effects (CPE). The FMD virus is fast cytocidal and CPE is characterized by a fast destruction of the monolayer cell and infected cells were disrupted and detached from the flask. Complete destruction of the cell, cell detachment and redistribution of internal cellular membrane sheet was mostly seen within 48 hrs of inoculation. When CPE appeared in the positive cases, supernatants of the homogenized clinical tissue materials were used in serotype differentiating antigen detection ELISA as per the recommended procedure (Bhattacharya *et al*., 1996) for confirmation of serotype of the virus involved in the outbreaks.

If no CPE was detected within 72 hours post infection, the cells were lysed by freezing and thawing in their growth medium at -80°C for at least one hour. Freezing and thawing were repeated for three cycles for lysis of cells and release of viruses (Xuan *et al*., 2011). The supernatants were then blindly passaged on fresh cell cultures and monitored for the presence of viral CPE for another three days. If no CPE was observed following three blind passages, the virus isolation results were declared to be negative for the presence of the virus in the sample (OIE, 2012). Once CPE was completed in the cultures, the fluids were tested for FMDV using ELISA and rRT-PCR testing. The cells were harvested when >85% of monolayer showed CPE development. The viral isolates were labeled using three-letter country code, isolate number, year of sample collection proceeded by initial letter from the study site. The three letter country codes were designated as outlined by the WRL for FMD expect at the study site initials (OIE, 2012).

3.10. Serological Diagnostic Tests

All sera were tested for the presence of antibodies produced against nonstructural proteins 3 ABC of FMD virus infection regardless of serotypes involved using a commercially available Chekit FMD 3 ABC bo-ov ELISA kit. Antibody to The assay

was performed according to manufacturer's instruction and results were analyzed and interpreted using:

$$\text{OD Value} = \frac{\text{OD sample} - \text{OD negative}}{\text{OD positive} - \text{OD negative}} \text{ X } 100$$

According to the ELISA test kit manual, the samples were categorized based on their optical density (OD values as negative if OD value < 20%, ambiguous if OD value is between 20-30 % positive if OD value is > 30%).

3.11. Serotyping of FMD Virus Isolates

Serotyping was performed by antigen detection sandwich ELISA with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies. The test was applied for detecting and typing of FMD viruses. The kit was designed for detecting and typing of FMD viruses serotypes such as 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 were also included in the kit to complement the specific typing and to detect FMD viruses which might have escaped binding to selected serotype-specific MAb. The micro plates were supplied with catching MAbs. The test was conducted as per the manufacturer's recommendation. Since six samples were needed to be tested on a microplate containing 96 wells, one positive control for each FMD types O, A, SAT1 and SAT2 and negative controls were included in each plate. These controls were already incorporated into the ELISA miroplate trapped by the respective catching MAb. First samples were diluted half in diluent buffer and 50µl of each diluted sample was distributed in 72 wells of A-F rows and two replicates of each-specific catching MAb and for the pan-FMDV MAb. Then, 50µl of diluents per well were added in all wells of G and H rows (positive and negative control, respectively) , then plates were incubated at 25^{o}C for 1hour. After incubation, all fluids on the plates were discarded and the remaining residual fluids were removed. Then 200µl of washing solution were added and incubated for 3min at room temperature, subsequently wells were emptied and the washing repeated twice (three washing cycles in total). Then all residual fluids were removed by tapping on clean absorbent paper and 50µl of conjugate

A was added from columns 1 to 8 and the same volume of conjugate B was added from columns 9 to 12. 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 20 minutes in the dark. The reaction was stopped by adding 50µl of stop solution (sulfuric acid (H2SO4)). Immediately after stopping, reading the optical density (OD) of each well was done at 450 nm wavelength using micro plate reader.

Criteria for test validity: The positive controls were expected to give OD values of 1.0 unit or higher in the type-specific reactions and in the pan-FMDV reaction, the negative control usually gives OD values lower than 0.1 in wells H1 to H8 and slightly higher in wells H9 to H12. The interpretations of the results of examined samples were indicated in (Appendix-8).

3.12. Molecular Characterization of FMD Virus

3.12.1. FMD viral RNA extraction

The cell culture grown viruses were used for RNA extraction that was amplified by RT-PCR using primers that can amplify the VP1 (Knowles and Samul, 2003). The presence of FMD viral genetic material was detected using rRT-PCR. The rRT-PCR was used to amplify genome fragments of FMD virus in diagnostic materials including epithelium, serum and probang samples (Amarel *et al*., 1993). Total RNA was extracted from 140 micro-liter original bovine epithelail tissues and oral swab suspension using Qiagen RNA extraction kit following manufacturer's instructions as (Kafeero *et al* ., 2016). Briefly, 140 mirco liters of original epithelail tissues and swab suspension was added to 560µl buffer AVL carrier RNA in the mirco centrifuge and vortexed for 15 sec to mix and then incubated at room temperature (25^0c) for 10 minutes. The tube was briefly centrifuged to remove drops from the inside of the lid. Then 560µl of ethanol (96%) was added to the sample and mixed by pulse vortexing for 15 seconds followed by brief centrifuging to

remove drops from the inside lid. Then 630µl of the solution were applied to the QIAMP Mini column in a 2ml collection tube and centrifuged at 6000g (8000rpm) for 1 minute. The filtrate was discarded and the column was placed in a fresh 2ml collection tube. Then 500µl of buffer AW2 were added to the column then centrifuged at 20,000 ×g (14,000 rpm) for three minutes and the filtrate was discarded. Then 65µl of Buffer AVE was added to the column equilibrated at room temperature for one minute then centrifuged at 6000g (800rpm) for 1 minute. Then after RNA sample was used for detection and molecular characterization.

3.12.2. Detection of viral RNA in field samples by rRT- PCR testing

RNA was also extracted from clinical samples and tested by real time PCR method targeting universal 3D regions of FMD virus specific primers whose forward primer sequence (5′- ACT GGG TTT TAC AAA CCT GTGA-3’) and reverse primer 5′ ˉGCG AGT CCT GCC ACG GA -3′) to determine the presence of viral RNA in clinical samples (Callahan *et al*., 2002). The cycle threshold or crossing point (Ct value) corresponds to the number of cycles required for a given sample to reach the threshold above is considered as positive. Using a positive cut-off cycle threshold (Ct) value of 32.0 (Shaw *et al*., 2007), FMDV genome was detected by rRT-PCR. The PCR cyclical cut-off point lies < 32 as strong FMDV positive sample while > 32 indicates negative samples.

3.12.3. Phylogenetic analysis

The PCR product of cell culture grown viruses VP1 were sequenced using Sanger method in WRL for FMD UK (Table 5). The generated sequences were used for phylogenetic tree reconstruction (Mishamo, 2016) and grouping of viruses according to their phylogenetic relationships using molecular evolutionary genetic analysis software (MEGA V-6; Tamura *et al*., 2013).

Table 5: Viral isolates submitted to WRL for confirmatory diagnosis and molecular characterization

Outbreak areas	Pas/kebeles	Species	Date of sampling	Type of samples
	Maru chobot	Cattle	31-10-2016	Epithelial tissues
Adea Berga	Bishan Dimo	Cattle	31-10-2016	Epithelial tissues
	Sire Berga	Cattle	31-10-2016	Epithelial tissues
Welmera	Menagesha kolobo	Cattle	11-11-2016	Probang sample
Addis Ababa	Kolfe keranyo	Cattle	18-11-2016	Epithelial tissues

3.14. Data Analysis and Management

Data generated from laboratory investigations were recorded and coded using Microsoft Excel spreadsheet and analyzed using STATA version 13 for Windows (Stata Corp. College Station, TX, USA) and Statistical Analysis System (SAS version 9). Odd ratio (OR) was used to assess the degree of association of risk factors with disease occurrence as indicated by 95% confidence intervals. Stepwise logistic regression model was used to analyze and regress those factors having significant putative effect on the occurrence of disease based up on p value < 0.05 as significance threshold for entries and removals. The association of potential risk factors (age, sex, breed type,) with FMD infection was computed by Pearson's chi square test. Cell culture results, CPE development and molecular characterization results were recorded and tabulated. The morbidity due to FMD was calculated by dividing the number of infected animals by the total animals examined in the study area, then multiplied by 100. Mortality was also calculated as the number of died animals during outbreak in the study area divided by the total animals included in the study multiplied by 100 (Dana *et al.*, 2001). In all the cases, 95% Confidence limit and P Values <0.05 was set for detecting statistically significant findings.

4. RESULTS

4.1. Findings from FMD Outbreaks

Among 586 animals observed in six districts during outbreaks, 226 (38.6%) of cattle were found to be clinically infected with FMD. The observed clinical signs in sick cattle were vesicle formations on oral cavity, erosions, profuse salivation, inter digital vesicles and lameness that were suggestive of FMD cases. The analysis of the outbreak statistics also indicated that out of 352 animals examined at Adea Berga dairy farm at the time of FMD outbreak, 130 clinical cases and 11 dead calves were encountered (Table 6).

Table 6: Summary of FMD outbreaks recently occurred in different areas.

Outbreak districts	Kebele level Outbreaks	No. of animals examined	Number of animals affected	No. of death occurred
Adea Berga	Maru cobot	51	20	-
	Bishan Dimo	19	8	-
	Sire berga	15	5	
	Adea berga dairy farm	352	130	11
Welmera	Menagesha kolobo 01	50	20	-
Addis Ababa	Kolfe keranio 01	20	7	-
Wuchale	Machala wartu	12	5	-
Mulo	Amuma Bubisa Dumburi	20	6	-
	Mulo siro	11	5	-
Kimbibit	Mogor gara daga	36	20	-
Total		**586**	**226 (38.6%)**	**11**

4.2. Risk Factor Analysis on FMD Outbreak at Adea Berga Dairy Farm

Upon clinical examination during outbreak, risk factor analysis showed that the overall morbidity, mortality and case fatality at Adea Berga dairy farm were 36.9%, 3.12% and 8.46% respectively (Table7 and 8).

4.2.1. Age related difference in the occurrence of FMD

The morbidity, mortality and case fatality were relatively higher in calves less than three months of age than other age groups (67.6%, 15.5%, 22.9%), respectively and this was found to be statistically significant ($\chi 2 = 38.4$; $P = 0.0001$) (Table 7 and 8).

4.2.2. Sex related difference in the occurrence of FMD

Results from the outbreak analysis also showed that morbidity in male and female animals was 73.3% and 31.6% respectively. There was statistically significant variation between sex categories ($p<5\%$) (Table 7).

4.2.3. Breed related difference in the occurrence of FMD

The breed specific morbidity was relatively higher in Jersey breed (38%) than Holstein Friesian breed (34%) but not statistically significant ($p>0.05$) (Table 7).

4.2.4. Body condition related difference in the occurrence of FMD

The morbidity proportion in different body condition scores were 63.6%, 34.2% and 34% in poor, moderate and good body conditioned animals respectively. The disease caused 15.2% mortality and 23% case fatality in poor body conditioned animals (Table 7 and 8).

Table 7: Association of putative risk factors with Calf morbidity and Mortality at Adea Berga dairy farm during FMD outbreak

Variables	No. examined	No. Sick	Morbidity percentage (%)	95% CI	χ2 (P-value)
Breed					
HF	94	32	34	25.6-44.5	0.46 (0.29)
Jersey	258	98	38	32-44.2	
Sex					
Female	307	97	31.6	26.4-37.1	29.4 (0.0001)
Male	45	33	73.3	58.1-85.3	
Age (M)					
<3	71	48	67.6	55.45-78.2	38.9 (0.001)
3-18	165	55	33.3	26.2-41.1	
> 18	116	27	23.3	15.9-32	
Body Condition					
Good	97	33	34.0	24.7-44.3	11.2 (0.004)
Moderate	222	76	34.2	28-40.8	
Poor	33	21	63.36	45.1-79.6	
Over all (%)	**352**	**130**	**36.9**	**31.8-42.2**	

M-Age in month, HF-Holstein Friesian

Table 8: Calf mortality and case fatality of calf at Adea Berga dairy farm

Variables	No. examined	No. Sick	No. died	Mortality (%)	95% CI	CF	95% CI
Age (M)							
< 3	71	48	11	15.4	7.9-26	22.9	12-37.3
3-18	165	55	-	-	-	-	-
>18	116	27	-	-	-	-	-
Body Condition							
Good	97	33	2	2.06	0.25-7.25	6.06	0.74-20.2
Moderate	222	76	4	1.8	0.49-4.54	5.26	1.45-12.9
Poor	33	21	5	15.2	5.1-31.9	23.8	8.21-47.2
Over all	**352**	**130**	**11**	**3.12**	**1.57-5.57**	8.46	4.29-14.6

CF-Case fatality , M-Age in month

The result showed that male animals were found to die (11.1%) more than female animals (2%) (Figure 7).

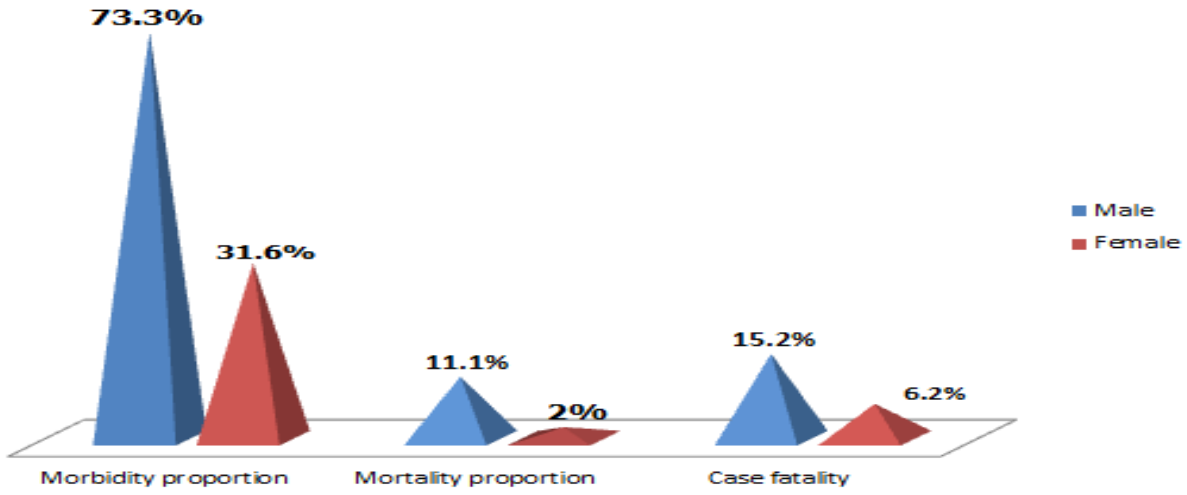

Figure 7: Sex wise morbidity, mortality and case fatality in Jersey breed at Adea Berga dairy farm

The mortality and case fatality were higher in Jersey breed of cattle (3.5%) and (9.18%) than in Holstein Friesian breed (2.1% and 6.25%), respectively (Figure8).

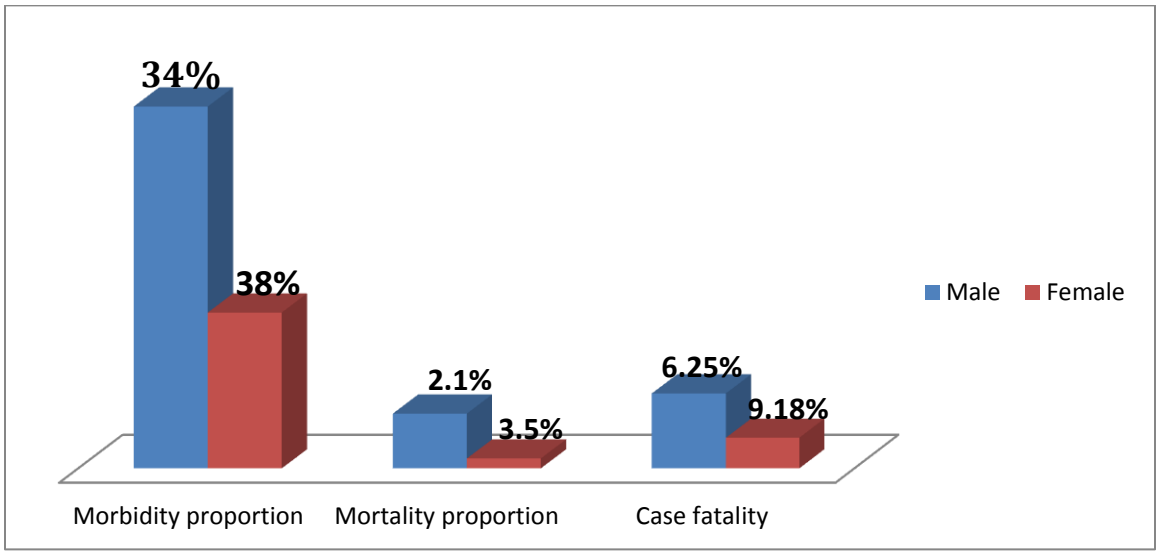

Figure 8: Breed wise mortality and case fatality in Jersey breed at Adea Berga state dairy farm

4.2.5. Stepwise selection model logistic regression analysis for FMD outbreaks at Adea Berga dairy farm

The results from analysis of the putative risk factors revealed that male animals were 3.2 times more likely to be infected by FMD infection (OR = 3.26, 95% CI: 1.52-8.59) than females. The results also indicated that the risk of FMD infection was increased by 4.1 times (OR = 4.12, 95% CI: 1.925-8.81) in calves less than three months of age than Adult cattle. Poor body conditioned cattle were found to be infected with FMD three times than good body conditioned (Table 9).

Table 9: Stepwise logistic regression analysis for the degree of association between risk factors and occurrence of FMD

Effects observed	**Effect category**	**No. of examined**	**No. of infected animals (%)**	**OR**	**95% wald CI for OR**	**Wald chi square**	**P value**
Sex	Male	45	33(73.3)	3.26	1.52-8.59	5.32	0.021
	female	307	97(31.6)	1.0			
Age (M)	< 3	71	48(67.6%)	4.12	1.925-8.81	13.42	0.001
	> 18	116	27(23.3)	1.0			
Body condition	Poor	97	33(63.3)	3	1.224-7.35	5.93	0.016
	Good	33	21(34)	1.0			

M - Age in month, OR- odds ratio, CI- confidence interval

4.3. Necropsy Findings during FMD Outbreak

Upon post mortem examination, the necropsy findings indicated grossly prominent lesions in the heart of calves which became dilated and cooked in appearance. The typical symptom of tigroid heart appearance was observed in heart of calf (Figure 9).

Figure 9: Death of Jersey calf at Adea Berga state dairy farm during FMD outbreak in October; 2016.

Careful postmortem examination inside the heart indicated lesions. Small foci of necrosis at the base of heart and friable areas were appeared as cooked and pale in color Figure 10).

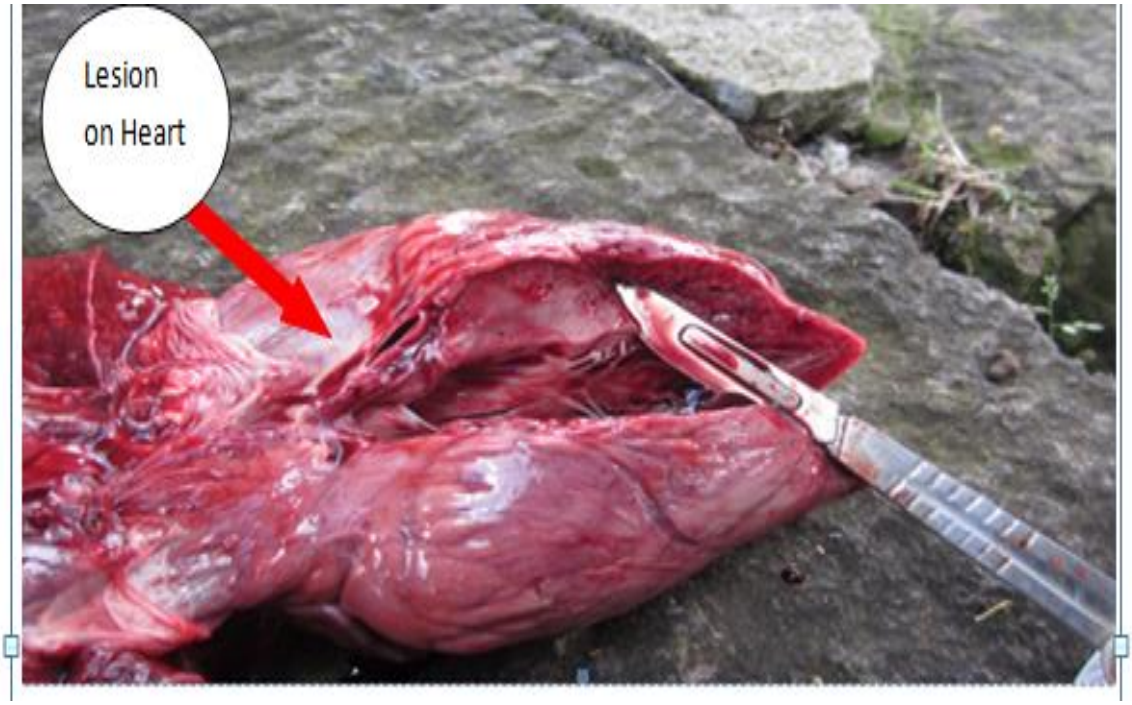

Figure 10: Lesions inside the heart of died calf

4.4. FMD Seropositivity

A total of 312 bovine sera were tested by CHEKIT FMD 3ABC Bo-Ov ELISA test for detection of antibody to 3ABC NSP of FMD virus of which , 30.8 % (n=96) cattle were found to be positive and the remaining were negative for the test. The results also indicated that seropositivity of FMD was observed to be higher in females (38.5%) than in males (15.8%) and this variation was statistically significant ($\chi2$= 16.93, p = 0.001). The associations of seropositivity of FMD in relation to body condition score, management system and animal breed were statistically significant ($\chi2$= 7.54, p = 0.023, $\chi2$= 26.7, p = 0.00, $\chi2$= 24.7, p = 0.001), respectively. Seropositivity was found to increase as age increases, however; this age group model was not statistically significant (Table 10).

Table 10: FMD seropositivity and associated risk factors by using chekit FMD 3ABC Bov-Ov ELISA test

Risk factors	Category	No. of Bovine sera tested	Seropositivity of FMD (%)	χ2	P value
Sex	Male	107	17(15.8)	16.93	0.001
	Female	205	79(38.5)		
Age	Young	103	28(27.2)	0.93	0.203
	Adult	209	68(32.5)		
BCS	Good	80	15(18.8)	7.54	0.023
	Moderate	85	28(32.9)		
	poor	147	53(36.1)		
Management system	intensive	113	55(48.7)	26.7	0.001
	Semi intensive	199	41(20.6)		
Breed	Cross	130	60(46.2)	24.7	0.001
	Local	182	36(19.8)		
Over all proportion (%)		**312**	**96 (30.8)**		

BCS- Body condition score

The logistic regression analysis identified that breed, sex, body condition and management systems were statistically significant (Table 11). Cross bred cattle were 3.5 more likely to be infected by FMD than local breeds (OR = 3.47, 95% CI: 2.10 – 5.74). The risk of FMD occurrence was also increased by 3.3 times (OR = 3.3, 95% CI: 1.84 – 5.98) in female animals than males. The results also showed that animals with poor body condition were2.4 times more FMD seropositive than animals with good body condition. Cattle that were kept under intensive management system were 3.6 times more prone to FMD infection than those managed semi intensively (OR= 3.65, 95% CI: 2.21-6.05) (Table 11).

Table 11: Logistic regression Analysis of FMD Seropositivity with various risk factors

Variables	Level of variables	OR	95% CI for OR	P value
Breed	Cross vs local	3.47	2.10-5.74	0.001
Sex category	Female vs Male	3.3	1.84-5.98	0.001
Management system	Intensive vs semi intensive	3.65	2.21-6.05	0.002
BCS	Poor vs Good	2.4	1.27-4.70	0.04

OR-Odd ratio, BCS-Body condition score, CI= confidence interval

4.4. FMD Virus Isolation and Characterization

Different types of samples (bovine epithelial tissues, oral swab and bovine probangs) that were collected from outbreak cases were inoculated in BHK-21 cell culture with three subsequent passages for virus isolation. The culture results indicated that out of 37 clinical samples processed and cultured, 56.8% (n=21) were isolated and exhibited morphological alterations (FMDV cytopathic effect (CPE) on BHK21 cell. The FMD virus CPE was observed on bovine-epithelial tissues collected from Adea Berga district, 5(13.5%) and the isolated virus were (ETH/02/2016, ETH/12/2016) while bovine probang sample was 1(2.7%) and the isolated virus was (ETH/3/2016). Similarly, bovine epithelia tissues and probang samples collected from outbreak cases at Addis Ababa Kolfe Keranyo showed cytopathic effect. Moreover, probang samples and bovine epithelial tissues collected from outbreaks at Wucale, Welmera and Kimbibit districts were isolated in BHK21 cell. The cell culture results also showed that bovine epithelial tissues collected from outbreaks occurred at Mulo district were isolated in the BHK-21 cell and the isolates that showed CPE was (ETH/11/2016). The current results indicated that virus isolated from clinical samples on BHK21 cell showed CPE which was characterized by a fast destruction of BHK-21 monolayer cell and infected cells were round (Table 12).

Table 12: FMD virus isolated on BHK21 cell culture from outbreak cases and the status of Viral CPE in different areas

Isolates code	District	Kebele level OBs	Date of OB onset	SPP	B	Date of sampling	Type of sample	Status of CPE on BHK21 cell and Name of the virus	
								With CPE development	Without CPE
Fl03	Mulo	A.B.dumburi	20/11/016	B	L	23/11/016	BET and OS	CPE detected, ETH/1/017	-
Fl5	Mulo	A.B.dumburi	20/11/016	B	L	23/11/016	BPS	CPE-detected, ETH/1/017	-
Fl3	Mulo	A.B.dumburi	20/11/016	B	L	23/11/016	BPS	-	ETH/1/017
FC3	Mulo	Mulo siro	20/11/016	B	C	24/11/016	BPS	CPE detected , ETH/12/016	-
FC2	Mulo	Mulo siro	20/11/016	B	C	24/11/016	BPS	-	ETH/12/016
FH5	AA	Kolfe keranio	15/11/016	B	C	18/11/016	BPS	CPE detected , ETH/02/017	-
FH6	AA	Kolfe keranio	15/11/016	B	C	18/11/016	BPS	-	ETH/01/017
FH1	AA	Kolfe keranio	15/11/016	B	C	18/11/016	BET and OS	CPE detected , ETH/02/017	-
FH2	AA	Kolfe keranio	15/11/016	B	C	18/11/016	BPS	CPE detected , ETH/02/017	-
Fm6	Wecale	Macala wartu	17/11/016	B	C	20/11/016	BET and OS	CPE detected , ETH/12/016	-
F11	Wecale	Macala wartu	17/11/016	B	C	20/11/016	BPS	-	ETH/12/016
Fm3	Wecale	Macala wartu	17/11/016	B	C	20/11/016	BPS	CPE detected , ETH/12/016	-
FB	Kimbibt	M.gara daga	14/12/016	B	L	18/12/016	BET	CPE detected , ETH/11/016	-
F6B	Kimbibt	M.gara daga	14/12/016	B	C	18/12/016	BET	-	ETH/12/016
F2B	Kimbibt	M.gara daga	14/12/016	B	C	18/12/016	BPS	-	ETH/12/016
F51	Kimbibt	M.gara daga	14/12/016	B	L	18/12/016	BET	-	ETH/12/016
FT1	welmera	M.kolobo	9/11/016	B	C	11/11/016	BPS	-	ETH/12/016
FT6	welmera	M.kolobo	9/11/016	B	C	11/11/016	BPS	-	ETH/12/016
FT9	welmera	M.kolobo	9/11/016	B	C	11/11/016	BET	CPE detected , ETH/11/016	-
FB1	A.Berga	Maru cobot	27/10/016	B	L	31/10/016	BET	CPE detected , ETH/11/016	-
FB8	A.Berga	Maru cobot	27/10/016	B	C	31/10/016	BET	CPE detected , ETH/11/016	-
FB3	A.Berga	Maru cobot	27/10/016	B	C	31/10/016	BET and OS	-	ETH/11/016
F23	A.Berga	Maru cobot	27/10/016	B	C	31/10/016	BET	-	ETH/12/017
F28	A.Berga	Maru cobot	27/10/016	B	C	31/10/016	BET	CPE detected ,ETH/11/016	-
F54	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BET and OS	-	ETH/11/016
F16	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BET and OS	-	ETH/11/016
F15	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BET	CPE detected , ETH/11/016	-
F07	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BPS	CPE detected , ETH/11/016	-

Table 12 (*Continued*)

Isolate code	District	Kebele level OBs	Date of OB onset	SPP	B	Date of sampling	Type of sample	Status of CPE on BHK21 cell and Name of the virus	
								With CPE development	Without CPE
F89	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BET	CPE detected , ETH/11/016	-
F10	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BET and OS	CPE detected , ETH/11/016	-
F15	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BOS	CPE detected , ETH/11/016	-
F70	A.Berga	Maru cobot	23/10/016	B	C	26/10/016	BOS	CPE detected , ETH/11/016	-
FU6	A.Berga	Sire berga	8/11/016	B	L	11/11/016	BPS	CPE detected , ETH/12/016	-
FU4	A.Berga	Sire berga	8/11/016	B	L	11/11/016	BET	-	ETH/12/016
FU3	A.Berga	Sire berga	8/11/016	B	L	11/11/016	BET	-	ETH/12/016
FE4	A.Berga	Bishan dimo	27/10/016	B	L	31/10/016	BET and OS	CPE detected , ETH/12/016	-
FE2	A.Berga	Bishan dimo	27/10/016	B	L	31/10/016	BET	-	ETH/12/016
Overall culture results							**37**	**21(56.8%)**	**16(43.2%)**

BES= Bovine epithelial tissues; Bos=Bovine oral swab; BET and OS-Bovine epithelial tissues and oral swab; B=Bovine; L=local; C= Cross; CPE= Cytopathic effect; SPP=species; OB=Outbreak

4.5. Characterization of CPEs of FMD Virus in Cell Culture

The prepared inoculum was inoculated into BHK21 cell to appreciate the development CPE of the virus. Most of the isolated virus that showed complete CPE was characterized by inducing fast destruction of BHK-21 monolayer cell and infected cells were round, swelling and formed singly. As time progress, there was sloughing of cells or monolayer detachment from the wall of cell culture flask and some cells were severely damaged within 72 hrs post inoculation and finally cell death that indicates the presence of virus. But samples that did not show CPE do not induce morphologic changes of cell. Complete destruction of the cell sheet was mostly seen within 48 hours of inoculation. The Figure indicated below illustrates the rounding and lysis (CPE) of BHK21 cells inoculated with FMD virus and uninfected cells (Figure 9).

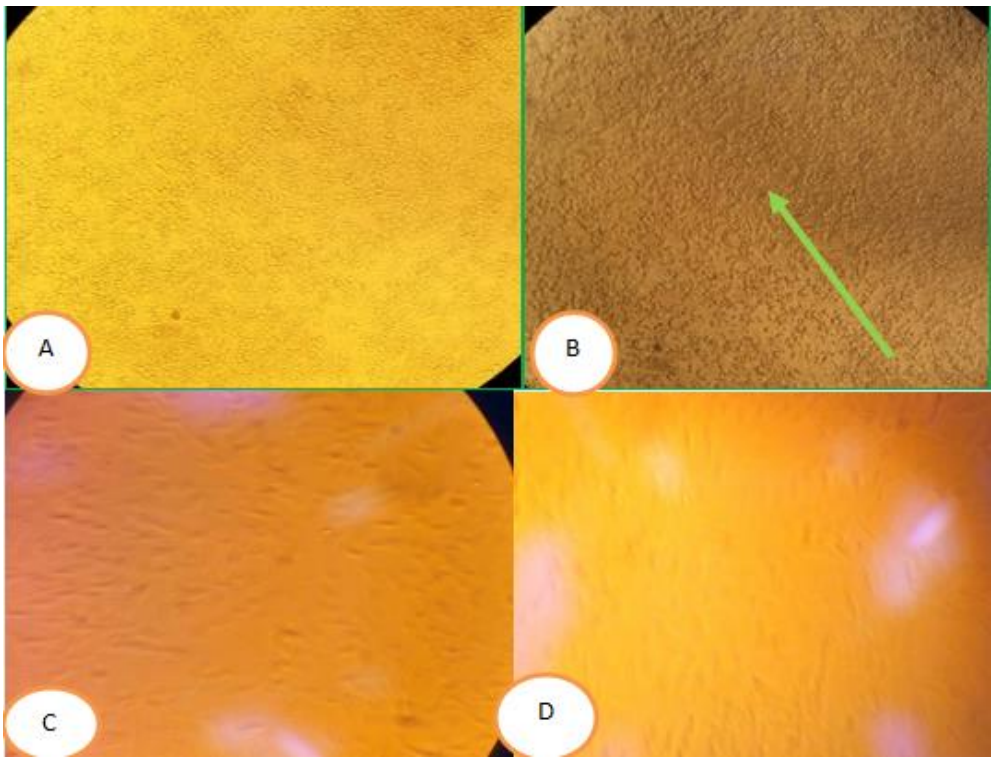

Figure 11: Characteristic CPE of FMDV inoculated on BHK21 cells exhibiting morphologic changes. (A and B) shows rounding and cell detachment from the flask and lysis of cells (indicated by arrow) against respective controls (C and D).

4.6. Serotyping of FMDV Isolates from Field Outbreak Cases

Out of 41 samples subjected for serotyping by sandwich ELISA, 16 (39.02%) samples were found positive and four types of serotypes were detected. The recent outbreaks were predominantly caused by FMDV O serotype (34.2%), followed by serotype A (17.1%), serotype SAT1 (4.9%) and serotype SAT2 (2.4%). Furthermore, bovine epithelial tissues collected during outbreak at Adea Berga district were serotyped into type O, A and SAT1 whereas probang samples collected from clinical cases of the same district were serotyped into type O field strain, respectively. Bovine epithelial tissues collected from outbreak of Welmera district were serotyped into type O and A field strains. However, probang samples collected from Addis Ababa Kolfe Keranyo were positive for serotype O. The current findings also showed that Mulo and Kimbibit districts had outbreaks due to FMDV serotype SAT2 and SAT1 strain, respectively. On the other hand, samples collected from Adea Berga and Welemra districts were found as positive for dual infection having Type O, A and SAT1 FMDV and this showed that the serotypes were circulating in the districts during the study period (Table13).

Table 13: Serotyping results of FMD virus isolates at NADHIC, Sebeta Ethiopia

Code	Outbreak sites	Kebele level Outbreaks	Date of OB onset	SPP	B	Date of sampling	Types of samples	Serotyping results	
								Serotypes detected	Not detected
F1454	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BET and OS	-	NVD
F216	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BET and OS	-	NVD
F215	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BET	O, A	-
F216	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BP	O	-
F1489	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BET	O,A	-
F1507	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BET and OS	O, A	-
F12	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BP	-	NVD
FAB1	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BOS	O, A	-
F470	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BP	O, A	-
F4	Adea berga	Maru cobot	23/10/2016	B	C	26/10/2016	BP	-	NVD
FB11	Adea berga	Maru cobot	27/10/2016	B	L	31/10/2016	BET	O, SAT1	-
FB8	Adea berga	Maru cobot	27/10/2016	B	C	31/10/2016	BET	O	-
FB3	Adea berga	Maru cobot	27/10/2016	B	C	31/10/2016	BET and OS	-	NVD
FB23	Adea berga	Maru cobot	27/10/2016	B	C	31/10/2016	BET	-	NVD
FB28	Adea berga	Maru cobot	27/10/2016	B	C	31/10/2016	BET	O	-
FU6	Adea berga	sire berga	8/11/2016	B	L	11/11/2016	BP	O, A	-
FU4	Adea berga	sire berga	8/11/2016	B	L	11/11/2016	BET	-	NVD
FU3	Adea berga	sire berga	8/11/2016	B	L	11/11/2016	BET	-	NVD
FBE4	Adea berga	Bishan dimo	27/10/2016	B	L	31/10/2016	BET and OS	O	-
FB	Adea berga	Bishan dimo	27/10/2016	B	L	31/10/2016	BET	-	NVD
FBE2	Adea berga	Bishan dimo	27/10/2016	B	L	31/10/2016	BET	-	NVD
FT10	welmera	M-kolobo	9/11/2016	B	C	11/11/2016	BP	-	NVD
FT6	welmera	M-kolobo	9/11/2016	B	C	11/11/2016	BP	-	NVD
FT9	Welmera	M-kolobo	9/11/2016	B	C	11/11/2016	BET	O, A	-
FT	Welmera	M-kolobo	9/11/2016	B	C	11/11/2016	BET	-	NVD
FH5	AA	Kolfe keranio	15/11/2016	B	C	18/11/2016	BP	O	-

Table 13 (*Continued*)

Code	Outbreak sites	Kebele level Outbreaks	Date of OB onset	SPP	B	Date of sampling	Types of samples	Serotyping results	
								Serotypes detected	**Not detected**
FH6	AA	Kolfe keranio	15/11/2016	B	C	18/11/2016	BP	-	NVD
FH1	AA	Kolfe keranio	15/11/2016	B	C	18/11/2016	BET and OS	-	NVD
FH2	AA	Kolfe keranio	15/11/2016	B	C	18/11/2016	BP	O	-
FM6	wechale	Macala wartu	17/11/2016	B	C	20/11/2016	BET	-	NVD
FM11	wechale	Macala wartu	17/11/2016	B	C	20/11/2016	BP	-	NVD
FM3	wechale	Macala wartu	17/11/2016	B	C	20/11/2016	BP	-	NVD
FLo3	Mulo	A.B. dumburi	20/11/2016	B	L	23/11/2016	BET and OS	SAT2	-
FL5	Mulo	A.B. dumburi	20/11/2016	B	L	23/11/2016	BP	-	NVD
FC3	Mulo	A.B. dumburi	20/11/2016	B	C	23/11/2016	BP	-	NVD
FL3	Mulo	A.B. dumburi	20/11/2016	B	L	23/11/2016	BP	-	NVD
FC2	Mulo	A.B. dumburi	20/11/2016	B	C	23/11/2016	BP	-	NVD
F55BT	kimbibit	M. gara daga	14/12/2016	B	L	18/12/2016	BET	SAT1	-
F2BH	kimbibit	M. gara daga	14/12/2016	B	C	18/12/2016	BET	-	NVD
F52B	kimbibit	M. gara daga	14/12/2016	B	C	18/12/2016	BP	-	NVD
F51	kimbibit	M. gara daga	14/12/2016	B	L	18/12/2016	BET	-	NVD
Total							**41**	**16 (39%)**	25

Proportion of serotypes; **O**, 14 (34.2%), **A:** 7(17.1%), **SAT1**: 2(4.9%), SAT2: 1(2.4%)

AA-Addis Ababa; OB-outbreak; A.B-Amuma Bubisa; M-Mogoro; Spp-species; B- Bovine; C-cross; L-local; BET-bovine epithelial tissue, Os-oral swab; BP-bovine probang; NVD-No virus detected

In proportion, serotype O was accounted for 34.2%, A, 17.1%, SAT1 4.9% and SAT 2 was 2.4% (Figure 12).

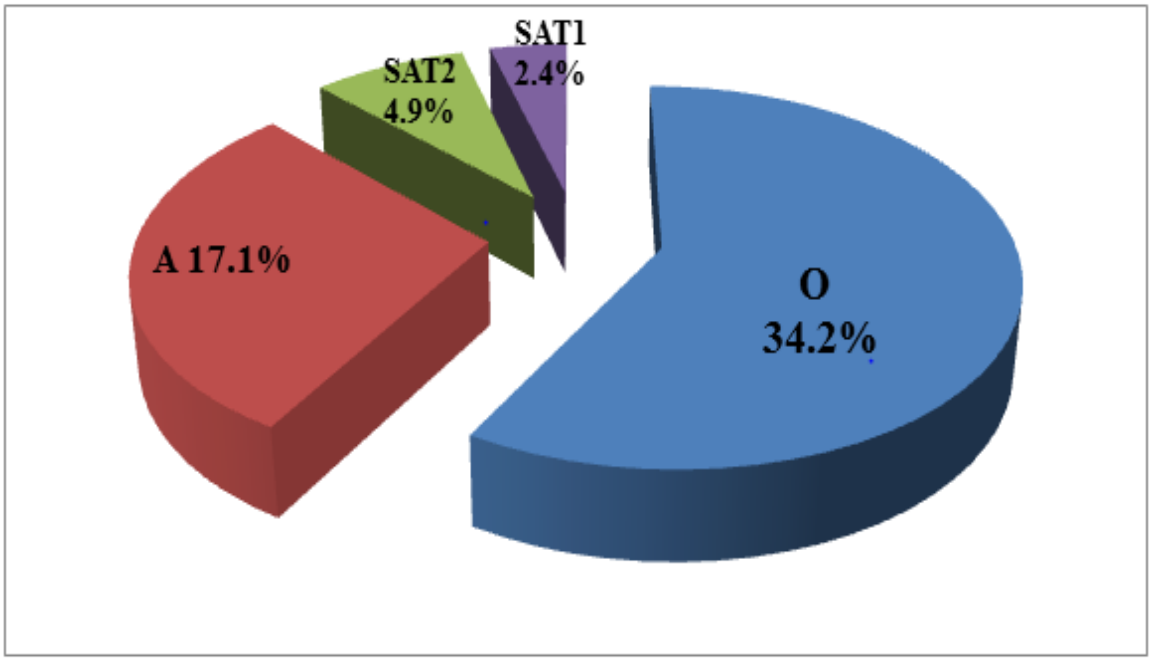

Figure 12: FMD virus serotypes identified from outbreak cases

4.5. Molecular Characterization of FMD Virus Isolates

4.5.1. Detection of viral RNA by rRT- PCR testing

The presence of FMD viral genetic material was detected using rRT-PCR method. The rRT-PCR was used to amplify and detect genome fragments of FMD virus in clinical materials including epithelium, serum and probang samples (Amarel *et al*., 1993). The extracted RNA from clinical samples was tested by real time RT-PCR method targeting the 3D regions of FMD virus genome to determine the presence of viral RNA in clinical samples (Callahan *et al*., 2002). The Ct 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 Ct value of 32.0 (Shaw *et al*., 2007), FMDV genome was detected by rRT-PCR with Ct values. The PCR cyclical threshold values Cut-off point lies < 32 as strong FMDV positive sample while > 32 indicates negative samples.

Out of 50 samples tested by RT- PCR method, 9 (18.1%) samples were positive for FMDV genome with Ct values ranging from 16.03 to 26. 98. The remaining samples that did not show Ct values were considered to be no virus detected (NVD). The lowest Ct value of 16.03 was recorded from bovine epithelial tissues whilst the highest Ct value (26.98) was recorded in bovine probang. Samples collected from Addis Ababa Kolfe Keranyo district showed lower Ct values than those collected from other districts. The Ct values ranged between 16.03 and 26. 98, indicating that higher levels of viral RNA in the samples collected from Adea Berga district than those collected from the Welmera district (Table 14). Lower Ct values were observed for most epithelial tissues than those from the probang tissues, indicating that there were higher levels of viral RNA in the epithelial tissues than in the OP samples (Table14) and (Figure 13).

Table 14: RT-PCR results obtained from suspected FMDV samples collected from cattle

Isolates code	Outbreak district	Sample types	Results		rRT-PCR result
			RT-PCR	rRT-PCR CT values	
F-L02B	Mulo	BP	+ve	18.39	FMDVGD
F-M1B	Wuchale	BP	+ve	26.98	FMDVGD
F-58B	kimbibit	ET	+ve	18.81	FMDVGD
F-Be11	Adea Berga	ET	+ve	16.03	FMDVGD
F-53B	kimbibit	ET	+ve	22.16	FMDVGD
F-58B	Adea Berga	ET	+ve	19.77	FMDVGD
F-57B	AA* .kolfe keranyo	BP	+ve	19.23	FMDVGD
F-H6A	AA*. kolfe keranyo	ET	+ve	16.83	FMDVGD
F-Lo1M	Mulo	ET	+ve	21.2	FMDVGD

AA*- Addis Ababa, FMDVGD- FMD viral genome detected, Ct- cycle threshold values, BP- Bovine probang, ET-Epithelial tissue; +ve- positive by RT-PCR; Samples with CT values of < 32 is considered positive and >32 is negative.

The representative samples from outbreak cases with real time results of Ct values < 32 were considered as strong FMD positive samples (Figure 11). The successfully amplified real time RT-PCR product gave an amplification curve and the Ct values at which the target samples were initially detected above the background fluorescent levels. Sample computer out of the Ct values FMD samples were shown in figure (13).

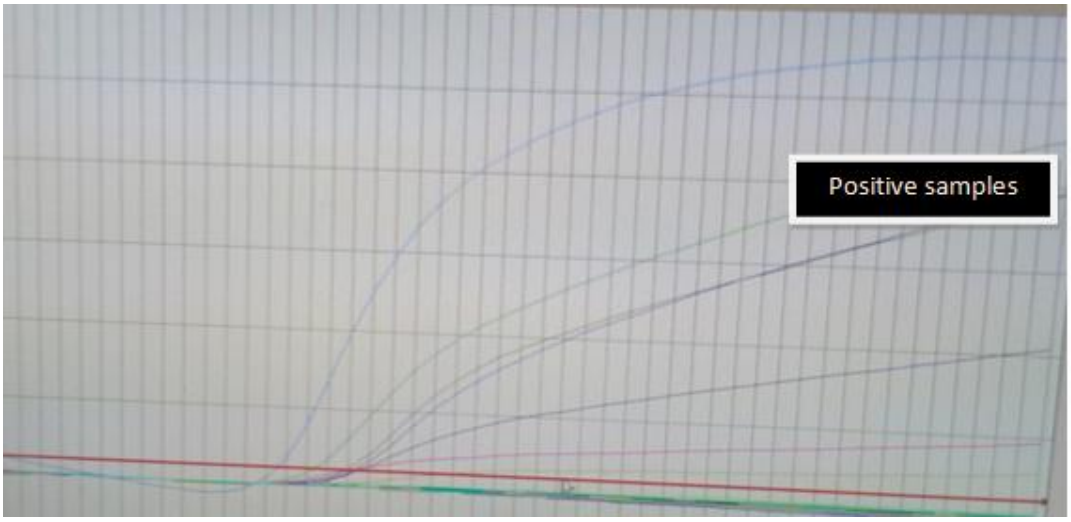

Figure 13: Amplification curves of FMD positive samples**.** Red color shows cut-off of point for Ct value positivity and negativity.

4.5.2. Phylogenetic analysis

The VP1 capsid of FMD virus genome sequences obtained from the Pirbright Institute, UK was used for reconstructing phylogenetic relationships of FMD virus isolates with reference viruses downloaded from Genbank to detect origins of FMD virus epizootics. Viruses from different topotype and published before were used for this purpose. Accordingly the Ethiopian isolates belonged to east African topotype 4 viruses (Figure 15)

4.5.3. Confirmation of FMD virus in suspected samples at WRL

From epithelial tissues and probang samples collected during FMD outbreaks in the study areas, serotype O FMD virus which falled under topotype EA-4 was identified at World Reference Laboratory for FMD (WRLFMD). The presence of FMD virus genetic material in the epithelial tissues and probang samples were also detected and confirmed by RT-PCR (Table 15).

Table 15: Results of FMD virus serotypes and topotype identified at WRL, Pirbright

Sample reference name	**Outbreak sites**	**Kebeles**	**Types of sample**	**Serotyping result**	**RT-PCR result**	**Topotype identified**
ETH/18/2016	AB	Maru chobot	ET	O	FMDVGD	EA-4
ETH/09/2016	AB	Bishan Dimo	ET	O	FMDVGD	EA-4
ETH/12/2016	AB	Sire Berga	ET	O	FMDVGD	EA-4
ETH/11/2016	Welmera	MK	Probang	O	FMDVGD	EA-4
ETH/13/2016	AA	Kolfe keranyo	ET	O	FMDVGD	EA-4

FMDV GD- FMD virus genome detected, EA-4 (East Africa Topotye 4), ET-Epithelial tissue, AA-Addis Ababa, AB-Adea Berga, MK-Menagesha Kolobo

4.5.4. Phylogenetic tree reconstruction

The VP1 capsid of FMD virus genome sequences obtained from the pirbright Institute was characterized by reconstructing phylogenetic relationships of FMD viruses with reference virus that have been downloaded from gene bank to detect origins of FMD virus epizootics. The VP1 gene characterization was used to study phylogenetic relationships between serotype O FMD viruses in Ethiopia as well as with other O type isolates from other countries of the continent.

The current isolates from Sire Berga of Adea Berga district west Shoa zone of Oromia region (O/ETH/12/2016) was compared based on 636 nucleotide sequence of VP1 with Serotype O FMD viruses in Ethiopia as well as with other O type isolates from other countries of the world. The isolate (O/ETH/12/2016) was 99.7-100% similarity with Ethiopian isolates of O/ETH/9/2016, O/ETH/11/2016, O/ETH/13/2016, and O/ETH/8/2016 respectively which are genetically closely related. The above mentioned isolates were also 88.1% similarity with Ugandan isolate (O/UGA/17/98), 87.1% similarity with Ethiopian isolate (O/ETH/58/2005), 86.6% similarity with Sudan isolate (O/SUD/2/86) and 86 % with Kenyan isolate (O/KEN/5/2002) respectively (WRL, 2016). The isolated serotype O in the current study falls into East African topotype (EA-4) and the virus was clustered with FMD viruses circulating in Sudan and Uganda (Figure 13).

The viral isolate (O/ETH/13/2016) virus isolated from Kolfe Keranyo subcity of Addis Ababa was compared based on 636 nucleotide sequence of VP1. The virus was most closely related and shared 98.3-98.6% genetic similarity with current isolates (O/ETH/8/2016, O/ETH/9/2016, O/ETH/11/2016 and the isolate (O/ETH/12/2016) from Maru chobot and Bishan Dimo (Adea Berga district), Menagesha kolobo (Welmera district) and Sire Berga of Adea Berga district, respectively. These viruses are very much closely related, geographically clustered and formed a single genetic lineage called topotype EA-4. The nucleotide sequence difference analysis confirmed that the nucleotide sequence difference of these viruses was ≤1.5%. The current serotype O

isolate from the mentioned area was 87.3% shared similarity with Ethiopian isolate (O/ETH/58/2005).

Furthermore, the isolate O/ETH/11/2016 virus isolated from Menagesha kolobo of Welmera district showed 99.7-99.8% genetically closely related with isolates (O/ETH/12/2016, O/ETH/9/2016 and O/ETH/8/2016) of viruses obtained from Sire Berga, Bishan Dimo and Maru chobot whilst it showed 98.2% showed genetic similarity with (O/ETH/13/2016) virus isolated from Kolfe Keranyo of Addis Ababa respectively. The nucleotide sequence difference analysis confirmed that the identified viruses had ≤2% sequence difference and hence these outbreaks were caused by cclosely related viruses. This isolate was 86.3- 88.7% similarity with Sudan isolate of SUD/2/86, Ethiopian isolate of O/ETH/58/2005, Ugandan isolate of O/UGA/17/98 and Kenyan isolate (O/KEN/5/2002) (WRL, 2016). The isolated serotype O in the current study falls in to East African topotype and the virus was clustered with FMD viruses circulating in Sudan, Uganda and Kenya. Generally, Serotype O isolated from outbreaks occurred at Adea Berga district in Oromiya regional state particularly in Maru-chobot (O/ETH/13/2016, O/ETH/8/2016), Bishan Dimo (O/ETH/9/2016) and Sire Berga O/ETH/12/2016) were closely related, and <2% nucleotide sequence difference was observed at 636 nt sequence(Figure 15).

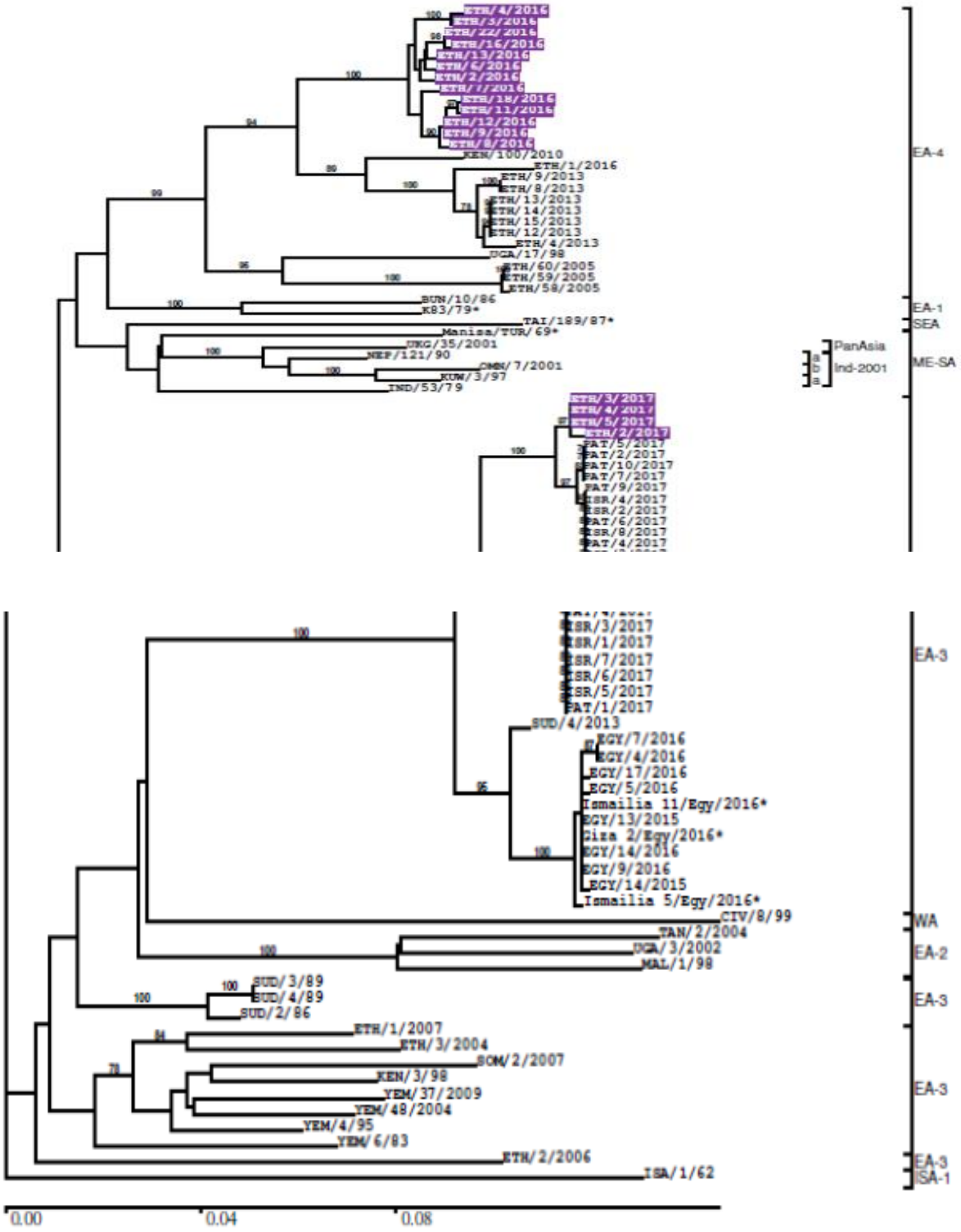

Figure 15: Phylogenetic tree depicting the clustering patterns of the FMDV O serotype isolated in the study areas

Table 16: Most closely related viruses with the current isolates

Virus name	Nucleotide Identity (%)	Topotype	Host
O/ETH/11/2016	99.8	EA-4	Bovine
O/ETH/9/2016	99.7	EA-4	Bovine
O/ETH/12/2016	99.7	EA-4	Bovine
O/ETH/18/2016	99.7	EA-4	Bovine
O/UGA/17/1998	87.7	EA-4	Bovine
O/ETH/58/2005	86.5	EA-4	Bovine
O/SUD/2/1986	86.3	EA-3	Bovine
O/ETH/13/2016	99.2	EA-4	Bovine
O/KEN/5/2002	86.4	EA-2	Bovine
O/ETH/8/2016	98.2	EA-4	Bovine
O/ETH/6/2016	99.5	EA-4	Bovine

5. DISCUSSION

5.1. FMD Field Outbreak Investigation and Clinical Examination

Foot and mouth disease (FMD) a contagious acute viral disease of animals characterized by formation of vesicles in the mouth, on the feet, teats and sudden death of young stock (Quinn *et al.,* 2005). The results of the present study indicated that out of the total number of 586 cattle examined during FMD outbreaks in all districts, 226 (38.6%) animals manifested clinical signs and lesions suggestive of Foot and mouth disease. The current findings were related with the previous clinical findings of Belachew (2014*)* who reported that during outbreak of FMD in Ethiopia, (36.9%) of animals manifested clinical sign of foot and mouth disease. Authors such as (Legesse, 2008) and (Nigussie *et al.*, 2011) reported that 53% and 28.2% clinically sick animals showed clinical signs after conducting outbreak investigations in different parts of the country, respectively. This was justified by earlier reports by Kitching *et al*. (2005) and McLaws *et al*.(2006) who described that variations in clinical severity and manifestations were associated with the virus strains, infection dose of the virus, species affected, susceptibility of the host, farming system and previous exposure of the animal.

Moreover, out of the total cattle examined for FMD cases, 352 animals were examined at Adea Berga district state dairy farm and the overall morbidity; mortality and case fatality in the farm were 36.9%, 3.12% and 8.46%, respectively. On the other hand, Nigussie *et al.* (2011) also reported that the morbidity of Foot and mouth disease was 21.1% in Akaki Kality sub-city, and the mortality rate was lower than 2% in the study districts. The works of Mersie *et al.* (1992) also indicated that the mortality and case fatality rates were 1.6% and 8.9% in calves during outbreak of FMD in Ethiopia. Studies conducted by Olabode *et al.* (2014) in Nigeria also showed that the morbidity, mortality and case fatality rates in young calves during the outbreak were 19.82%, 0.92% and 4.63% respectively. This could be supported by the fact that mortality in young calves might be due to cardiac involvement, and frequent death occurred due to acute heart failure. The

findings of this study also showed that the breed specific morbidity rate (38%) and mortality rate was relatively higher in Jersey breed (3.5%) than Holstein Friesian and it was statistically insignificant (P > 0.05). This result was lower than the reports of Nigussie *et al.* (2011) who reported (8.5%) morbidity rate in indigenous breed of cattle. This might be associated with genetic factors that are involved in the susceptibility of animal breed. The works of Fiebre (2015) also indicated that during outbreak, the morbidity in cattle reaches 100% while the mortality can be up to 40% in younger animals. Male calves were more likely to die than females possibly because of lack of care due to their low economic value. The mortality seems to be very high in this outbreak cases showing that the form of disease was acute despite the disease is endemic in the area.

The result of necropsy findings from clinically infected calves showed that there was prominent lesion in the heart which became dilated and flabby with epicardiac hemorrhage. This finding was most likely justified by the earlier works of (Radostitis *et al.,* 2007) who reported that FMD in new born animal causes high mortality as it frequently results in lesion and necrotizing myocarditis with some virulent strains of the virus. This could be due to the peculiar behavior of the virus strain variation and the change of antigenic character between emerging serotype as well as the virulence of the virus which may cause acute myocardial failure and death of young calves (Radostitis *et.al.,* 1994).

5.2. FMD Seropositivity and Potential Risk Factors

The results of the present study indicated that the overall seropositivity of FMD was 30.8% in the study area and this finding was in close agreement with the findings of Ayelet *et al. (*2012) who reported 32.7% and 30 % in Guji zone of Oromia region and Yeka district of the city of Addis Ababa, respectively. The earlier studies of Nigussie *et al*. (2011) also indicated 28.3% seropositivity in Akaki-kality sub-city. Compared to the current result, lower seroprevalences of 8.18% (Molla, 2009) and 9% (Beyene *et al*., 2015) were reported from Afar Regional State, South Omo Zone of SNNPRS and

Western Ethiopia, respectively. Besides this, relative to the present finding, higher sero-prevalence was previously reported in samples collected from the eastern zone of Tigray with 41.5% (Ayelet *et al*., 2012).

Previous studies conducted by Hafez *et al*. (2014), in Saudi Arabia and Namatovu *et al.* (2015) in Uganda also showed FMD seropositivity of 53% and 77% in infected cattle. A report from neighboring Sudan also indicated that, after an active outbreak of the disease, the seroprevalence of FMD was 79% in cattle (OIE, 2012). This observed seropositivity variation might be resulted from differences in individual animals breed, immune status, interaction of cattle with other animals and production system as well as differences in geographical area or the way sampling was conducted based on the existence of recent outbreak and this could also contribute to higher seropositivity recorded in this study area.

The study also revealed that age-specific seropositivitity in adult was 32.5% and 27.2% in young cattle. This was in accordance with earlier studies of Murphy et al. (1999) who reported that seropositivity increment was due to the cumulative experience of the population with the agent. The reaserch works conducted by Bayissa (2009) indicated rel ative low seropositivity in age group less than two years that might be indicative of the existence of passive maternal immunity and low frequency of exposure. Age association with FMD seropositivity was also consistent with the previous study of (Olabode *et al*., 2013). The probable reason might be, aged animals might have acquired the infection from multiple serotypes, and could produce antibodies against serotypes of FMD. In the study areas, young animals were often managed separately at around homestead and have less exposure to the disease.

The results also showed that seropositivity of FMD was observed to be higher in females (38.5%) than in males (15.8%) and this variation was statistically significant ($p<5\%$). This finding was higher than the previous findings in Ethiopia, 8.27% in male and 15.07% in female cattle (Gelaye *et al*., 2009) and Lower than the previous works in Kenya, 67% in female and 33% in male as reported by (Chepkwony *et al*., 2012) .The

significant seroprevalence variation observed between the sex of cattle may be related to the effect of the higher number of females included in the study than males. This might also be related with the personal observation where dairy farm owners are usually keeping few males only for breeding purpose. The greater percentage of seropositivity in females might be due to physiological stresses which include oestrus, pregnancy and lactation which are known to affect their resistance to infection (Susan and Asamays, 1998). Cross breed cattle was more affected (46.2%) than local breed (19.8%). This could be related to differences in management practices. Those genetically improved cattle are more prone to FMD infection than local breeds since they may suffer from deficient housing and malnutrition.

The management factors which influenced the seroprevalence of FMD in the study areas of cattle were intensive (48.7%) and semi intensive (20.6%) management system. In line with this result, Vosloo *et al*. (2002) reported that intensive livestock production is highly vulnerable to the effect of FMD. This could be attributed to crowding of animals that can facilitate frequency of direct contact and hence enhances chances of transmission. The present study also identified those cattle with poor body condition scores had more FMD infection (36.1%) than moderate (32.9%) and good (18.8%) body condition which was also reported by (Fraser *et al.,* 1991). The probable reason could be related to the weak protective immune response in poor body conditioned cattle as animals with good body condition have relatively good immunological response to infection (Radostatit *et al.,* 2007).

5.3. FMD Virus Isolation and Characterization in Cell Culture

In this study from, a total of 37 field samples subjected to cell culture, about 56.8% of samples showed FMDV cytopathic effect (CPE) and infected cells were found as rounding. This finding was in agreement to the previous studies by (Huang *et al*., 2011; Nigussie *et al*., 2010) and (Tesfaye, 2014) who reported that FMDV cytopathic effect was characterized by inducing fast destruction of BHK-21 cell monolayer and infected cells were round and sloughing as well as monolayer detachment from the wall of cell culture flask. Other authors such as (Shawky *et al*., 2013) also described that FMDV isolated from clinical samples and inoculated on BHK-21 cell-culture results in infected-cell that showed specific CPE within 24-48 hours post infection was characterized by rounding of cells and distortion of the monolayer and cell detachments. The remaining samples did not show CPE since some outbreaks occurred in areas where vehicle is inaccessible and some may be due to death of the virus during transportation.

5.4. Serotyping of FMD Virus Isolates from Outbreak Samples

The overall serotyping results of the current findings disclosed that 16 (39.02%) samples were positive for FMD virus and four types of serotypes were identified. The dominant identified serotypes were FMDV O serotype (34.2%), followed by A serotype (17.1%), serotype SAT1 (4.9%) and serotype SAT2 (2.4%), respectively. These results were corroborated with previous reports from Ethiopia (Gelaye *et al.*, 2007; Ayelet, 2009) who indicated that serotype O (73.3%) followed by serotype A (19.5%), SAT-2 (4.1%), SAT-1 (1.8%) and C(1.4%), during outbreaks in Ethiopia. This statement was also supported by the studies of (Tesfaye, 2014) who reported that serotype O (65.63%) was the dominant serotype prevailed through Addis Ababa, Debre Berhan and Bishoftu. In support of this findings, studies conducted by Noureldin and Elfadil (2014) in Khartoum state of Sudan indicated that serotype O (82.6%) and SAT-2 (40%) were the main circulating FMDVs in cattle. Habiela *et al.* (2010) in Sudan added that the main

circulating virus in cattle was A (78.1%), O (69.4%), SAT-2 (44.0%) and SAT-1 (20.2%).

Serotype O was highly prevalent serotype causing an outbreak in Ethiopia which agreed with the survey of SPS-LMM (2008) that there is a tendency for type O strain to occur most frequently in the outbreak. This similarity of the serotype in one outbreak might be due to the presence of uncontrolled animal movement. The works of Ekboir (1999) also showed that movements of infected animals are by far the most important dissemination and transmission means for FMD. Those statements were 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. Studies conducted in Uganda indicated that serotype O and SAT-2 were responsible for most of FMD outbreaks (Balinda *et al*., 2009).

Moreover, Serotype O was isolated from Adea Berga and Welmera district (Menagesha kolobo) and Kolfe Keranyo of Addis Ababa. This was supported by studies of (Gelaye *et al.,* 2005; Ayelet *et al.,* 2009; Nugussie *et al.,* 2010; Menda *et al.,* 2014) who reported that serotype O was the dominant serotype causing an outbreaks in Ethiopia. Other studies also indicated that serotype O was the most common cause of outbreaks globally followed by serotype A (Klein, 2009; Rweyemamu *et al*., 2008). Serotype O was also isolated from bovine epithelium, probang samples and oral swabs at Adea berga, Welmera, and Addis Ababa. Previous reports by Knowles (2010) have indicated outbreaks of FMDV SAT 1 in 2009 and FMDV serotype O in 2010 in the same areas.

It was also found that serotype SAT-2 FMD virus was circulating in Mulo district of North Shoa zone of Oromiya region. This result was corroborated with earlier studies of (Gelaye *et al.,* 2005), (Ayelet *et al.,* 2009), (Tefera, 2010) and (Mishamo, 2016) who reported serotype SAT2-2 virus in Borean pastoral area, Benishangul-Gumuz, Gambella, Addis Ababa and Adama, respectively. Serotype SAT-2 was also previously reported from many sub-saharan African countries (Sangare, 2005; Sahle *et al.,* 2007) suggesting

endemicity of serotypes in these countries. On the other hand, serotype SAT1 FMD virus was isolated in samples collected from Kimbibit district (Mogoro gara daga) and Adea berga district during outbreak. This might be the probable existence of numerous types of virus during outbreak.

In the current study, the type SAT2 virus was the least prevalent serotype of FMD virus and causing about 2.4% of the outbreak in Kimbibit district of North Shoa zone. This observation was consistent with previous studies of (Kasanga *et al*., 2014) who reported the presence of serotypes O, A, SAT 1 and SAT 2 in various locations in Tanzania and indicated that the epidemiology of FMD in the country was complicated by the presence of multiple and single serotypes.

In these recent findings, mixed serotypes of FMD virus infection (O and A, O and SAT1) were recorded in Adea Berga and Welmera districts. This finding was similar to the earlier works conducted in Pakistan by (Jamal *et al.,* 2011) who reported dual serotypes (O and A) in a single sample collected from the same area. Other author such as (Knowles, 2010) in Zambia also reported the presence of serotype SAT-1 and O in the same area during outbreak. This situation could be associated with the presence of endemic state of disease in the area. There is a possibility of having more FMDV serotypes circulating in the area that could not be detected during the study period.

5.5. Molecular Characterization

5.5.1. Real time PCR testing results

In this study, out of 50 samples tested by real-time PCR for the presence of FMDV genetic material in the sample, 18.1% (n=9) were found positive. The result also showed that bovine epithelial tissues were accounted for 12% (n=6) and had the lower Ct values which suggests higher concentrations of the virus whereas bovine probang samples were accounted for 8% (n=4). This could be related to the evidence that epithelial tissues have

been obtained from clinical cases while probang samples were taken from asymptomatic animals sharing the same farm with symptomatic animals. This was also justified by (OIE, 2012) as the preferred samples for virus detection is epithelial tissue in which the virus multiplication starts from the pharynx epithelium and spreads to the oral mucosa for producing vesicles.

Other studies also indicated that FMD virus can also be obtained from oral swabs (Alexanderson *et al*., 2002). The lowest Ct values of 16.03 was recorded in bovine epithelial tissues collected from Adea Berga district whilst the highest (26.98) Ct value was examined in bovine probang samples collected from Wuchale district. This finding was verified the presence of higher levels of viral RNA in the epithelial tissues than in the probang samples. This was also justified by the fact that low number of rRT-PCR-positive samples and small samples yielding infectious virus could have been due to virus degradation during transfer from the field. Studies conducted by (Mwiine *et al.,* 2009) also indicated that the low rate of virus, despite healing lesions suggestive of FMDV, was probably due to long transportation times for samples and difficulties in maintaining the cold chain during transportation and storage.

5.5.2. Phylogenetic analysis

The VP1 gene characterization was used to study phylogenetic relationships between serotype O FMD viruses in Ethiopia as well as with other O type isolates from other countries of the continent. Phylogenetic analysis showed that serotype O virus from the current study belonged to East Africa-4 (EA-4) topotype and this virus was clustered with FMD viruses circulating in Sudan and Uganda. This is in agreement with the previous study on molecular epidemiology of serotype O by (Ayelet *et al*., 2009) and (Nigussie *et al*., 2010) who demonstrated the existence of EA-3 and EA-4 topotypes in Ethiopia. It was also supported by the previous report on samples from Mizan Teferi indicated the emergence of a new topotype within serotype O has fallen to East Africa 4(EA-4), apart from the dominant topotype within serotype O of EA-3 encountered in Eritrea, Ethiopia

and Sudan (Ayelet *et al*., 2009). On the other hand, serotypes were distinguished on the basis of nucleotide sequence differences of 30- 50% and high bootstrap support (> 70%) while a divergence of 15% to 20% distinguishes topotypes. The existence of eight serotype O topotypes within samples collected around the world based on the comparison of sequence data of the VP1 of gene. Among those, two topotypes were found in Africa, one in East Africa and one in West Africa (Knowles and Samul, 2003). This is in agreement with previous study on molecular epidemiology of serotype O by Asfaw and Sintaro (2000) who demonstrated the existence of EA-3 and EA-4 topotypes in Ethiopia.

In the current findings, O isolate from Adea Berga district shoa zone of Oromia region (O/ETH/12/2016) during the study period was 99.7-100% similarity with other isolates of O/ETH/9/2016, O/ETH/11/2016, O/ETH/18/2016, an O/ETH/8/2016 and shared 88.1% similarity with Ugandan isolate (O/UGA/17/98), 87.1% Ethiopian isolate (O/ETH/58/2005) and 86.6% similarity with Sudan isolate O/SUD/2/86) respectively (WRL, 2016). The virus isolated in kolfe keranyo of Addis Ababa (O/ETH/4/2016) was more closely related to the virus isolated from Menagesha of Welmera district (< 1% nucleotide difference).These indicated that outbreaks due to these isolates were from the same epizootics. This might be due to free movement of livestock and livestock products among various markets in different regions which plays an important role in the dissemination of the virus. These viruses were closely related, and <2% nucleotide sequence difference was observed at 636 nt sequence. This also showed that outbreaks due to these isolates were from the same origin. This isolate was 86.3- 88.7% similar with the Sudan isolate of SUD/2/86, Ethiopian isolate of O/ETH/58/2005, and Ugandan isolate of O/UGA/17/98 (WRL, 2016). These indicated that outbreaks due to these isolates were the same epizootics. This might be due to free movements of livestock and livestock products. This was also indicated as recent common origin, isolates with >85% nucleotide sequence identities have been placed within groups or topotypes, which tend to be restricted in their geographic distribution (Knowles and Davies, 2004).

6. CONCLUSION AND RECOMMENDATIONS

FMD is endemic in most parts of Africa including Ethiopia and it is rampant in the current study areas. This was verified through clinically, necropsy, serologically, virologi cally and molecular diagnostic techniques. FMD is a major barrier to international trade in animal and animal products that results in tremendous economic losses. Thus, the output of the current study indicated an overall 38.6% of cattle showed clinical signs during FMD outbreak and young calves were mostly suffered from the disease. In this study, the dominant serotypes identified from outbreak cases were FMDV serotype O (34.2%), followed by serotype A (17.1%), serotype SAT1 (4.9%) and serotype SAT2 (2.4%). The outbreaks were mostly caused by serotype O and it was confirmed to be under East Africa 4 (EA-4) topotype. In conclusion, the presence of huge susceptible animals, animal age variability, and breed susceptibility, lack of prophylactic vaccination, free (cross border) movement, communal grazing places and high contact of animals at common points were incriminated as major risk factors that could contribute to the occurrence of FMD. Therefore, based on the current findings, the following recommendations are forwarded:

- A comprehensive active assessment and serotyping of FMD outbreak field strains are required in the disease endemic areas to detect the emergence and circulation of new serotypes and variants involved in the outbreaks
- Detailed studies on molecular characterization of field strains of FMD virus circulating in the area and their genetic closeness with the vaccine strains need to be conducted to institute the nature of their diversity on the tree and to know the evolutionary situation of emerging variants.
- In vitro vaccine matching test should be conducted at certain intervals covering wide areas and targeting animal species since the virus serotypes and subtypes might be changed as they differ antigenically, genetically and epidemiologically.
- More attention should be given to control FMD through regular vaccination and animal movement restriction prior to outbreaks.

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8. LIST OF APPENDICES

Appendix 1: Number of PAs/kebeles selected during the study period

S/No.	Districts	PAs/Kebeles included in the study
1	Adea Berga	Bishan Dimo, Sire Berga, Maru Chobot
2	Welmera	Menagesha Kolobo (01)
3	Addis Ababa	Kolfe keranyo (01)
4	Wuchale	Machala wartu
5	Mulo	Amuma Bubisa Dumburi, Mulo siro
6	Kimbibit	Mogoro gara daga

Appendix 2: Study animals age determination during study period

Species	Animal Age categories

Bovine	Calves up to three months
	Young stock from three to Eighteen month
	Adult cattle –aged over Eighteen month

Appendix 3: Desription of Body condition scores in dairy cattle (BCS)

S/No.	Interpretation	Description
1	Prominent spines with sharp process at tail head and deep cavity with no fatty tissue under sjin at loin region	poor
2	Shallow cavity with prominent pain bonesat tail head and horizontal process can be identified individually with rounded end at loin region	Moderate
3	Fat covered with entire area but pelvic can be felt and the end of horizontal process can only be felt with pressure slight depression at loin region	Good

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

Appendix 4: FMD field outbreak investigation and data collection recording sheet

1. FMD outbreak location:

- Region_____ Zone _____ District ____PA _____Date ___/ ____/ ____

A. Name of animal owner_____

B. Number of animals clinically examined for FMD cases

Code	Species	Breed		Sex		Age		Total
01	Bovine	Local	cross	Male	Female	Adult	Young	
02								

C. Number of animals clinically affected during FMD Outbreak

Code	Species	Breed		Sex		Age		Total
02	Bovine	Local	cross	Male	Female	Adult	Young	
03								

D. Number of Animals died during FMD Outbreak

Code	Species	Breed		Sex		Age		Total
02	Bovine	Local	cross	M	F	Adult	young	
03								

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

- Profuse salivation _____ Lameness _____ interdigital lesion _____
- Udder lesion _____ Oral and tounge lesion _____ other signs _____

Appendix 5: FMD Outbreak sample collection sheet

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

Code	Species	Breed	Age	Sex	Sample type			
					Tissue	Probang	Oral swab	Pool
B1								
B2								
B3								

Appendix 6: Parameters used for blood sample collection and associated risk factors

- Region _____Zone____Distric____Kebele____Date ____ /____ /____

code	Breed		Sex		Age		Body condition			Management system	
01	L	C	F	M	Y	A	G	M	P	intensive	semintenisve
02											

Hint: L-local, C-cross, F-female, M-male, Y-young, A-adult, G-good, M-moderate

Appendix 7: Plate layeout for FMDV detection and serotyping ELISA

Catching MAbs	Type O		TYPE A				Pan-O,A,C ,As		TYPE SAT 1		TYPE SAT 2	
	MAb 3B11		MAb 4D12		MAb5F6		MAb 1F10		Pool 2 MAbs		Pool 2 MAbs	
	1	2	3	4	5	6	7	8	9	10	11	12
Sample 1 A	1	1	1	1	1	1	1	1	1	1	1	1
Sample 2 B	2	2	2	2	2	2	2	2	2	2	2	2
Sample 3 C	3	3	3	3	3	3	3	3	3	3	3	3
Sample 4D	4	4	4	4	4	4	4	4	4	4	4	4
Sample 5 E	5	5	5	5	5	5	5	5	5	5	5	5
Sample 6 F	6	6	6	6	6	6	6	6	6	6	6	6
Pos Ctr G												
Neg conr H												

MAb=Monoclonal antibodies, As1. = Asia 1

Appendix 8: Interpretation of OD values as recommended by Sandwich ELISA

Negative for FMDV	OD <0.1 with all catching MAbs, after subtracting the OD of the respective negative control.
FMDV positive type O	OD ≥ 0.1 with the type O MAbs and the pan-FMDV O, A, C, Asia 1 MAb. Some samples may cross react with MAb A 4D12, but OD values with MAb O are higher.
FMDV positive type A	OD ≥ 0.1 with at least one of the two type A MAbs and with the pan-FMDV O,A,C,Asia1 MAb
FMDV positive type SAT1	OD ≥ 0.1 with the type SAT1 catching MAbs, after subtracting the OD of the respective negative control
FMDV positive type SAT2	OD ≥ 0.1 with the type SAT2 catching MAbs, after subtracting the OD of the respective negative control
FMDV positive (un-typed)	OD ≥ 0.1 with the pan-FMDV MAb and <0.1 with the type-specific MAbs, after subtracting the OD of the respective negative control

Appendix 9: Master Mix composition of PCR

- 2x PCR Buffer with Taq Polymerase
- Forward primer
- Reverse primer
- Nuclease free water
- Extracted template

Appendix 10: Miscellaneous pictures


Salivation, lesion on guma and Hoof distrotion

Appendix 11: Sequences of identified O serotypes, Adea Berga, Maru cobot : **O/ETH/8/2016**

ACCACCTCCCCAGGTGAATCAGCCGACCCCGTGACCGCCACCGTTGAGGACT
ATGGTGGCGAGACACAGGTCCAGAGGCGTCAACACACGGACGTCTCGTTCAT
CCTTGACAGATTTGTGAAGGTAACACCGAGAGAGGACCTAATTAATGTTTTG
GACCTGATGCAGATTCCTGCCCACACGCTGGTGGGGGCGCTCCTCCGTGCTG
CCACCTACTACTTCGCTGATCTAGAGGTGGCGGTCAAGCACGAAGGGAACCT
CACGTGGGTCCCGAACGGAGCGCCCGAGTCAGCACTGGACAACACCACCAA
CCCAACGGCTTACCACAAAGCACCACTTACCCGTCTTGCTCTGCCCTACACAG
CGCCCCACCGCGTTTTGGCAACCGTTTACAACGGGAACTGCAAGTACGGAGA
GACACCAGTGGCCAATGTGAGGGGTGATCTCCAAGTGTTGGCCCAGAAGGCA
GCTAGGACGCTGCCCACCTCCTTCAACTACGGTGCCATCAAGGCCACCCGGG
TGACCGAGCTGCTCTACCGCATGAAGAGGGCTGAGACGTACTGTCCCAGACC
CCTCCTAGCTATTCACCCAAGCGAGGCCAGACACAAACAGAAGATTGTGGCA
CCTGTGAAACAGCTCTTG

Welmera district,Menagesha kolobo: O/ETH/11/2016

ACCACCTCCCCAGGTGAATCAGCCGACCCCGTGACCGCCACTGTTGAGAACT
ATGGTGGCGAGACACAGGTCCAGAGGCGTCAACACACGGACGTCTCGTTCAT
CCTTGACAGATTTGTGGAGGTAACACCGAGAGAGGACCTAATTAATGTTTTG
GACCTGATGCAGATTCCTGCCCACACGCTGGTGGGGGCGCTCCTCCGTGCTG
CCACCTACTACTTCGCTGATCTAGAGGTGGCGGTCAAGCACAAAGGGAACCT
CACGTGGGTCCCGAACGGAGCGCCCGAGTCAGCACTGGACAACACCACCAA
CCCAACGGCTTACCACAAAGCACCACTTACCCGTCTTGCTCTGCCCTACACAG
CGCCCCACCGCGTTTTGGCAACCGTTTACAACGGGAACTGCAAGTACGGAGA

GACACCAGTGGCCAATGTGAGGGGTGATCTCCAAGTGTTGGCCCAGAAGGCA
GCTAGGACGCTGCCCACCTCCTTCAACTACGGTGCCATCAAGGCCACCCGGG
TGACCGAGCTGCTCTACCGCATGAAGAGGGCTGAGACGTACTGTCCCAGACC
CCTCCTAGCTATTCACCCAAGCGAGGCCAGACACAAACAGAAGATTGTGGCA
CCTGTGAAACAGCTCTTG

Addis Ababa, Kolfe Keranyo: O/ETH/13/2016

ACCACCTCCCCAGGTGAATCAGCCGACCCCGTGACCGCCACTGTTGAGAACT
ATGGTGGCGAGACACAGGTCCAGAGGCGTCAACACACGGACGTCTCGTTCAT
CCTTGACAGATTTGTGAAGGTAACACCAAGAGAGGACCTAATTAACGTTTTG
GACCTGATGCAGATTCCTGCCCACACACTGGTGGGGGCACTCCTCCGTACTG
CCACCTACTACTTCGCTGATCTAGAGGTGGCGGTCAAGCACGAAGGGAACCT
CACGTGGGTCCCAAACGGAGCGCCCGAGTCAGCACTGGACAACACCACCAA
CCCAACAGCTTACCACAAAGCACCACTTACCCGTCTTGCTCTGCCCTACACAG
CGCCCCACCGCGTTTTGGCAACCGTTTACAACGGGAACTGCAAGTACGGAGA
GACACCAGTGGCCAACGTGAGGGGTGATCTCCAAGTGTTGGCCCAGAAGGCA
GCTAGGACGCTGCCCACCTCCTTCAACTACGGTGCCATCAAGGCCACCCGGG
TGACTGAGCTGCTCTACCGCATGAAGAGGGCTGAGACGTACTGTCCCAGACC
CCTCCTAGCTATTCACCCAAGCGAGGCCAGACACAAACAGAAGATTGT
GGCACCTGTGAAACAGCTCTTG