Thesis Ref. No. ----------

ISOLATION, MOLECULAR CHARACTERIZATION AND SERO-PREVALENCE STUDY OF FOOT-AND-MOUTH DISEASE VIRUS CIRCULATING IN CENTRAL ETHIOPIA

MSc Thesis

By

Mishamo Sulayeman Issa

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Microbiology, Immunology and Veterinary Public Health

June, 2016

Bishoftu, Ethiopia

ISOLATION, MOLECULAR CHARACTERIZATION, SERO-PREVALENCE STUDY OF FOOT-AND-MOUTH DISEASE VIRUS CIRCULATING IN CENTRAL ETHIOPIA

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

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Mishamo Sulayeman Issa

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Department of Microbiology, Immunology and Veterinary Public Health.

As member of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: **Mishamo Sulayeman Issa,** titled: **Isolation, Molecular Characterization and Sero-Prevalence Study of Foot-And-Mouth Disease Virus Circulating in Central Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the degree of master of Science in Veterinary Microbiology.

	Signature	Date
Dr.	----------	----------
Chairman		
Dr.	----------	----------
External Examiner		
Dr.	----------	----------
Internal Examiner		
1. Dr. Fufa Dawo	----------	----------
Major advisors		
1. Daniel Gizaw	----------	----------
2. Dr. Bedaso Mamo	----------	----------
Co- advisor		
Dr. Gezahegne Mamo	----------	----------
Department chairperson		

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STATEMENT OF AUTHOR

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

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Name: MISHAMO SULAYEMAN ISSA Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

ACKNOWLEDGMENTS

Almighty GOD should receive glory for his endless mercy and life he provided me through his one and only son Jesus Christ. Next, I would like to thank my thesis advisors Dr. Fufa Dawo, Dr. Daniel Gizaw, and Dr. Bedaso Mamo for their unreserved help, constructive advice and time commitment to correct this manuscript. I also thank them for their genuine and energetic encouragement, suggestion, insight and scientific and professional guidance.

I want to express my deepest gratitude and appreciation to National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Ethiopia for providing me valuable support and assistance during research work in the field as well as laboratory. My special thanks go to Dr. Dereje Shegu for his encouragement, professional and material support. I am grateful to the staff members of the center, especially to Ayelech Muluneh, Membere Kidane, Aynalem Fente and Genet Bogale for their positive cooperation during my laboratory work. Also I would like to extend my thanks to Demeke Sebhat and Abebe Garuma for their positive cooperation in providing me information while collecting the sample from dairy cattle and outbreak areas.

I am deeply grateful to World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD), England for the laboratory support in molecular aspect of my research.

Finally, my wholehearted and deepest gratitude goes to my mother w/ro Tegesech Cheyago, my father, Sulayeman Issa and my brothers and sisters specially my elder sister Lemaye Sulayeman for their moral and financial support throughout my educational career.

LIST OF ABBREVIATIONS

BHK21	Baby hamster kidney 21 day
CPE	Cytopathic effect
ELISA	Enzyme Linked Immunosorbant Assay
FAO	Food and Agriculture Organization
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GDP	Gross domestic production
ICTV	International Committee on Taxonomy of Viruses
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kb	kilo base pair
Koh	Potassium hydroxide
MAbs	Monoclonal antibodies
MEM	Minimum essential media
NAHDIC	National Animal Health Diagnostic and Investigation Centre
Naoh	Caustic soda
NCR	Non-coding region
NVI	National Veterinary Institute
OD	Optical Density
OIE	World Animal Health Organization
PAs	Peasant Association
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
RI	Replicative intermediates

LIST OF ABBREVIATION (Continued)

RNA	Ribonucleic Acid
RT-PCR	Reveres Transcriptase Polymerase Chain Reaction
SAT	Southern African Territories
SNNPR	Southern nation nationality and people republic
ssRNA	Single stranded Ribonucleic Acid
SVD	Swine vesicular disease
TCID	Tissue culture infective dose
UK	United Kingdom
VN	Virus Neutralization
VP1	Viral capsid protein one
Vpg	Viral genomic Protein
Vpg	Viral genomic protein
VS	Vesicular stomatitis
WRL	World Reference laboratory
χ^2	Chi –square

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ABSTRACT

A cross sectional study was conducted to determine sero-prevalence and to isolate and characterize causative agents of foot-and-mouth disease (FMD) in selected areas of Central Ethiopia from September 2015 to May 2016. In addition, the study was meant to assess epidemiological risk factors associated with FMD in dairy cattle. A multistage random sampling for the sero-prevalence study was implemented to select the required sample size. Blood sample was collected in vacutainer tubes for sera separation and the sera were tested by 3ABC-Ab ELISA. Outbreak cases were investigated by collecting epithelial tissues and by using oro-pharyngeal fluids samples for FMD virus isolation and characterization. The isolated viruses were used for antigen typing by sandwich ELISA. Molecular characterization was done by VP1 gene sequence generation and phylogenetic tree reconstruction. Laboratory results were analyzed using descriptive statistics, chi-square test and logistic regression. The overall sero-prevalence of FMD was 24.22% (139/574) for the dairy cattle. Sero-prevalence of 26.84% and 22.91% was recorded in Adama and Asella, respectively but not significantly different ($P>0.05$). In logistic regression analysis, breed, age and animal composition were significantly associated with FMD sero-prevalence ($p<0.05$). From the total of 378 cattle clinically examined during an outbreak, 109 (28.8%) of cattle showed signs and lesions suggestive of FMD infection. In this study, three serotypes (A, O and SAT-2) were isolated and serotyped FMD virus. From the isolated serotypes A and SAT2 viruses were characterized at molecular levels by phylogenetic reconstruction. The SAT2 virus shared greater than 90% genetic similarity with other SAT2 isolates reported previously from Ethiopia. The serotype A isolate has 87.79- 88.26% genetically similar with Cameron, Eritrea and Sudan isolates. This study generated useful information on FMD sero-prevalence, circulating serotypes, molecular characteristic of the isolates and associated risk factors. The isolated viruses clustered differently from the previously known isolates suggesting that they are variant viruses. Detailed molecular analyses coupled with protection potential of the existing vaccines against these isolates are recommended.

Key words: *Ethiopia, dairy cattle, FMD virus, molecular characterization, sero-prevalence, risk factors*

1. INTRODUCTION

Ethiopia is one of few countries in Africa with huge livestock resources that play a crucial role in the livelihoods of the majority of Ethiopians. Animal rearing is an integral part of the agricultural production in Ethiopia and animals represent the major drought power (95%) for crop production. The country is believed to have the largest livestock population in Africa comprising approximately 56.7 million cattle, 29.3 million sheep and 29.1 goats (CSA, 2015). The agricultural sector constitutes about 45% of the gross domestic production (GDP), more than 90% of foreign exchange earnings, 85% of employment opportunities and most of the domestic food supply (CSA, 2009).

Animal diseases are currently widespread in all agro-ecological zone of the country and annual mortality rates due to diseases is estimated at 8-10% for cattle herd and 15% and 12% for sheep and goats flock respectively. It is estimated that animal diseases reduce the production and productivity of livestock by 50 to 60% per year (Ganeshkumar, 2012). Among the livestock diseases hampering productivity of the sector foot and mouth disease (FMD) is considered as a bottleneck to the livestock production (Mansley *et al*., 2011).

FMD is an extremely contagious, acute viral disease of all cloven-hoofed animals/ungulates and pigs. The disease is characterized by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats, and sudden death of young stock (Quinn *et al.,* 2005). It is also one of the most important trans-boundary animal diseases that cause severe economic losses due to high morbidity and livestock export trade restrictions imposed on affected countries (Knight *et al*., 2013). FMD is caused by FMD virus (FMDV) and the virus has seven recognized serotypes (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3), with distinct immunologic properties. The seven serotypes also differ in distribution across the globe (FAO, 2007).

Formerly, five of the seven serotypes of FMDV (O, A, C, SAT 2, SAT 1) were endemic in Ethiopia (Rufael *et al.,* 2008; Ayelet *et al.,* 2009; Negusssie*,* 2010; Admassu *et al*., 2015). But serotype C was not identified after 1983; except that, a serotype C specific

antibody was detected in cattle (Sahle, 2004; Gelaye *et al.,* 2005; Legess, 2008; Rufael *et al.,* 2008). Studies undertaken on FMD so far revealed the existence of the disease in different parts of the country, with sero-prevalence that vary from 8.18% in south Omo (Molla, 2009) to 44.2% in different parts of the country (*Negusssie,* 2010).

FMD affects animals' performance directly through reduction of milk yield, a high number of deaths among young animals and fertility impairment due to increased abortion rate (Admassu *et al.,* 2015). Moreover, the economic importance of the disease is also related to the reaction of veterinary services to the presence of the disease and to the restrictions on the trade of animals both locally and internationally (Knight *et al*., 2013). The recovered animals remain in poor physical condition over long periods of time leading to economic losses for livestock industries (Sangare, 2002).

Currently, in Ethiopia there is no government strategy in FMD control through vaccination and movement control. Lack of vaccination strategies (quality, coverage and timing) and presence of free animal movement without certification are thus the main factors that could increase the spread of FMD along the cattle market chain. For the development of adequate FMD control and prevention determining the status of FMD through serological study, virus isolation and characterization of the serotype is needed. The study areas were known with the abundance of dairy farms that constituted the known milk sheds border and thus implementing sound epidemiological study is essential to generate important baseline information about the study disease.

Therefore, the main objectives of this study were;

- To determine the sero-prevalence of FMD in and around Adama and Asella dairy cattle.
- To assess potential risk factors associated with FMD in and around Adama and Asella adairy cattle.
- To isolate, identify and molecularly characterize FMDV circulating in central Ethiopia.

2. LITERATURE REVIEW

2.1. Definition

FMD is an extremely contagious, acute viral disease of all cloven-hoofed animals/ungulates and pigs, characterized by 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 of the disease (Knight *et al*., 2013).

2.2. Etiology

2.2.1. Taxonomy

FMD virus was defined in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as belonging to the genus *Aphthovirus*, family *Picornaviridae*. The name, *Picornaviridae* is derived from the Latin word 'Pico' meaning small and 'rna' meaning RNA (ribonucleic acid), 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, 2004).

2.2.2. Physicochemical properties

FMDV is a small RNA virus that is enclosed with a non-enveloped protein shell (capsid). The capsid consists of polypeptides, which are devoid of lipo-protein, and hence is stable to lipid solvents like ether and chloroform (EWCA and GNRP, 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 but all strains are rapidly inactivated below pH 4 and above pH 11 (Domingo *et al.,* 2002; Greering and Lubroth, 2002). 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 solution of caustic soda (NaOH) or KOH and 4% Na_2CO_3 in combination with detergent are effective disinfectants for FMDV contaminated objects, but the virus is resistant to alcohol and phenolic quaternary ammonium disinfectants. The FMDV is also sensitive to other chemicals like trypsin, which causes cleavage and denaturation of the virtal capsid protein (VP1) (Rueckert, 2006).

At temperature below freezing point, the virus is also stable almost indefinitely. Exposure to 56°C for 30 minutes is sufficient to destroy most strains, although there is some variation between strains in resistance to temperature and/or pH stress (Geering *et al.,* 1995). 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 (Geering and Lubroth, 2002). 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, e.g. in epithelial fragments (Donaldson, 2000).

2.2.3. Virus morphology

Picornaviruses 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 proteins (VP1-4). VP1-VP3 is exposed on the surface, whilst VP4 is located internally at the pentametric apex of the icosahedrons and contains a myristic acid molecule attached to the amino terminal glycine (Robert & Bruce, 1981).

2.2.4. Genomic organization

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 polyprotein 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. All encoded Vpg variants have been shown to be attached to the 5' terminus of viral RNA (King *et al.,* 1982). It is roughly spherical in shape and about 25–30 nm in diameter. It 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 VP1, VP2 and VP3 are exposed on the surface of the virus while VP4 is located internally.

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. Firstly, the intermediate 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. The mature virion is produced after the encapsidation of the virion RNA that is accompanied 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).

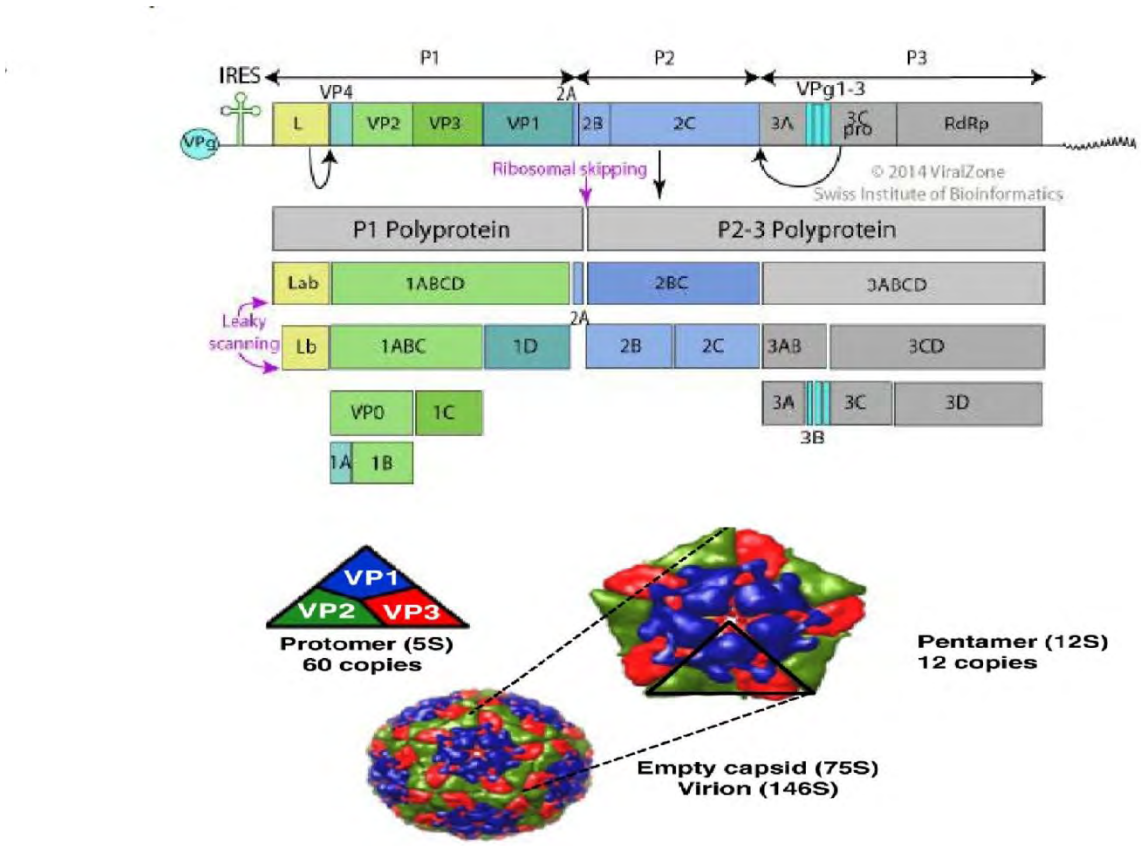

Figure 1: Foot-and-mouth disease virus genome. Source: Adapted from Viral Zone (2014)

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 the absence of proofreading in the 3D-encoded RNA dependent RNA polymerase. Secondly, competitive selection is continuously acting on the genome. Thus, those mutants with a selective advantage in the prevailing environment will be better represented than those with a selective disadvantage (Sahle, 2004).

Mutation

FMDV undergoes a high (10^{-5} to 10^{-3}) rate of mutation during replication. This is mainly due to a lack of replication error checking mechanisms (proof reading). RNA viruses that exhibit such a deficiency mutate at the rate of one nucleotide base change per 103 bases

per replication cycle (Holland *et al.,* 1982). It is also estimated that a mutation rate of up to 10−8 to 10−9 nucleotide substitution per year during an epizootological cycle of FMD viruses can occur. Therefore, new variants of FMD viruses are continuously arising after each replication cycle, which constitute an intratypic population of FMD viruses with different degrees of genetic relationships, previously described as the quasi species phenomena (Domingo *et al.,* 1990). This may result in the generation of viral diversity. Changes in the nucleotide compositions of the capsid genes are responsible for the genetic or antigenic variability of the virus (Sangar *et al.,* 2005). Thus, the generation of new variants is considered as one of the major problems in the control of FMD by vaccination.

Immune selection pressure

One of the evolutionary mechanisms employed by RNA viruses is the profile mutant production, as detailed above. In addition, the immune system of an infected animal, which presumably provides a power full selective force, is another driving force in viral evolution (Domingo *et al.,* 1990) for the reason that the viruses are exposed to immune sera. Under this condition, only neutralization escape variants will be able to grow.

Recombination

Recombination is another important process driving viral biology and evolution. In RNA viruses, recombination involves the exchange of genetic material between two non-segmented RNA genomes resulting from polymerase 'jumping' during RNA synthesis. It has been shown that genetic recombination occurs between viruses of the same serotype as well as between serotypes. 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 (King *et al.,* 1982).

2.2.6. Antigenic variation

Changes to the genes encoding capsid proteins through mutation can result in antigenic variation and evolvement of new subtypes (Haydon *et al.,* 2001). This may give 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 Vallée and Carré in 1922 that an 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: O, 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 (Brooksby, 1982).

Serological studies and the observation in the degree of virulence of the virus in recovered animals have shown that there are significant differences between strains within each serotype (subtypes) (Brooksby, 1982). 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). Progress made in the understanding of the genetic differences underlying observed antigenic variation, has played a major role in the epidemiology of the disease. Today, 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).

2.2.7. FMD virus replication

In the initial event of the replication process, FMD virus uses among other picornaviruses its 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 virally-encoded RNA polymerase. Further synthesis of (+) RNA strands leads to the formation of multi-stranded replicative intermediates (RI) with a 3' poly [A] which are transcribed from the poly [U] tract in the RI. The RI generates a poo1 of (+) RNA for translation and some for synthesis of additional (-) RNA. As the protein level increases, some (+) RNA is packaged into virions (Rueckert, 2006). Proteolytic cleavages occur during shell assembly in a poly protein precursor of structural and non-structural proteins. Complete virus particles are released by cell lysis (Rueckert, 2006; Belsham, 1993). The cycle is completed in 5 to 10 hours (Sangare, 2005).

2.2.8. Serotypes and subtypes

Currently there are seven serotypes of 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 also been described using biochemical and immunological tests; and new subtypes occasionally arise spontaneously. However, at a 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, 2004). FMD 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).

Infection with any one serotype does not confer immunity against another (OIE, 2004). 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).

2.3. Epidemiology

2.3.1. Global distribution of the disease

FMD has occurred in most parts of the world except Greenland, Iceland, New Zealand and the smaller islands of Oceania. Outbreak was not reported in Australia, USA, Canada

and Mexico since 1970, 1929, 1952, and 1954, respectively. Europe has experienced a number of sporadic outbreaks since the cessation of vaccination on the continent during 1990 - 1991 (Samuel and Knowles, 2001; 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; Sumption *et al.,* 2008) (Table 1).

Table 1 Serotypes of FMD isolated from certain geographical regions

Continent	Virus serotypes
Europe (historically)	O, A, C
Asia	O, A, C, Asia 1
Africa	O, A, C, SAT 1, SAT2, SAT 3
South America	O, A, C

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

2.3.2. Distribution of the 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 (i.e. all but 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 (Valarcher *et al.,* 2004; FMD Homepage-Maps, 2006; Rweyemamu *et al.,* 2008). Type O is the most widely prevalent serotype in the world flowed 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. Historically 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 (*Syncerus caffer*) 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 (Bastos and Sangare, 2001).

Due to globalization, FMD epidemics were changed from local and regional spread to wide international spread (Knowles *et al.,* 2005; Cottam *et al.,* 2006). It should be noted that with globalization of trade even areas where FMD is endemic can suffer from introduction of virus strains that are exotic to the region (Brückner *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 products is a significant issue and the probable main route of virus introduction into FMD free areas (WRL, 2007).

2.3.3. The disease situation in sub-Saharan Africa 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 intra-typic variants occur. Furthermore, wildlife plays a unique and important role in the epidemiology of the disease in Africa although this aspect has been adequately investigated only in Southern Africa (Hargreaves *et al.,* 2004; FAO, 2006). Regardless of the disease endemicity in nearly all countries of sub-Saharan Africa, the majority of outbreaks remains unrecorded and is not notified in a timely fashion due to trade restrictions and pastoral systems where inadequate in disease surveillance and sometimes absent; the transport of sampling material is difficult and expensive, few African laboratories are able to confirm the diagnosis of FMD (FAO, 2006; Rweyemamu *et al.,* 2008). Trans-boundary live animal movements are part of the

main characteristics of husbandry systems in many African regions; drought conditions, refugee movements and frequent cross-border trade have probably increased the risk of the entry and spread of FMD. In most pastoral areas of the continent livestock trade is active across border areas, dynamic and predominantly driven by price differential, and hence, livestock movement (both formal and informal) plays an important role in the spread and epidemiology of FMD (Vosloo *et al.,* 2002; Sahle, 2004). 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 (Sahle, 2004; FAO, 2006; Rweyemamu *et al.,* 2008).

The situation of FMD in infected areas indicates that FMD types continually spread within endemic regions, and periodically and unpredictably 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; Hargreaves *et al.,* 2004).

2.3.4. Susceptible hosts

Foot and mouth disease is highly contagious and affects over 70 domestic and wild life species of animals; however, not all FMD viruses have the same host range (Hedger, 1981; Sáiz *et al.,* 2002). As a whole the sensitive species belong to the mammalian order of *Artiodactyls.* Of the domesticated species, cattle, pigs, sheep, goats, camels and llamas and buffalo are susceptible to FMD. Similarly, many species of wild life, such as deer, antelope, wild pigs, Warthogs, elephants, giraffes, hedgehogs and tapirs 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 (Moutou, 2002; OIE, 2004). Human infections have been reported but are extremely rare and mild (Geering and Lubroth, 2002).

The susceptibility of cloven-hoofed animals varies with species of animal and strain of the virus. The disease is considerably less obvious or sub-clinical in 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; Kitching and Hughes, 2002; Kitching and Alexanderson, 2002).

2.3.5. 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 (Vosloo *et al.,* 2002; Bastos, 2001; Bastos *et al.,* 2003; Knowles and Samuel, 2003; Sangare *et al.,* 2003; Sangare, 2005).

Currently, DNA 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 subgroups (genotypes, topotypes) 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). Virus isolates from the same epizootic differ by ≤ 1%, viruses belonging to the same epizootics differ by < 7%, viruses of the same genotype differ up to 15% and viruses from different genetic lineage, differ by ≥ 20% (Sahle, 2004). Sequence data has also been instrumental in identifying outbreak resulting from improperly inactivated vaccine and for refuting

vaccine involvement in outbreaks (Murphy *et al.,* 1999).

2.3.6. Source of infection and mode of transmission

Foot and mouth disease is very contagious because a small dose of the virus is infectious and several routes of FMD virus 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 a 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 (Geering *et al.,* 1995; 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 extremely rapidly because of the high level of challenges from infected animals. FMD virus also readily spreads indirectly by a variety of inanimate objects including animal food staff, beddings, farm equipment, livestock holding areas, transport vehicles and so on that have been contaminated with acutely infected animals excretions and secretions such as saliva, milk, faeces and urine. Outbreaks of FMD 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 (Kitching *et al.,* 2007). 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 (Geering and Lubroth, 2002). 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 (Kao, 2001; Gloster and Alexanderson, 2004).

Airborne spread of the virus is determined by the numbers and species of animals affected, the virus strain, the environmental conditions, and the species and number of animals located downwind. Infected pigs emit high levels of airborne virus which can be as high as 4 x 10^6 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 emit or excrete less airborne virus between 10^3 to 10^8 infectious virus units per day as an aerosol, but are highly susceptible to airborne infection of respiratory route and a dose of 20 TCID50 (Tissue culture infective dose) of virus is sufficient to establish infection (Gloster and Alexanderson, 2004). Sheep and goats excrete airborne virus at levels similar to cattle but are thought to be less susceptible to airborne infection than cattle due to their smaller respiration volume (Gloster and Alexanderson, 2004). 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).

2.3.7. Immune response

The protection of a susceptible host against FMD virus correlates with the neutralizing antibodies level. Infection with one-serotype produces complete protection against homologous virus, but little or no protection against heterologous viruses (Samina *et al.,* 1998). Serotype specific immunity is based on the presence of neutralizing antibodies to one of the viral capsid proten, VP1, develops 7 to 21 days after exposure to the virus. The immunoglobulin M (IgM) is most prevalent in the early convalescent serum and is less specific to the different serotypes than Immunoglobulin G (IgG). IgG is produced in the later stage during the FMD infection and the reaction between the serotype and the homologous antibodies is highly specific. It 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 (Mulcahy, *et al.,* 1990).

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

2.3.8. Clinical signs

The clinical signs of the disease can range from a mild or in apparent in sheep and goats to a severe disease in cattle and pigs. The severity of clinical signs of the disease varies with the strain of the virus, the exposure dose, the age and breed of the animal, the host species and their status of immunity (OIE, 2004). 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. The incubation time may be even shorter in experimental infections. Viremia in cattle lasts for 3-5 days and the lesions develop 2-14 days post infection (Kitching, 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 (Woodbury, 1995; Sahle, 2004). Pregnant cows may abort, presumably as a consequence of fever (Murphy *et al.,* 1999) and young calves up to 6 months of age may die suddenly before the appearance of any clinical signs of the disease due to virus infection of the developing heart muscle (myocarditis) (Radostits *et al.,* 2000).

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. A

chronic panting syndrome characterized by dyspnoea, anorexia, and hair overgrowth and heat intolerance has been reported as a sequel of cattle recovered from FMD associated with pituitary gland damage (Burrows *et al.,* 1981). In sheep and goats the clinical signs of FMD are very mild or in apparent (Donaldson and Sellers, 2000). The disease can easily be overlooked until and unless individual animals are carefully examined for disease lesions. In sheep and goats if clinical signs occur, it may include dullness, fever, and small vesicles (0.5-2 mm) or erosions on the dental pad, lips, gums, and tongue (Uppal, 2004). Mild lameness may be the only sign. 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).

In swine, lameness is often the first sign. Other signs include fever about 40-40.6^{o}C, anorexia, reluctance to move, and squeal when forced to move. These signs are followed by formation of vesicles on the coronary bands, heels, snout and in the inter-digital space (foot involvement is usually severe). Mouth lesions are not too common and when they occur they are smaller and of shorter duration than in cattle and tend to be a dry-type lesion; there is no drooling; sows may abort; and piglets may die without showing any clinical sign (Radostits *et al.,* 2000).

2.3.9. Morbidity and case-fatality rate

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 generally very 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 (MacLauchlan and Dubovi, 2011). Mortality in suckling pigs and lamps ranges from 20-75% in most extreme cases and it is highly age dependent, infect for animals under 4 weeks of age, mortality is high and decrease rapidly as animals get older (>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).

2.3.10. Pathogenesis

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). Following 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 frank 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). In cattle, virus may be detectable for periods up to 2 years after exposure to infection, in sheep for about 6 months (Knipe and Howely, 2001).

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 generally 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).

2.4. Diagnosis

In cattle, FMD should be considered whenever salivation and lameness occur simultaneously and when a vesicular lesion is seen or suspected. Due to highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a virus secure laboratory (OIE, 2004). Diagnostic samples include vesicular fluid epithelium, blood in anticoagulant,

serum and esophageal/pharyngeal fluids collected with a Pro-probing (Quinn *et al.,* 2005). When epithelium tissue is not available from ruminant animals e.g. in advance or convalescent cases and infection is suspected in the absence of clinical sign, samples of oesophageal-pharyngeal (OP) fluids is collected by means of a probang and used for virus isolation (OIE, 2004).

For virus isolation specimens suspected to contain FMDV are inoculated into cell culture (primary pig, calf, and lamb kidney), BHK-21 and IB-RS-2 cells, incubated at 37ºC and examined for Cytopathic effect (CPE) after 48 hours (OIE, 2004). The serological tests, virus Neutralization (VN) and liquid phase blocking ELISA are prescribed for trade (Blood *et al.,* 1994). The antibody detection by 3 ABC ELISA can be used on a herd basis to detect FMDV infection in vaccinated and unvaccinated population (OIE, 2004).

The polymerase chain reaction (PCR) can be used to amplify the genome fragments of FMDV in diagnostic material (Quinn *et al.,* 2005). Differential diagnosis for FMD should include vesicular stomatitis, rinderpest, malignant catharal fever, the bovine herpes 1 infections, swine vesicular disease, vesicular exanthema of swine and bluetongue (Blood *et al.,* 1994).

Diagnosis based on clinical signs alone is complicated by the fact that at least two other viral disease of livestock, vesicular stomatitis (VS) and swine vesicular disease (SVD) produce lesions which are identical to those of FMD. Horses are affected by vesicular stomatitis but not by FMD or SVD. Swine vesicular disease affects only pigs. Lesions induced by bovine papular stomatitis, bovine herpes mammilitis, infectious bovine rhinotracheitis, bovine mucosal disease, malignant catarrhal fever, rinderpest in cattle, bluetongue, parapox-virus, peste des petits ruminants and footroot in sheep may also be mistaken for FMD and all these listed diseases should be considered during the diagnosis (Re´mond *et al.,* 2002).

2.5. Prevention and control

Foot and mouth disease is subject to national and international control and the measures taken depend on whether the country is free from the disease, is subject to sporadic

outbreaks or has endemic infection (Aitken, 2007). 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 generally directed at protecting high yielding dairy cattle by a combination of vaccination and control of animal movement (Quinn and Markey, 2003).

Preventive measures in the absence of disease should be implemented as follows: Control of national borders to regulate or prevent significant movement of animals and livestock products from non-free neighbors or trade partners. For officially free countries, prohibition of imports of animals and livestock products from non free countries in accordance with the OIE standards, prohibition to distribute untreated catering waste (human food) to pigs. Emergency measures in the event of outbreaks through: Rapid slaughter of infected animals, in contact animals and herds considered to have received infection by contact, to reduce the quantity of virus released policy of "stamping-out" (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 containment measures for such herds or villages, depending on the risk identified. And also possible emergency vaccination is important (Ding, 2013; Radostits *et al.,* 2007).

Immunity to one serotype provides protection only against the homologous viruses. In some cases, inactivated bi-, tri-, or polyvalent vaccine, which contains the representative strains of the serotypes that are in circulation in the region, must be used; therefore, 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, 2004).

In Ethiopia context the control of FMD is practiced by involvement of quarantine, restriction of animal movement, isolation of infected animals, vaccination programs,

proper disposal of infected carcass and other methods which are feasible to Ethiopian economy (Tassew, 2011). Currently there is no country-wide vaccination program aimed to control FMD and a ring vaccination is carried out around an infected area. Considering the wide prevalence of serotypes O, A and SAT 2 the National Veterinary Institute (NVI) is producing an inactivated trivalent vaccine (Tesfaye, 2014). The procedures commonly used are; control by eradication and control by vaccination or a combination of the two (Radostits *et al.,* 2007).

2.6. Economic impact of FMD

Foot and mouth disease remains one of the most important livestock diseases of the world, given its highly infectious nature, its broad economic impacts on animal wellbeing and productivity, and its implications for successful access to domestic and export markets for livestock and products (Perry *et al.,* 2006; Perry and Rich, 2007). The impacts of the disease vary markedly between developed and developing countries, and also within many developing countries depending on the species involved, the genotype of animal, the level of productivity, the significance of livestock to livelihoods, and the effectiveness of indigenous coping mechanisms for controlling the effects of FMD (Perry *et al.,* 2002b; Catley *et al.,* 2004).

At farm or individual level, 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 substantially lower farm incomes, particularly in susceptible highly productive animals (James and Rushton, 2002). Overt clinical disease affects animals' performance directly and causes reduction of milk yield in dairy cattle by 20%, growth rate of beef animal by 10-20%, pig meat production up to 20% per annum and inflict fertility impairment due to increased abortion rate up to 10% (Knight *et al*., 2013). Moreover, death of very young animals, culling of unproductive and chronically infected animals, and loss of valuable breeding stock and disruption of livestock improvement programs are also attributed to the direct effect of the disease (Catley *et al.,* 2004).

In less commercialized production systems in Africa and Asia where indigenous breeds predominate, these direct effects may be minor (Randolph *et al.,* 2002). However, livestock often serve multiple purposes in these systems, such as providing draught power, so that the temporary lameness reported up to 60-70% of working buffalo and cattle caused by FMD can also lower farm income by reducing crop production or transport services. Furthermore, if the disease affects livestock kept by the poor who have few such productive assets, the impact on their livelihoods can be especially devastating and similarly pastoralists severely suffer by the direct impact of the disease since their livelihood directly depend on livestock production (Perry *et al.,* 2002a; Doel, 2002; Rufael *et al.,* 2008; Bayissa, 2009).

From a current global perspective, the indirect effect and the risk of the disease has a much greater impact than that of the disease itself. Much of the burden (cost) of containing outbreaks, as well as maintaining the ongoing capacity and measures intended to reduce the risk of FMD such as vaccination, surveillance, movement control and so on can incur an extremely 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. Measures taken to contain an outbreak can also affect other sectors, such as the impact on tourism in the UK outbreak (Thompson *et al.,* 2002). The presence of FMD has also a negative impact on the integration of livestock and wildlife based activities, which is believed by many to be ecologically and financially desirable. Thus the disease can exert considerable influence on the development of integrated land use policies in South African countries (Sutmoller *et al.,* 2000).

Currently, the single largest impact of FMD is undoubtedly its critical role as a restriction to international trade. The highest value markets for livestock products are in FMD free countries, and these countries are allowed to restrict or ban imports of livestock products and sometimes other products as well as a potential risk of introducing FMD (Gulati *et al.,* 2005). The presence of FMD in a country is considered as binding constraint to developing an export sub-sector within the livestock industry, and, therefore, contributes

to unavoidable loss. 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 (Rweyemamu and Astudillo, 2002; Rich and Winter, 2007).

2.7. Foot and Mouth Disease Situation in Ethiopia

2.7.1. Disease status

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 also frequently noted in the highlands of the country (Tefera, 2010).

2.7.2. Spatial distribution

FMD is widely distributed in all areas of Ethiopia, although the level of the disease prevalence may show significant variations across the different farming systems and agro-ecological zones of the country. The national picture of FMD status by summarizing the outbreak data reported to Ministry of Livestock and Fishery (MoLF)/ formerly said Ministry of Agriculture and Rural Development (MoARD) from 2009-2015 is presented in (Figure 2).

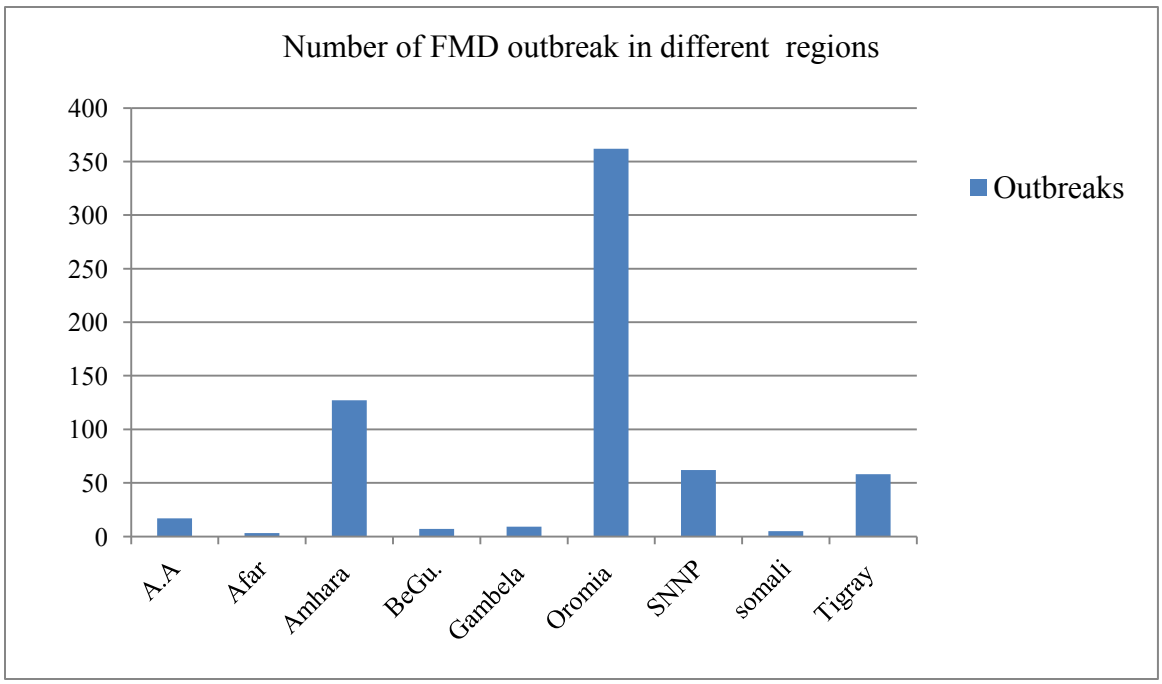

Figure 2: FMD outbreaks reported to MoLF from 2009-2015 from different Regional States of Ethiopia

2.7.3. Temporal distribution

According to MoLF, division of epidemiology directorate disease outbreaks report summary, FMD occurs at any time of the year however, the highest outbreaks of the disease are observed extreme dry seasons the years (Figure 3). Various researchers reported that this might be associated with factors such as drought. During dry seasons especially pastoralists are obliged to move their herds long distances in search of pasture and water and thereby transmission of highly contagious diseases like FMD exacerbated at herd gathering sites or communal points (Rufael, 2006; Legesse, 2008; Molla, 2009; Bayissa, 2009). On the other hand in most rural parts of the country during rainy seasons of the year wide areas of farm land is planted with crops. During this time huge numbers of domestic animals are kept on confined small plots of communal grazing lands that could favor occurrence and transmission of the disease (Personal observation).

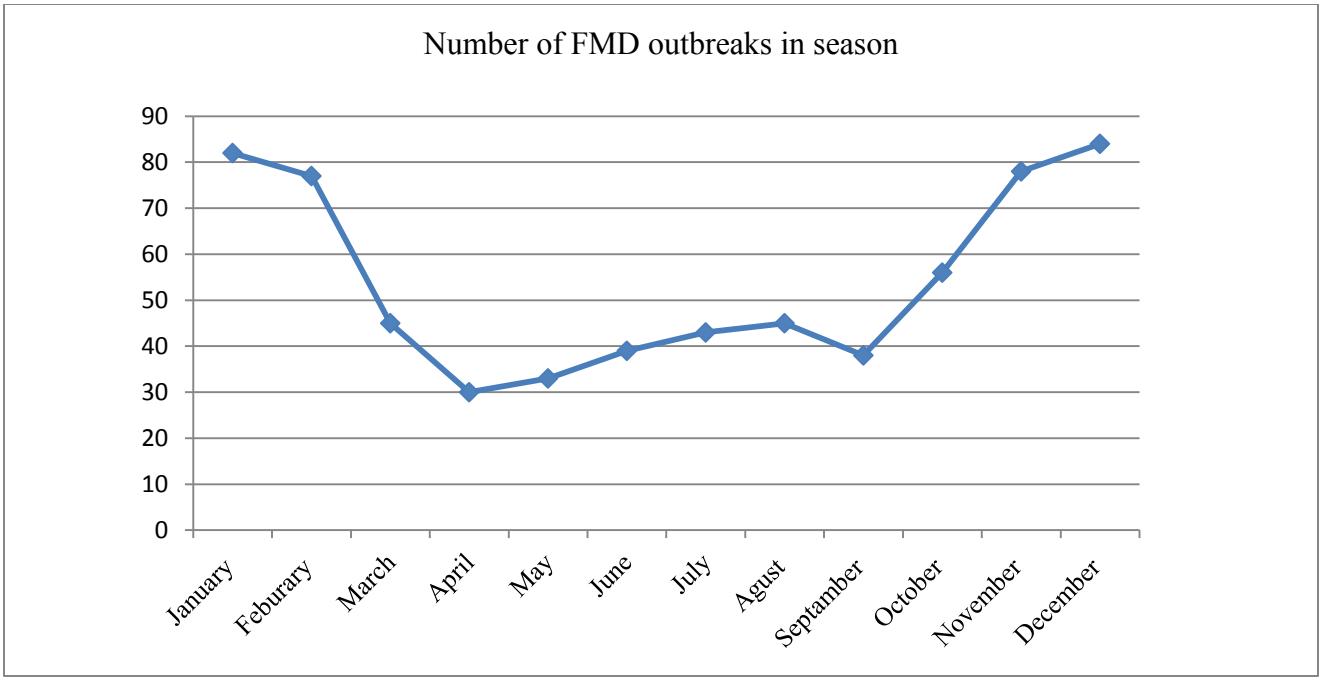

Figure 3: FMD outbreaks reported to MoLF by monthly bases, from 2009-2015

2.7.4. Disease occurrence frequency

FMD outbreaks are reported frequently throughout the country (Asfaw and Sintaro, 2000). In the past seven years (2009-2015) on average 93 numbers of FMD outbreaks were reported to MoLF annually. The outbreaks occurred every year, but most were reported in 2011 and 2012 each 124 and 205 outbreaks, respectively (Figure 4). However, considering the figures provided are definitely underestimated and do not reflect the reality of the epidemiological situation in the country due to endemic nature of the disease and the unreported cases by farmers (MoLF, 2016).

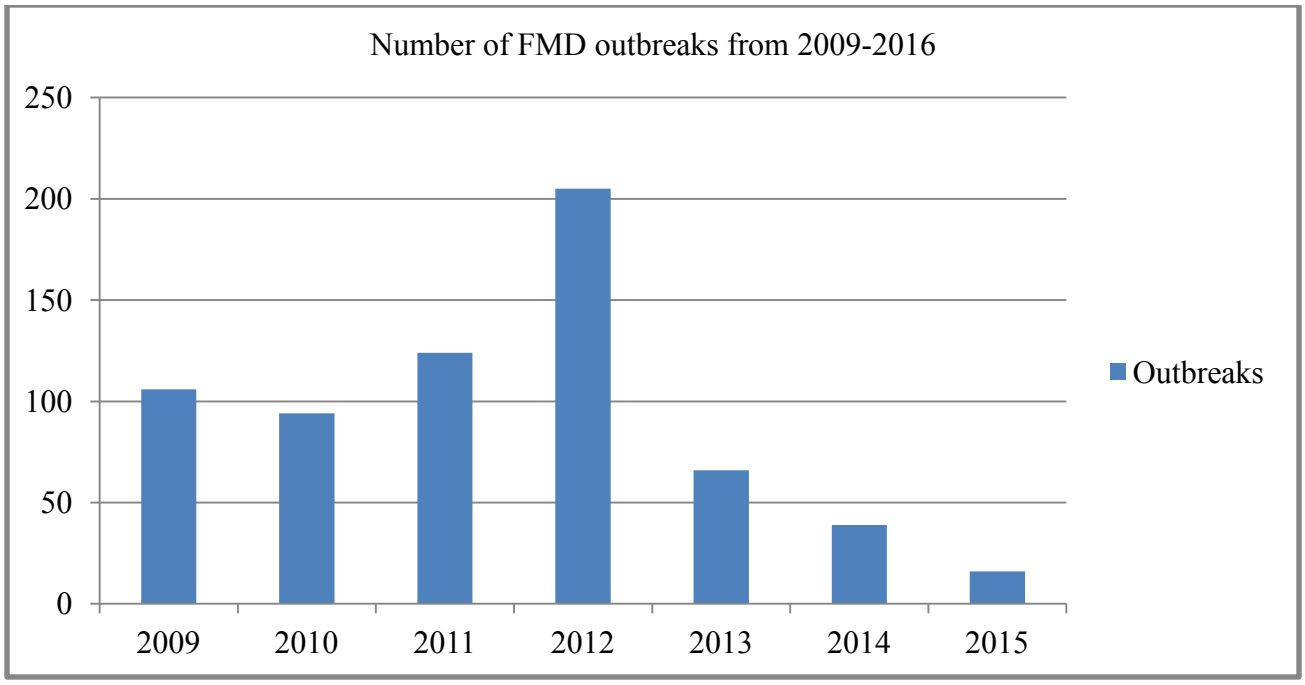

Figure 4: Numbers of FMD outbreaks reported to MoLF from different part of the country from 2009-2015

2.7.5. Disease prevalence

Despite the widespread occurrence of the 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 (Table 2), and the 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 (Sahle, 2004).

Table 2: Overall sero-prevalence of FMD in different parts of the country

Location	No. of cattle tested	Sero-positive	Prevalence (%)	References
Borana	920	193	21.0	Rufael *et al.,* 2006
Afar	765	43	5.6	Jembere *et al.,* 2008
Borana	768	177	23.0	Bayissa *et al.,* 2009
South Omo	770	63	8.2	Molla *et al.,* 2009
Somalia Regional State	384	59	14.05	Mohamoud *et al.,* 2011
Dire dawa	986	79	8.01	Abunna *et al.,* 2013
Kellem Wollega Zone	384	82	21.4	Desissa *et al.,* 2014
Central Ethiopia	38,187	5,536	14.5	Alemayehu *et al.,* 2014
Western Ethiopia	1,144	103	9	Beyene *et al.,* 2015

2.7.6. FMD virus serotypes identified

Research findings and records from National Animal Health Diagnostic and Investigation Centre (NAHDIC) and National Veterinary Institute (NVI) of Ethiopia indicate that five of the seven FMDV serotypes (O, A, C, Southern African Territories SAT-1, and SAT-2) were identified in Ethiopia 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 the outbreak areas. Cattle were found to be infected with all circulating serotypes of FMDV, where as swine had only serotype O (Sahle, 2004; Gelaye *et al.,* 2005; Legesse, 2008; Nigussie, 2010; Ayelet *et al.,* 2009).

During the period of 1957-73, 62 outbreaks of serotype O, 24 of serotype C and 12 of serotype A were recorded (Berson *et al.,* 1972). Following this period, until 1992, FMD outbreaks due to serotypes O and A were common (Haileyesus, 1988; Roeder *et al.,* 1994). Serotypes O and SAT-2 viruses were identified as the major causative agents of FMD during 1988-1991 (Roeder *et al.,* 1994). FMDV serotypes O and C were first recorded in Ethiopia in 1957 while serotype A was identified in 1969 (Martel, 1974;

1975). Serotype C was not identified after 1983. It seems to have disappeared from Ethiopia. However, a recent report of serotype C specific antibodies in cattle in Ethiopia indicates that circulation of serotype C viruses in the country may have gone unnoticed (Rufael *et al.,* 2008).

The first isolation of SAT-2 in Ethiopia was in 1989 in a sample collected from cattle raised in Leben Ranch, Borena Zone, southern Ethiopia (Roeder *et al.,* 1994); the virus was detected for the subsequent 2 years in 1990 and 1991. After an apparent gap of 16 years, serotype SAT-2 was recorded in 2007 from a bovine sample collected from 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 the 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 the sample collected from Bovine and small ruminants of Itang, Gambella, Abobo and Lare Districts, Gambella region (Tefera, 2010) and serotype O was also isolated from the out breaks occurred in Addis Ababa, Debre Berhan and Debreziet (Tesfaye, 2014).

The presence of FMDV serotype SAT-1 in Ethiopia was isolated and reported for the first time in 2008 (Legesse, 2008), from three species of animals; cattle, sheep and goats. Although SAT-1 has not been previously reported in Ethiopia, it might be circulating within wildlife and infrequently transmitted to domestic animals (Martel, 1974; 1975; 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 (19.50%), SAT-2 (4.10%), SAT-1 (1.80%) and C (1.40%), respectively (Gelaye *et al.,* 2005, 2007; Ayelet *et al.,* 2009).

2.7.7. Risk factors for FMD in Ethiopia

Risk factors for FMD may include factors that may change the level of risk (e.g. new serotypes or biotypes, or changing epidemiological or live stock 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 factor in Ethiopia. Hence from 58 FMD outbreak occurred in different regional state of country showed all the virus serotypes and topotypes were similar, i.e. O serotypes, EA-3 except the Makelle 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 an apparent gap of sixteen years (Ayelet *et al.,* 2009).

2.7.8. FMD control program in Ethiopia

At present, FMD is considered as one of the most important livestock diseases demanding urgent control intervention that should result in minimizing the impact of FMD to the level that won't be a major cause of international trade barrier. Conversely, the complex nature of the disease, its wider distribution across the country and absence of proper vaccination program with FMD vaccine within Ethiopia demanded that control strategies be implemented progressively on a short and medium to long-term basis. Measures such as disease free zone establishment and mass vaccination of the national cattle herds may have important contributions to minimize the impact of the disease. However, these measures will require huge financial and logistic resources that their consideration should be viewed from long term perspective (MoARD, 2006).

Therefore, the short term FMD vaccination program give emphasis to the control of all outbreaks occurring in the country through ring vaccination and vaccination of all export cattle before entering the quarantine stations. To protect export animals from contracting the disease while being kept in quarantine sites cattle found within 10 km radius of these sites could be vaccinated. All dairy animals should also be vaccinated (MoARD, 2006). Currently, a trivalent vaccine manufactured from locally isolated FMDV serotypes SAT2, A and O is produced by the NVI (Tesfaye, 2014). The virus is propagated from cell culture and absorbed into aluminium hydroxide gel and inactivated with 0.3% formaldehyde and adjuvinated with saponin. The recommended dosage 4ml per head is administered to cattle subcutaneously, preferably in the dewlap region. In order to protect the cattle, two injections at 6 months interval are recommended. Immunity develops 2-3 weeks after vaccination and may last for one year (DACA, 2006).

3. MATERIALS AND METHODS

3.1. Study Areas

The study was conducted in sites where outbreak of FMD occurred in Ethiopia from September 2015 to May 2016 to isolate and characterize virus responsible for the outbreak. Outbreaks have been reported at Addis Ababa (kolfe), Arsi zone (Guna and Lude Hitosa) and East shewa zone (Adama and Boset). Across sectional study was also conducted to study sero-prevalence of FMDV in and around Adama and Assela dairy farm animals. These study areas were selected based on the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes Inc., 2010).

Addis Ababa has an altitude of 2300 meter above sea level with a subtropical high land climate. The average annual rainfall and average maximum and minimum temperature for the area are 1180 mm and 22.8ºC and 10.6^{o}C, respectively. It is found between 9°1′48″N latitude and 38°44′24″E longitude. It has a humid subtropical mild summer climate that is mild with dry winters, mild rainy summers, and moderate seasonality. The city is fully surrounded by Oromia Regional Government in all directions (CSA, 2009).

The second study area was Arsi zone, which is found in Oromia regional state; with Asella city being the administrative center. Asella is located at distances of 175 kilometers south-east of Addis Ababa. This has a latitude and longitude of 7°57′N and 39°7″E, respectively with an elevation of 2,430 meters above sea level. The zone is located at altitude ranging from 1500 to 4245 meters above sea level and is known as the crop belt of Ethiopia due to its optimal agro-ecology and flat terrains. The total number of cattle found in Arsi Zone is 2,528,903 and area of the Zone is approximately 19,825.22km^2. It receives biannual rain falls and the average temperature ranges from 10 to 25°C (CSA, 2015).

The third study area was East Shewa Zone, which is located in the central parts of Oromia regional state of Ethiopia; with Adama city being the administrative center. Absolute location of East Shewa Zone extends from 7^0 33′0″ to 9^{0}08′56″N and 38^{0}24′10″E to 40^0 05′ 34″E which indicate that this zone is located in tropical climatic zone though the

climate is influenced by altitudinal variation. The total number of cattle found in East Shewa Zone is 1,147,173 and the Zone covers approximately 9,633.52km^2. The altitude ranges from 500 to 4307meter above mean sea level. The Zone can be categorized under rift valley system of Oromia since about 93% of the total area of the zone is completely located in rift valley system. The mean annual temperature varies between 18^{0}c and 30^{0}c and its mean annual rainfall is 410mm-820mm (CSA, 2015).

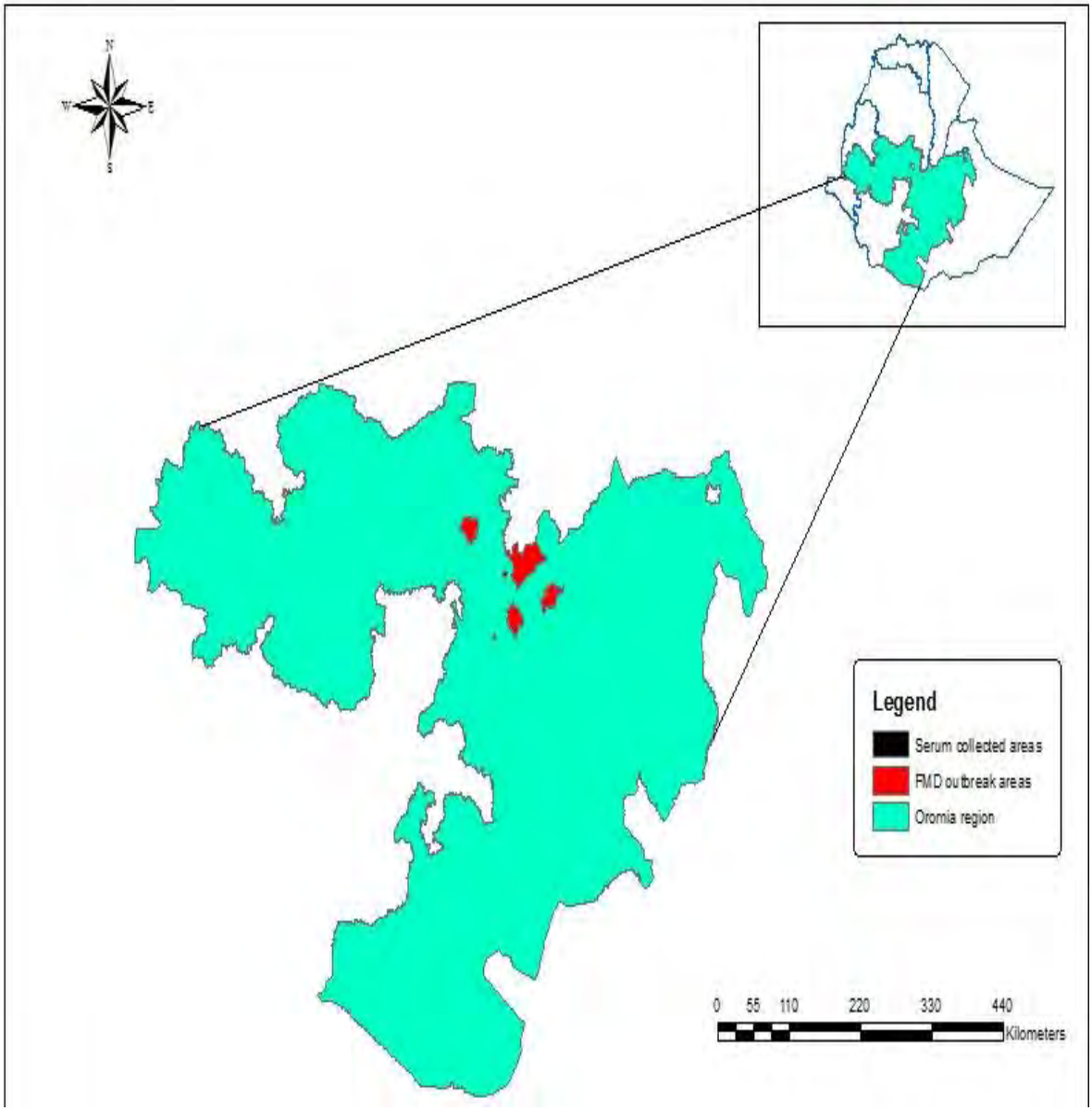

Figure 5: Map of Ethiopia showing the study areas

3.2. Study Animal Population

The study was conducted in 378 Cattles that had experienced outbreaks of FMD. Cattles of all age and breed reared under intensive, semi-intensive and extensive production and management system were included in the outbreak investigation. In addition, for sero-prevalence study of FMD, the study population was composed of households which keep dairy cattle from individual farm to highly organized dairy farm level. Peasant association (PA) in the study area was selected randomly. Number of animals sampled for this study was determined by using standard sampling procedures (Thrusfield, 2005).

3.3. Study Design

Cross sectional study design was used for outbreak investigation so as to isolate and characterize the serotypes of FMD virus circulating in that particular ecology. For this specific task, tissue and oro-pharyngeal (OP) fluid specimens were collected from infected and apparently healthy in contact animals for diagnostic testing. Moreover, a cross-sectional study design was also implemented to determine the sero-prevalence of FMD in dairy cattle. Blood for serum separation was collected from animals and the serum was tested using 3ABC-ELISA (Louis Pasteur, Grables, France). Breed, age, sex, animal composition and herd size history of sampled animals were considered as major risk factors to the occurrence of the disease. Sampled animals were grouped into three age categories based on their dental eruption status (Appendix 2).

3.4. Sample Size Determination

The sample size for dairy cattle in Adama was calculated on the basis of previous report of 14.5% sero-prevalence of FMD (Alemayehu *et al.,* 2014). Therefore to determine the sample size of dairy cattle in this area, 14.5% was used as expected prevalence (P_{exp}) and 95% confidence interval and 5% required precision (Thrusfield, 2005).

$$n = \frac{1.96^2 \times P_{exp} \times (1-P_{exp})}{d^2}$$

$$n = \frac{3.84 \times 0.145 \ (1-0.145)}{(0.05)^2} = 190$$

In Asella, since there was no previous study done in the area, by considering 50% expected prevalence, 95% confidence interval and 5% required precision, 384 cattle were selected for this study (Thrusfield, 2005).

$$n = \frac{1.962 \times P_{exp} \times (1-P_{exp})}{d2} \quad \text{(Thrusfield, 2005)}$$

Where n= required sample size, d= desired absolute precision, P_{exp}= expected prevalence (50%). Hence, a total of 574 dairy cattle (384 from Asella and 190 from Adama) were considered for this study.

3.5. Sampling Technique

Multistage sampling technique was applied for the determination of sero-prevalence of the disease. Adama and Asella were purposively selected sites for sero-prevalence study of FMDV based on their accessibility, geographical location and on the abundance of dairy farms that constituted the known milk sheds. From each District 30% of *Kebeles*/PAs were selected randomly (Appendix 1). From each *Kebele*/PA, 20% of privately owned herds were selected randomly to obtain primary sampling unit. An effort was made to include different herd sizes and composition in the PA. Finally, 10% from Adama and 20% from Asella, individual animal from each herd were selected randomly as secondary sampling unit to attain the required number of sample size (Thrusfield, 2005). During FMD outbreak, a field investigation was conducted purposively at the specific site of the outbreak within the study Districts and animals with the clear signs, symptoms and suspected to be diseased with FMDV were sampled.

3.6. Ethical Consideration

Ethical clearance for this study was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Minutes of Animal Research Ethics and Review committee. A seven page request for explanation of the purpose of carrying out the studies and all possible care planed to reduce animal suffering due to sampling was given to the committee. After the committee evaluated the significance of this research, approval was given (minutes number AREC001/2016). Before sampling all animals

included in this study, consent was asked from the owners and only animals from volunteer owners were sampled.

3.7. Study Methodology

3.7.1. Field observation

In order to arrive at sound and unbiased conclusions with respect to this study, undertaking visual assessment of the target areas was of paramount importance. Therefore, field observations and exploration have been considered in all the representative sample areas.

3.7.2. Clinical examination

Soon after arrival at the specific outbreak sites during disease outbreak investigation, the animals were examined from a distance for evidence of salivation and lameness in each individual animal owner's homestead since there was no crush in the area. Salivating and/or limping animals were restrained for thorough examination and sampling. The mouth cavities of salivating animals were opened and examined for evidence of intact and/or ruptured vesicles, erosions and ulcers on the tongue, dental pad and mucosa of the oral cavity. The hooves of lame animals were thoroughly washed with water and then carefully examined for similar lesions particularly on the coronary bands and inter-digital spaces.

3.7.3. Sample collection

Serum

A total of 10ml blood was collected from the jugular veins of randomly selected cattle into sterile vacutainer tubes and stored overnight at room temperature for serum collection. The serum was then transferred into a sterile cryovial bearing the identification number, species, village, age and sex and transported in icebox to Asella Regional Veterinary Laboratory and then stored at -20°C. Finally, at the end of each sampling sera were transported in cold chain to NAHDIC, Sebeta, and stored at -20^{o}C (OIE, 2004).

Tissue and oro-pharyngeal (OP) fluid samples

Active tissue samples were collected from the gum and tongue of cattle in one outbreak case. Epithelial tissue was collected from un-ruptured or freshly ruptured vesicles and placed in a bottle with transport medium composed of equal amount of glycerol and 0.04M phosphate buffer with antibiotics (OIE, 2004). Samples of OP fluid were collected from apparently healthy cattle that were found in close proximity to the affected herd. The OP fluid was collected by a probang cup and poured into a 20 ml bottle. 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, 2004). Then, the samples were labeled and transported to NAHDIC, Sebeta, in similar fashion as for serum transportation. Both tissue and OP fluid samples were kept at -80^{o}C until ready for laboratory investigation at NAHDIC, Sebeta (OIE, 2004).

Specimen shipment process to World Reference Laboratory for FMD (WRL)

Preserved virus isolates of different outbreak areas were further processed before shipment to WRL, Pirbright England. A total of 3 pooled viral samples were prepared in 2 ml cryotubes and sealed with Para film (Table 3). The isolates were sent in dry ice to the WRL. Sample submission to the WRL was facilitated by NAHDIC, Sebeta.

Table 3: Viral isolates submitted to WRL for virus characterization

Outbreak locations		Species	Number of samples	
District	PAs/Kebele		Viral isolate	pool
Guna	Chire Anole	Bovine	8	1
Ludehitosa	Boruwodecha	Bovine	5	1
Adama	01	Bovine	4	1
Total	3		17	3

3.7.4. Serological diagnostic tests

Sera collected from bovine species was tested by FMDV 3ABC-Ab ELISA (ID Screen® Louis Pasteur, Grables, France) for the detection of antibody to 3ABC poly protein which is a useful indicator of FMD virus infection regardless of the serotype involved

(Mackay *et al.,* 1998). Antibody to 3ABC (non structural protein) is found only in virus infected animals but not in vaccinated animals (Ding, 2013). Therefore, in this study the test was undertaken for screening and determination of positive and negative proportions of the test sera.

Briefly, the test was carried out stepwise as per the manufacturer's manual. First all reagents were kept at room temperature and homogenized by vortexing. The test was carried out in 96 well micro plates. Then 50µl of dilution buffer 18 were added in to each well. Thirty µl of positive control were added in to wells A1 and B1 and the same volume of negative control were also added to wells C1 and D1 while, the rest wells were filled by 30µl of test sera. Then incubated at 37^{o}C for 2hours, after incubation the wells were washed 5 times with adding 300µl of wash solution. After washing 100µl of the conjugate 1X were added in to each wells and incubated for 30min at 21^{o}C. After incubation the wells were washed 5 times with 300µl of wash solution, then 100µl of the substrate solution (TMB) was added in to each wells and incubated at 21^{o}C for 15minutes in dark. To stop color reaction 100µl of stop solution was dispensed into each well. Finally the optical density (OD) readings were recorded using a spectrophotometer at wavelength of 450nm. Plate lay out used for the test is indicted on Appendix 5.

Validation

The test result was validated if:

- ✓ The mean value of negative control O.D. (OD_{NC}) was greater than 0.7.

  $OD_{NC} > 0.7$
- ✓ The mean value of the positive control O.D. (OD_{PC}) is less than 30% of the OD_{NC}

  $OD_{PC}/OD_{NC} < 0.3$

Interpretation

For each serum sample, the competition percentage was calculated (S/N %):

$$S/N\ \% = OD_{sample}/OD_{NC} \times 100$$

Sample presented S/N %:

- ❖ Less than or equal to 50% were considered positive
- ❖ Greater than 50% were considered negative

3.7.5. FMD virus isolation

Virus isolation was established under laminar air flow class II cabinet. A suspension was prepared by grinding the sample with sterile sand in a sterile pestle and mortal with small volume tissue culture made and antibiotics (penicillin, streptomycin and Amphotericin B solution) medium was added, so that the final volume was 10X that of the epithelial tissue, producing 10% suspension (OIE, 2012). The suspension was centrifuged at 2000 rpm for 10minutes. The suspension was filtered by Millipore filter of 0.22µm pore size. About 1ml of filtered tissue suspension was inoculated on confluent cultured baby hamster kidney (BHK-21) monolayer cells grown on 25cm^2 tissue culture flask and incubated at 37°C for 1hour for adsorption of the virus. Then infected cell was added with 8ml of maintenance media (2% MEM) and incubated at 37°C and 5% CO2 in a humidified incubator for 24-48hours. Cytopathic effect (CPE) was observed after 48 hours (or even less) in positive cases. If no CPE was detected, the cells were frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours before the samples were declared to be negative (OIE, 2012). Samples not exhibiting CPE by 72hours post infection on the second passage were considered virus negative. The cells were harvested when >85% of monolayer showed CPE. The viral isolates were labeled using the following format: three-letter country code/isolate number/year (e.g., ETH/02/2016). The three letter country codes were designated as outlined by the WRL for FMD.

3.7.6. Antigen detection ELISA

The assay was a sandwich ELISA (Brescia, Italy) performed with selected combinations of anti-FMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies. The test was applied for detection and typing of FMD virus. The kit was designed for detection and typing of FMD viruses of type O, A, SAT 1 and SAT 2. A pan FMD test, detecting any isolates of serotypes O, A, C, Asia 1 and some of the SAT serotypes was

included in the kit to complement the specific typing and to detect FMD viruses which might have escaped binding to selected serotype-specific MAb.

The test was conducted as per the manufacturer's recommendation. Since 6 samples were feasible to test on a micro plate containing 96 wells, one positive control for each of FMD types O, A, SAT 1 and SAT 2 and negative control were included in each plate. These controls were already incorporated into the ELISA micro plate (trapped by the respective catching MAb) (Appendix 6). First samples were diluted ½ in diluent buffer and 50µl of each diluted sample was distributed in 72 wells of A-F rows: 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 (positive and negative control respectively) rows; then plates were incubated at 25^{o}C for 1hour. After incubation, all fluids on the plates were discarded and all remaining residual fluids were removed by tapping. 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 were added from columns 1 to 8 and the same volume of conjugate B were added from columns 9 to 12. Plates were covered and incubated at room temperature for 1hour. After incubation 50µl of substrate (TMB) per well were added to all wells and plates were covered and left at room temperature for 20minutes in the dark. Reaction was stopped by adding 50µl of stop solution (sulfuric acid (H_2SO_4)). Immediately after blocking, 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.

Interpretation of the results

Results of the samples examined were interpreted as indicated in the table 4.

Table 4: 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 (untyped)	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

3.7.7. Reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing

The cell culture grown viruses were used for RNA extraction that was amplified by RT-PCR using primers that can amplify the VP1 (Ahmed *et al*., 2012). All RNA extraction, RT-PCR, and sequencing were conducted in WRL for FMD, The Pirbright Institute, UK (Ahmed *et al*., 2012). The VP1 sequences of FMD serotypes A and SAT 2 obtained from the WRL were used for molecular characterization.

3.7.8. Phylogenetic analysis

A total of one VP1 sequences of serotype A and two sequences for serotype SAT 2 FMD viruses were obtained from WRL for FMD, Pirbright Institute, UK. One sequence from each FMD serotype viruses were used for online blast search to retrieve closely related sequences from Genbank using Molecular Evolutionary Genetic Analysis software (MEGA, V 6.06). In addition some sequence accession numbers were obtained from published article (Ahmed *et al*., 2012) and the sequences were retrieved from Genbank.

The VP1 nucleotide sequences were aligned by using Clustal W program inbeded in the MEGA software. The alignment sequences were used to construct phylogenetic tree analysis using midpoint-rooted neighbor-joining tree and Kimura 2-parameter nucleotide substitution model using MEGA 6.06. The robustness of the tree topology was assessed with 1000 bootstrap replicates as implemented in the program.

3.8. Data Analysis

Data generated from laboratory investigations were recorded and coded using Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). To identify association of sero-positivity with the potential risk factors (origin, age, sex, management system, breed type, herd size and animal composition) were computed by Pearson's Chi-square test and logistic regression. In all the analyses, 95% confidence level and $p < 0.05$ was set for detecting significance results.

4. RESULTS

4.1. FMD Sero-prevalence

From the total of 574 sera collected from dairy animals and tested by 3ABC-Ab ELISA, 24.22% (139/574) were found positive. The higher prevalence was observed in and around Adama town compared to Asella (Table 5) and the difference was not statistically significant ($p > 0.05$).

Table 5: Prevalence of FMD in and around Adama and Asella dairy cattle

Origin	Sera tested	Test (+)	Prevalence (%)	95%CI	χ^2	p-value
Adama	190	51	26.84	0.20-0.33	1.06	0.304
Asella	384	88	22.91	0.18-0.27		
Total	574	139	24.22	0.20-0.27		

(+) = Positive, CI = Confidence Interval

4.1.1. Prevalence in relation to host intrinsic risk factors

FMD prevalence among different age groups

FMD sero-prevalence comparison was done in different age groups of dairy cattle. An increasing sero-prevalence trend was observed with increasing age (Figure 6) and the difference was statistically significant among age groups ($\chi2 = 37.43$; $p = 0.000$).

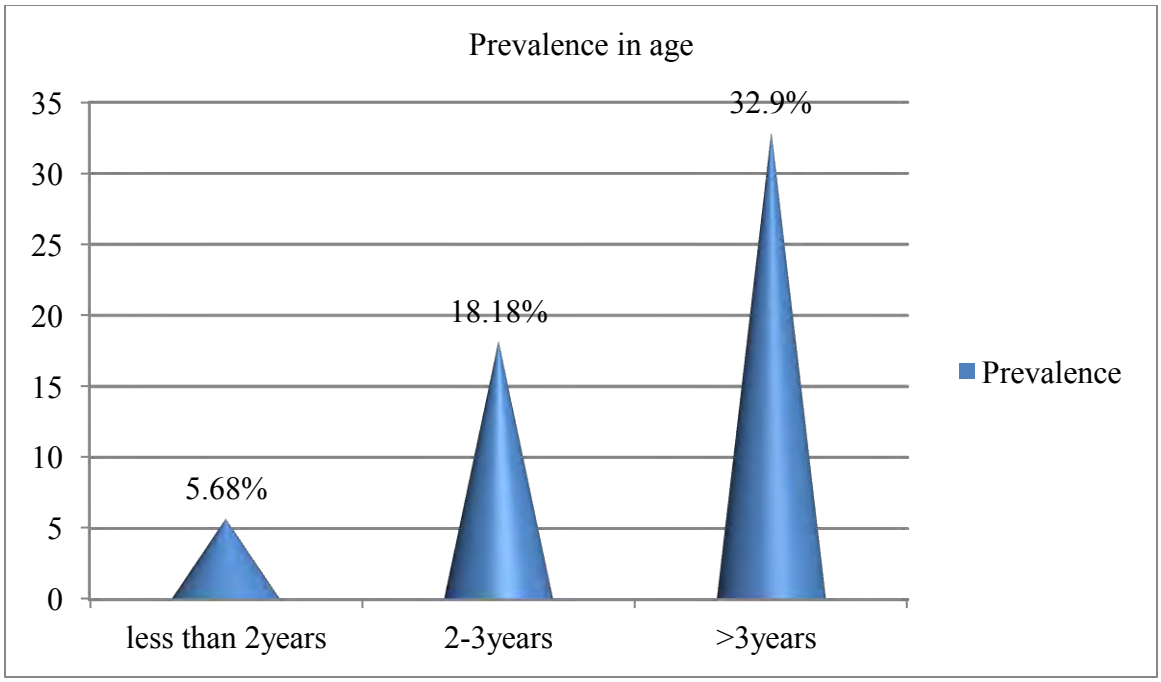

Figure 6: FMD prevalence within age groups in and around Adama and Asella dairy cattle.

FMD prevalence between sex groups

The prevalence estimate for female and male cattle was 25.26% and 18.95% respectively, (Table 6). The higher prevalence was observed in females than males, but the difference was not statistically significant ($p > 0.05$).

Table 6: FMD prevalence within sex groups in and around Adama and Asella dairy cattle

Sex	Sera tested	Test (+)	Prevalence (%)	95%CI	$\chi 2$	p-value
Female	479	121	25.26	0.21-0.29		
Male	95	18	18.95	0.09-0.38	1.80	0.179
Total	574	139	24.21	0.21-0.28		

FMD prevalence between breeds

Breed specific prevalence of 12.69% for local and 27.73% for cross was observed (figure 7). The difference between breeds was statistically significant ($\chi 2 = 14.02$; $p = 0.000$).

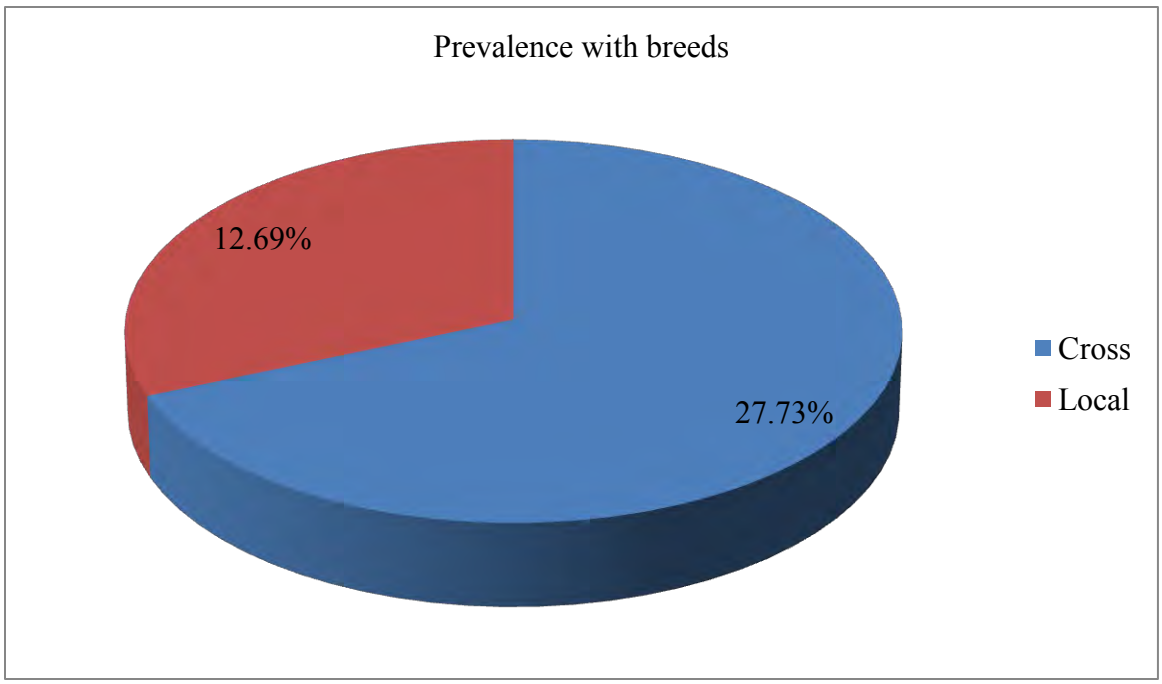

Figure 7: FMD prevalence within breeds groups in and around Adama and Asella dairy cattle

4.1.2. Prevalence in relation to host extrinsic risk factors

FMD prevalence in relation to herd size

Sero-positivity was assessed in herd size and an increasing sero-prevalence of FMD occurrence was observed in larger herds (Table 7). But the difference was not statistically significant for herds of cattle ($p > 0.05$).

Table 7: FMD prevalence in relation to herd size in and around Adama and Asella dairy cattle

Herd size	Sera tested	Test (+)	Prevalence (%)	95%CI	$\chi 2$	p-value
Small	438	98	22.37	0.18-0.26	4.76	0.092
Medium	77	20	25.97	0.16-0.36		
Large	59	21	35.59	0.23-0.48		

FMD prevalence in relation to animal composition and management system

Furthermore, other risk factors such as herd composition and managements were considered. Higher sero-prevalence was recorded in cattle that were kept together with small ruminants than herds of cattle without and the prevalence was significantly varied ($p < 0.05$). Animals managed semi-intensively have manifested greater FMD prevalence than those intensively managed (Table 8) and the prevalence was not varied significantly ($p > 0.05$).

Table 8: FMD prevalence in relation to animal composition and management in and around Adama and Asella dairy cattle

Risk factors	Tested	Test +ve	Prevalence (%)	95%CI	$\chi 2$	p-value
Animal composition						
Only cattle	422	93	22.04	0.18-0.26	3.99	0.040
Cattle and shoats	152	46	30.26	0.23-0.38		
Management						
Intensive	254	55	21.65	0.17-0.27		
Semi-intensive	320	84	26.25	0.21-0.31	1.64	0.200

4.1.3. Logistic regression analysis

The univariable and multivariable logistic regression analysis included effects of breed, history of herd contact with small ruminants, and age of animals (Table 9 and 10). Univariable logistic regression revealed cross-bred was found 2.64 times more likely to be infected by FMD than local breed. The risk of FMD occurrence was increased (odds ratio (OR) = 1.54) in herd of cattle and small ruminant combinations compared to herd of cattle that were kept alone. Animals greater than 3 years old were found, to be 8.14 times more FMD sero-positive than young animals (those found below 2 years old).

Table 9: Univariable logistic regression analysis of potential risk factors for cattle sero-positivity

Explanatory variable	Variable categories	OR	95% CI	p-value
Breed	Cross vs. local	2.64	1.52-6.67	0.001
Animal composition	With small ruminants vs. without	1.54	1.01-2.33	0.046
Age	Old *vs.* young	8.14	3.20-20.70	0.000
	Adult *vs.* young	3.69	1.38-9.83	0.009

OR, odds ratio; vs. versus; CI, confidence; old > 3 years, adult 2-3years, young < 2 years old.

Table 10: Multi-variable logistic regression analysis of potential risk factors for cattle sero-positivity

Explanatory variable	OR	SE	95% CI	p-value
Breed	2.79	0.81	1.59-4.92	0.000
Animal composition	1.54	0.34	0.99-2.38	0.048
Age	2.55	0.44	1.81-3.58	0.000

OR, odds ratio; SE, standard error; CI, confidence interval

4.2. Clinical Examination of FMD Outbreak

Out of 378 cattle clinically examined 109 (28.8%) of animals showed lesions and signs typical of FMD infection (Appendix 8). The major important clinical signs observed during the outbreak investigation were salivation and lameness. Mouth lesions consisted of erosions and ulcers on tongue and dental pad, whereas foot lesions comprised erosions on the inter-digital space and the coronary bands. In severe cases the hooves of affected animals tended to separate from the coronary bands. Reluctant to move and lagging behind herds and refusal of grazing were also seen as common features of clinically affected animals.

4.3. FMD Virus Isolation and Characterization

A total of 34 epithelial tissue and OP fluid samples collected from the three study sites, were subjected for virus isolation using BHK21 cell culture and CPE was developed on 28 samples while virus did not grow from the rest of 6 samples (Table 11).

From the 34 field epithelial tissue and OP fluids samples, 28 (82.2%) were showed CPE in the cell culture. Most of the isolated virus showed CPE which was characterized by a fast destruction of BHK-21 monolayer cell and infected cells were round and formed singly as indicated in arrow (Figure 8). Complete destruction of the cell sheet was mostly seen within 48 hours of inoculation.

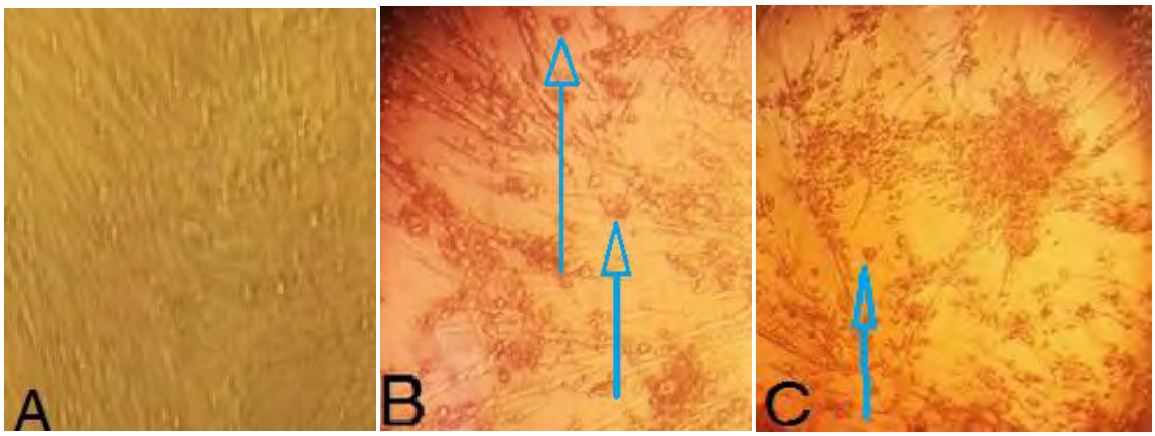

Figure 8: BHK21 cells infected with FMD viruses

A shows BHK21 cell control (cell without FMDV infection), B and C showed CPE (indicated by blue arrows) developed as a result of FMDV inoculations, ETH/08/15 and ETH/20/15 respectively.

Table 11: FMD virus isolated from outbreak cases from different parts of Ethiopia

No	Site of outbreaks			Species sampled	No. of sample	Suspected FMDV sample	Date of sample collection	Name of the virus	
	Region	Zone	District					With CPE	Without CPE
1		Arsi	Guna	Bovine	8	6TE, 2OPF	20/12/2015 08/01/16	ETH/01/15,ETH/03/15,ETH/06/15, ETH/08/15,ETH/11/15,ETH/14/16, ETH/19/2015, ETH/22/15	
					4	3 TE, 1OPF	21/11/2015		ETH /05/15, ETH/12/15, ETH/09/15 ETH/13/15
	Ormoia		Ludehitosa	Bovine	5	4 TE, 1OPF	22/12/2015 15/01/2016	ETH/02/15,ETH/04/15,ETH/15/16 ETH/16/16, ETH/20/2015	
		E.shewa			1	1OPF			ETH/17/15
			Adama	Bovine	4	4TE	18/12/2015 03/01/2016	ETH/07/15,ETH/10/15, ETH/18/2015 ETH/21/2015	
			Boset	Bovine	4	3TE, 1OPF	03/03/2016	ETH/23/16,ETH/25/16,ETH/26/16, ETH/27/16	
					1	1OPF			ETH/24/16
2	Addis Ababa	A.A	Kolfe	Bovine	7	5TE, 2OPF	29/03/2016	ETH/28/16,ETH/29/16,ETH/30/16, ETH/31/16, ETH/32/16, ETH/33/16 ETH/34/16	
	Total				34			28	6

Note: TE (Tissue epithelium), OPF (oro-pharyngeal fluids).

4.3.1. FMDV serotype identification

Further laboratory investigation was conducted from the 28 samples that showed CPE to characterize the viruses using sandwich ELISA. Serotypes (SAT2, A and O) were identified using sandwich ELISA at NAHDIC, Ethiopia (Table 12).

Table 12: FMDV serotypes and their topotype identified in different sites of outbreak investigated

Site of outbreaks			No. of samples	CPE positive	Serotype		Topotype
					Sandwich ELISA result	RT-PCR result	
Region	Zone	District					
Oromia	Arsi	Guna	12	8	A	GD	Africa
		Ludehitosa	6	5	SAT 2	GD	VII
	East shewa	Adama	4	4	SAT 2	GD	VII
		Boset	5	4	SAT 2	-	-
Addis Ababa	Addis Ababa	Kolfe	7	7	O,A& SAT 2	-	-

GD: genome detected

4.3.2. Molecular Characterization (phylogenetic analysis)

The isolated SAT2/ETH/18/2015 and SAT2/ETH/20/2015 viruses were compared based on 648 nucleotide sequence of VP1. The viruses were shared 99.07% genetic similarity with each other. The WRL, nucleotide sequence difference analysis confirmed that the nucleotide sequence difference of the two identified viruses was ≤1% and hence these viruses were isolated from the same outbreak. The two viruses were also showed >90% genetic similarity with virus isolates of SAT2/ETH/15/2015, SAT2/ETH/10/2015 and SAT2/ETH/14/2015 from Sidama (SNNPR), Awi (Amahara) and North Shoa (Oromia) respectively

and SAT2/SAU/6/2000 Saudi Arabian isolate. These viruses are homologous, geographically clustered and formed a single genetic lineage called topotype VII and genotype Alx-12. The genetic relationship of the isolates with the other SAT-2 serotypes is displayed on phylogenetic tree (Figure 9).

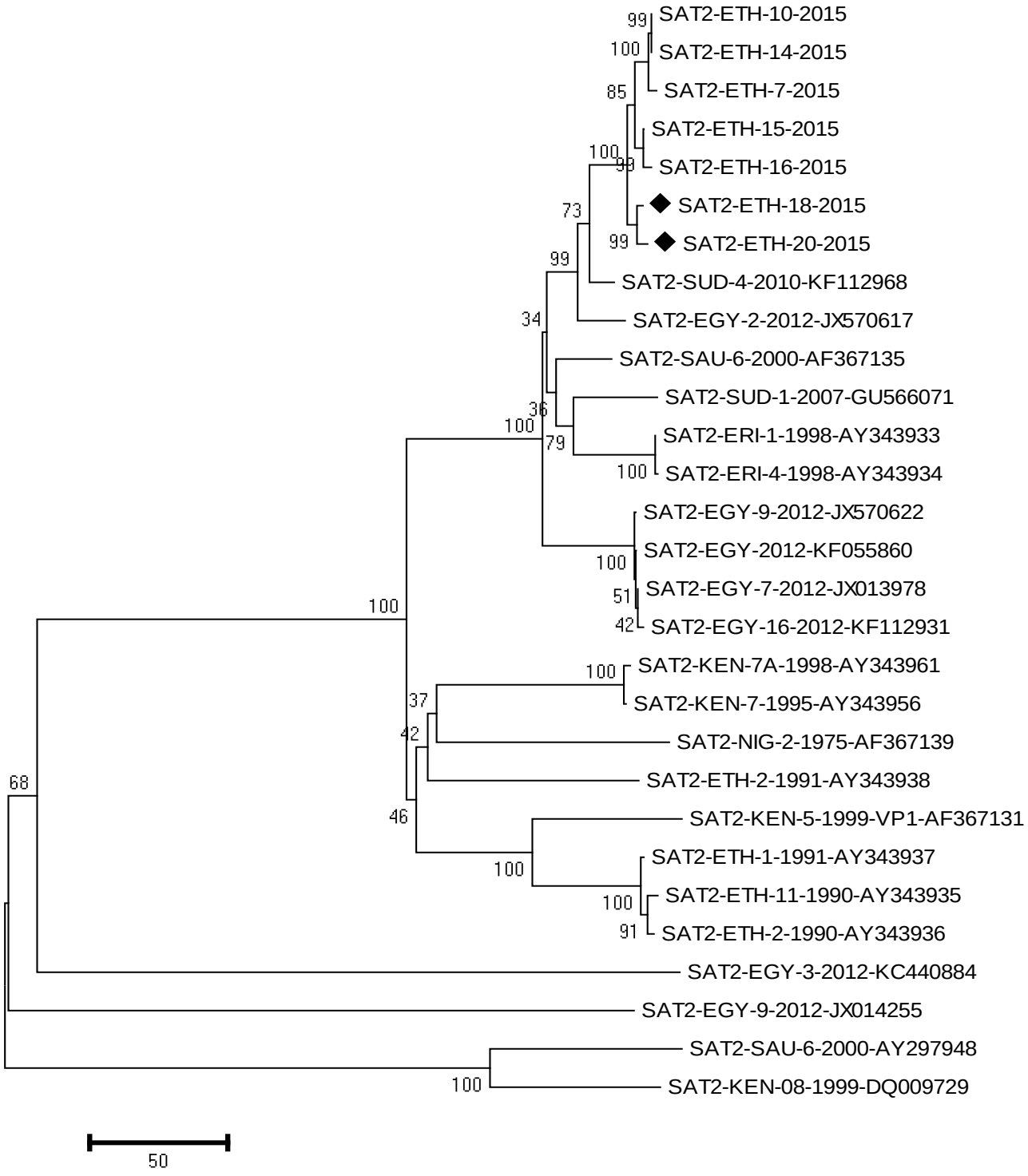

Figure 9: Midpoint-rooted neighbor-joining tree showing the relationships between the SAT2 virus isolates from Ethiopia and reference viruses

Numbers indicate the percentage occurrence of the branches by the bootstrap resampling method (GenBank Accession numbers are shown). This (◆) indicates the new isolates obtained from the outbreak cases from Ludehitosa and Adama, Arsi and East Shewa zone respectively, Oromia.

Serotype A isolated from Guna District, Arsi zone of Oromia region, during the study period was also compared with other countries type A isolates. This isolate was 87.79-88.26% similar with Cameron isolate of A/CAR/15/2000, Sudan isolate of A/SUD/3/2006 and Eretria isolate of A/ERI/3/1998 (WRL, 2016). The isolated serotype A in the current study falls in to African topotype (Figure 10). The genetic relationship of the isolates with the other A serotypes is displayed on phylogenetic tree. Molecular characterization of the single serotype A FMD virus showed as it belongs to genotype IV of the serotype A FMD virus. The virus clustered with FMD viruses circulating in Sudan and Eritrea.

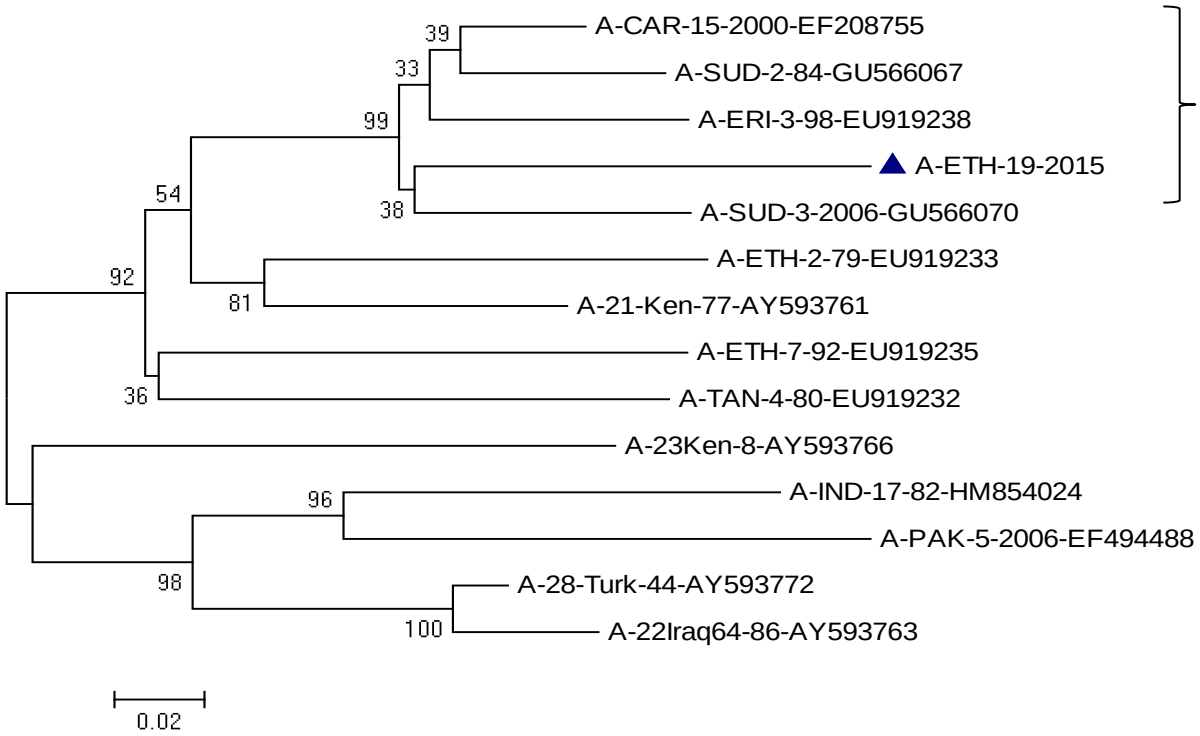

Figure 10: Midpoint-rooted neighbor-joining tree showing the relationships between the A virus isolates from Ethiopia and with reference viruses from other East African, middle East and Asian countries (GenBank Accession numbers are shown)

The▲ indicates the new isolate obtained the outbreak cases from Guna area (Arsi Zone).

5. DISCUSSIONS

5.1. FMD Sero-prevalence and Associated Risk Rectors in Dairy Cattle

The overall sero-prevalence rate of 24.22% reported in this study was in agreement with the previous finding from Ethiopia (Sahle, 2004) in which sero-positivity of 26.5% was reported. Compared to the present findings lower prevalences of 5.6% (Jembere, 2008), 8.01% (Abunna, 2013) and 9% (Beyene *et al.,* 2015) were reported from Afar Regional State, Dire Dawa and western Ethiopia, respectively. On the other hand relatively higher sero-prevalence was previously reported in samples from the Eastern zone of Tigray with 41.5%; followed by the Guji zone of Oromia and Yeka district of Addis Ababa city, with 32.7% and 30% respectively (Ayelet *et al.,* 2012). Abu-Elzein *et al*. (2007) in Sudan, Hafez *et al*. (2014), in Saudi Arabia and Namatovu *et al*. (2015) in Uganda also reported sero-prevalences of 16%, 53% and 77% respectively, from FMD virus infected cattle. The observed prevalence variation may be resulted from differences in individual animals breed, immune status, interaction of cattle with other animals like small ruminants and production system.

The significant prevalence variation was observed in keeping cattle and small ruminants together and associated with high risk of FMD sero-conversion as the result of infection. Previous study conducted in 4 selected Districts of Gambella Regional State reported a similar effect (Tefera, 2010). Similar studies in Nigeria (Obi and Newman, 1988) and South America (Barnett and Cox, 1999), where communal farming of cattle, sheep and goats occur, identified a high prevalence of antibodies against the virus infection associated with sheep and goats in the absence of clinical signs. This suggests that small ruminants have an important role in the epidemiology of FMD. Higher sero-prevalence was observed on semi-intensively managed cattle than those intensively managed. Studies of cattle in semi-intensive production systems in different regions of Ethiopia have reported a direct association between FMDV infections and management type (Jenbere *et al.,* 2011).

The potential risk of herd size variation in FMD occurrence showed that an increment of

sero-positivity with herd size expansion. This implies the importance of herd size in the epidemiology of the disease. Herds with large number of animals were found more at risk of contracting FMD as compared to small and medium herd sizes. This could be attributed to crowding of animals caused by large herd size that can facilitate frequency of direct contact and hence enhances chances of transmission. The study conducted around South Omo Zone and Borana pastoral area reported a similar effect (Bayissa, 2009; Molla, 2009).

The study revealed a significant variation on sero-positivity of foot and mouth disease among the three age groups. The significantly higher sero-prevalence of FMD in old and adult animals than in young observed in the current study is in agreement with the previous reports of Rufael *et al*. (2008) in Borena pastoral area, Megersa *et al*. (2009) in Gamo gofa and Sidama zones and Mohamoud *et al*. (2011) Awbere and Babille districts of Jijiga zone. This may be due to the cumulative exposure of cattle population to the FMD viruses (Murphy *et al.,* 1999). Aged animals might have acquired the infection from multiple serotypes, and could have produced antibodies against those serotypes. Relatively low sero-prevalence in animal groups below two years old might be indicative of the existence of passive maternal immunity and low frequency of exposure (Bayissa, 2009; Negussie, 2010). Higher sero-prevalence was observed in females than males. These Variation may be related to the effect of the higher number of females included in the sample than males due to the fact that most dairy farm owners usually keep few males only for breeding purpose (personal observation).

These higher percentages of FMD sero-positivity in cross-bred cattle might be associated with the genetic difference among cattle breeds. These indicated that cross-breds appeared more susceptible to the FMD viruses endemic to Ethiopia. Direct impact of FMD on livestock in sub-Saharan Africa depends on the breeds of animals used (Hunter, 1998). Quinn *et al.* (2005) also reported that FMD is more severe in European breeds of cattle than the other breeds.

5.2. FMDV Isolation, Identification and Molecular Characterization

In this study from the total number of 378 cattle examined 109 (28.8%) animals manifested clinical sings and lesions suggestive of FMD. In agreement with this finding Nigussie (2011) reported 28.2% clinically sick animals after conducting a number of outbreak investigations in different parts of the country. This is supported by the works of Kitching *et al*. (2005) and McLaws *et al*. (2006) who justified that variations in clinical severity and manifestation are associated with the strains and infective dose of the virus, species affected, individual susceptibility of the host and the farming system.

From a total of 34 field samples 28 (82.2%) showed CPE, which appeared as rounding in cells culture and the result obtained was in agreement to previous work by Huang *et al*. (2011), Negussie *et al*. (2011) and Tesfaye (2014) that the CPE was characterized by a fast destruction of the cell monolayer and infected cells with detaching of the cells from flask surfaces.

In the current study, three serotypes (A, O, and SAT2) of FMD viruses were isolated and antigen typed. From the three serotypes, two of them (A and SAT2) where characterized at molecular levels.

Serotype O was isolated from the samples collected from Kolfe district (Addis Ababa). Our results agree as indicated that serotype O was prevalent and a serotype causing an outbreaks in Ethiopia (Gelaye *et al.,* 2005; Ayelet *et al.,* 2009; Nugussie *et al.,* 2010, Menda *et al.,* 2014) and the most prevalent serotype worldwide (Kitching *et al.,* 2007).

Serotype A was isolated from the samples collected from kolfe and Guna district of Addis Ababa and Arsi zone respectively. Most likely the viruses were responsible for the occurrence of the investigated outbreak in these areas. Previously serotype A was reported from bovine samples collected from Hadiya and Yabello areas (Ayelet *et al.,* 2013) and Konso (Tesfaye, 2014); similarly from bovine and swine samples obtained from different outbreak areas of Ethiopia (Gelaye *et al.,* 2007).

Serotype SAT 2 FMD viruses were identified from cattle found in Ludehitos district (Arsi zone), Adama and Boset district (East shewa zone) and Kolfe district (Addis

Ababa). The viruses were responsible for the occurrence of the investigated outbreak in these areas. Previously serotype SAT 2 was isolated and reported from cattle in Addis Ababa (Dejene, 2004), Borana pastoral area (Gelaye *et al.,* 2005), Benshangul-Gumz (Ayelet *et al.,* 2009) and Gambella (Tefera, 2010). The presence of serotype SAT 2 specific antibodies in cattle in Borana pastoral area was reported (Rufael *et al.,* 2008). Serotype SAT 2 FMDV also previously reported from many sub-Saharan African countries (Bastos and Sangare, 2001; Sangare, 2005; Sahle *et al.,* 2007) suggesting the endemicity of the serotype in these countries.

Serotype A identified in the current study, in the samples collected from Guna District was clustered to genotype IV of African topotype. This genotype was not reported before and more detailed analysis need to be conducted using advanced software's. In general, previous studies also indicated that serotype A was prevalent in Ethiopia (Negusssie *et al*., 2011). This is in support of the findings of Negusssie *et al*. (2011), who reported A virus, belonged to African topotype and all viruses from Ethiopia belonged to the Afrcan topotype (knowel and Samuel, 2003).

The presently identified SAT 2 serotype was clustered to topotype VII. This is in agreement with the findings of Ayelet *et al*. (2009), who reported SAT 2 viruses circulating in Ethiopia belonged to topotype VII along with the Sudan isolates. All these collective facts can obviously indicate that SAT 2 was introduced to the present study Districts; the possible way of entry of the virus might be related to communal resource utilization and free movement of animals across the regions because SAT 2 is endemic to different regions of the country (Gelaye *et al.,* 2005; Ayelet *et al.,* 2009; Tefera, 2010) and neighboring African countries like Sudan republic (OIE, 2012; Vosloo *et al.,* 2002). The present isolates seem to be different from previously published isolates as they cluster differently on the tree. Further detailed genetic analysis like genetic distance determination is needed to have more insight about these viruses.

This suggested that the outbreaks due to these isolates were most probably spread by uncontrolled trans-boundary movement of animals, and these have a big risk on the transmission of the virus across the border in both directions because there is no strong

animal movement regulation across the border and the ability of the virus to transmit with the wind. This statement is supported by Samuel *et al.,* (1999) who demonstrated that closely related viruses could either be from the same outbreak or from viruses temporally closely related. Sangare (2005) also reported on the presence of trans-boundary and trans-continental transmission of viruses in west and southern Africa.

6. CONCLUSION AND RECOMMENDATIONS

FMD is prevalent in the study Districts as conformed clinically, serologically, virologically and by molecular characterization and reported to be endemic in Ethiopia. The presence of this disease in the country is a major obstacle to the development of livestock resource because of its adverse effects on production and their product exports. In Ethiopia, factors such as the presence of high numbers of susceptible domestic animals, herd composition or the involvement of multiple hosts (cattle, sheep and goats), herd size and individual animal age variability, lack of prophylactic vaccination, absence of regulation for prohibition of animal movement, high contact of animals at marketing and common grazing place as well as at watering points could contribute to the occurrence of FMD and create the difficulty in controlling the outbreaks. During the study period serotype A, O and SAT 2 were identified, with highest prevalence of serotype SAT2. Serotype SAT2 and A isolated in Ethiopia were lies on genotype Alx-12, topotype VII and genotype IV, African topotype respectively. But these molecularly characterized serotypes seem to be emerging viruses as they cluster differently with the previously reported FMD viruses from Ethiopia.

Therefore, the followings are offered as recommendations:

- An extensive regular surveillance and serotyping of the outbreak isolates throughout the country should be conducted to check the introduction and circulation of new serotype in the country and to ensure that circulating viruses are protected by existing vaccines and/or look for alternative vaccines.
- During dairy cattle rearing keeping animals with variety age and mixing shoats with dairy cattle should not be practiced.
- Government strategy in FMD control through regular vaccination and movement control should be implemented.
- Further detailed studies on the molecular characterization of the viruses and their genetic closeness with the existing vaccines need to be conducted to establish the nature of their diversity on the tree.

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8. APPENDICES

Appendix 1: Number of PAs/*Kebeles* randomly selected for the study within each District

District	Total number of PAs/*Kebeles*	30% of the total PAs/*Kebeles* randomly selected for the study
Adama	18	6
Asella	18	6
Total	36	12

Appendix 2: Study animals age estimation on the basis of dental eruption

Species	Eruption of teeth	Age estimation in years
Bovine	One incisor	Less than 2
	Two incisors Three incisors	From 2 - 3
	Canine teeth Wear of teeth	Greater than 3

Source: (Merck veterinary manual, 1998)

Appendix 3: Bovine blood sample and associated risk factors recording format

Region ____ Zone____ District____ PA ____ Village____ Date ____ /____ /____

Animal		sex		Age in years			Herd size			Herd composition	
owner's name	ID. No.	M	F	< 2	2-3	> 3	< 50	50-100	> 100	With small ruminants	Without Small ruminants
	01		X		X		X				X
	02	X		X				X		X	
	.										
	.										
	574										

Appendix 4: Tissue and oro-pharyngeal (OP) fluid sample collection sheet

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

Species	No	Sex		Sample			
		F	M	Tissue	OP fluid	Number	Pool
Bovine	01		X		X	1	
	02	X		X	X	3	X
	.						
	.						
	34						

Appendix 5: Plate layout of ID Screen® FMDV 3ABC-Ab ELISA test

OD	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	05										
B	PC	.										
C	NC	.										
D	NC	.										
E	01	etc										
F	02											
G	03											
H	04											

PC = Positive control serum; NC = Negative control serum; 01, 02, 03, etc. = Tested serum sample

Appendix 6: Plate layout for FMDV detection and serotyping ELISA

Catching MAbs	Type O MAb 3B11		Type A MAb 4D12		Type A MAb 4F6		Pan-O,A,C,As. 1 MAb 1F10		Type SAT 1 Pool 2 MAbs		Type SAT 2 Pool 2 MAbs	
	1	2	3	4	5	6	7	8	9	10	11	12
Sample1 A												
Sample2 B												
Sample3 C												
Sample4 D												
Sample5 E												
Sample6 F												
PC G												
NC H												

MAb= monoclonal antibody, As. 1=Asia 1

Appendix 7: Foot and mouth disease outbreak investigation recording sheet

1. Location of the outbreak:

Region _____

Distance from Addis Ababa _____

Zone _____

District_____

Village _____

PA/kebele _____

Date/month/year_____

2. Animal owner's name_____

3. Number of animals clinically examined owned by individual farmers:

Species	No	Sex		Age categories			Total
		M	F	< 2 years	2-3 years	> 3 years	
Bovine	01						

4. Number of animals affected (sick):

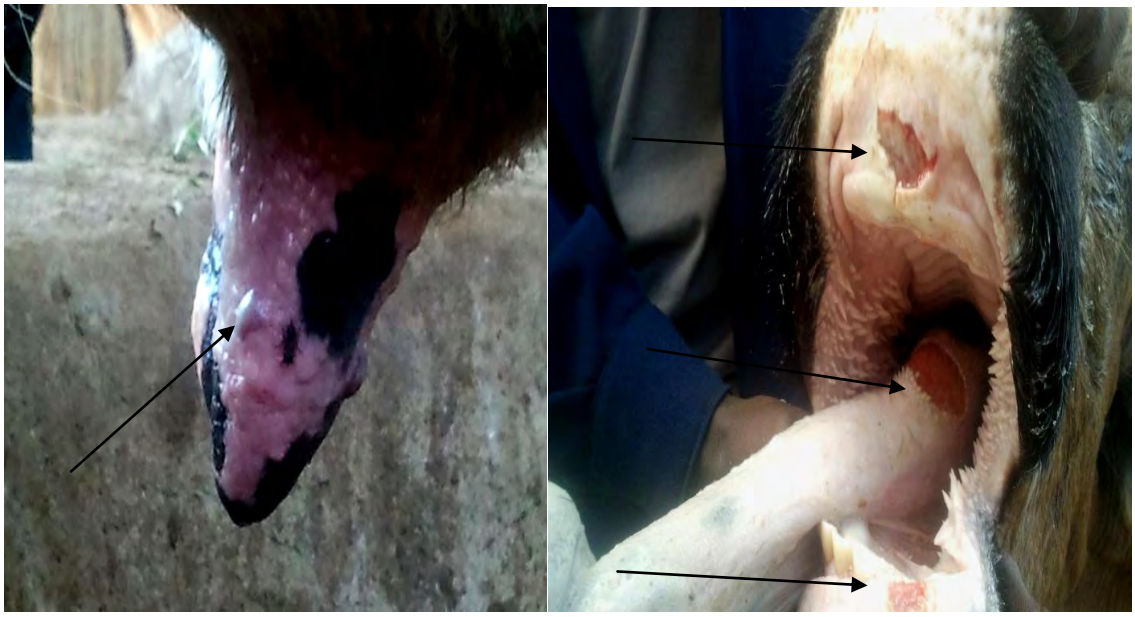
Species	No	Sex		Age categories			Total
		M	F	< 2 years	2-3 years	> 3 years	
Bovine	01						

5. Clinical signs observed (tick):

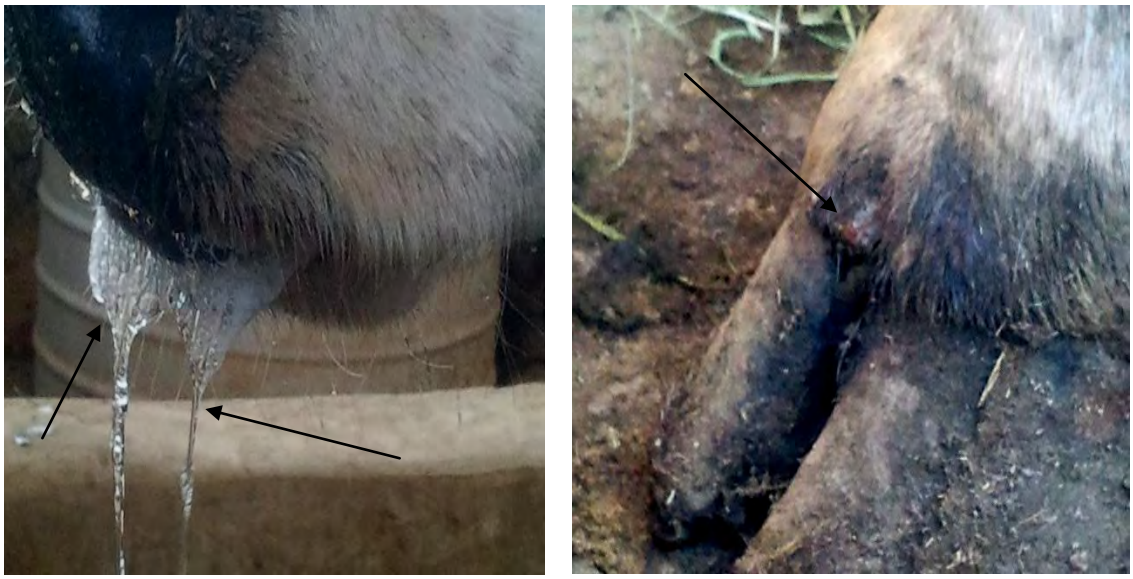
Salivation _____ Lameness _____ Interdigital _____ Udder lesion _____ Oral lesion _____ Others _____

Appendix 8: FMD clinical signs observed in cattle during outbreak investigation

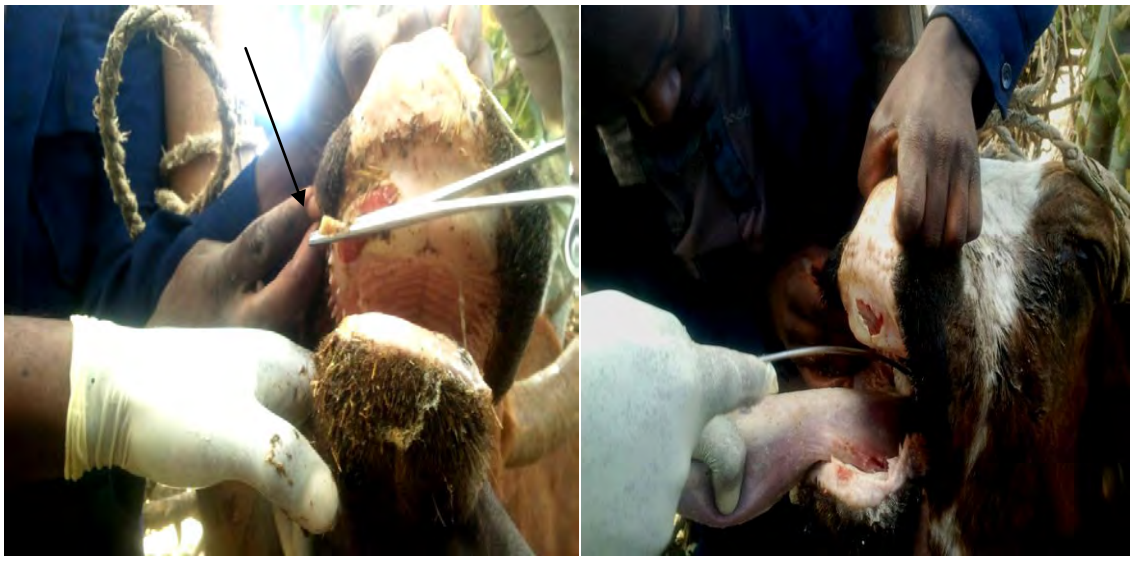
Lesion and vesicular swellings in gum, muzzle and teat



Salivation and hoof distortion due to acute FMDV infection



Sampling (tissue and oro-pharngeal fluid)



Appendix 9: Miscellaneous pictures

Work at cell culture room

Work at viral serology room

OD value reading

Appendix 10: Sequences of identified serotypes

Serotype A

A/ETH/19/2015

ACCACTGCAACGGGGGAGTCTGCAGACCCTGTCACCACCACCGTGGAGAACTACGGCGGTGA
GACACAACCCCAGCGACGGCACCACACGGACGTTGGCTTCATAATGGACAGATTTGTGAAAG
TGAACAGTTCTACTCCCACACACGTCATTGATCTCATGCAAACCCACCAACACGGGCTGGTAG
GTGCGTTGTTGCGTTCAGCCACCTACTATTTCTCTGATCTGGAGGTCGTGGTAAGACACCAGG
GGAACCTGACCTGGGTGCCCAACGGCGCCCCGGAGGGCGCTCTCTTGAACACGAGCAACCCC
ACAGCTTACCACAAAGCGCCGTTCACGAGGCTGGCACTTCCCTACACCGCGCCACACCGCGTG
CTGGCGACAGTGTACAACGGGACGACCAAGTACACGGCAGATACTCCAACCAAGCGAGGTGA
CCTGGGGGCCCTTGCGGCGAGAGTCGCTACACAGCTCCCCTCCTCTTTCAACTATGGGGCGCT
GCGCGCTGAGACCATCCACGAGCTTCTCGTGCGCATGAAGCGGGCCGAGCTTTACTGTCCCCG
ACCGCTGTTGTCAACAGAGGTGAGTTCAGCGGACAGGCACAAACAGAAGATCATTGCGCCCG
CCAAACAGCTCCTC

Serotype SAT 2

SAT2-ETH-18-2015

ACCACTTCAGCGGGAGAAGGCGCGGATGTCGTCACCACGGACCCGTCTACACACGGCGGGAA
CGTGCAGGAGGGTCGACGCAAACACACCGACGTTGCGTTCCTCCTTGACCGCAGTACACACGT
CCACACAAACAAAACAACCTTTGTTGTGGACCTCATGGACACAAAGAAGAAAGCGCTCGTGG
GCGCAATCCTGCGGGCTTCCACCTACTACTTTTGTGATCTTGAAATTGCATGTGTGGGCGACCA
CACGAGGGCCTTCTGGCAGCCTAACGGGGCTCCACGAACCACCCAGCTTGGCGACAACCCCA
TGGTCTTTGCCAAGGGCGGTGTGACCCGCTTTGCCATCCCGTTTACAGCCCCACACAGGTTACT
GTCCACTGTCTACAATGGTGAGTGTGTATACAAGAAAACCCCCACCGCCATCCGCGGGGACCG
TGCAGTGCTCGCGGCAAAGTACGCTGGCGCCAACCACACTTTGCCATCAACCTTCAATTTCGG
GTTCGTGACCGTCGACAAACCAGTCGATGTTTACTACCGGATGAAGAGAGCTGAGTTGTACTG
CCCACGCCCACTGTTGCCAGCTTATGAACACGCTGACAGAGACAGATTCGACGCACCCATCGG
CGTCGAAAGACAGACCCTG

SAT2-ETH-20-2015

ACCACTTCAGCGGGAGAAGGCGCGGATGTCGTCACCACGGACCCGTCTACACACGGCGGGAG
CGTGCAGGAGGGTCGACGCAAACACACCGACGTTGCGTTCCTCCTTGACCGCAGTACACACGT
CCACACAAACAAAACAACCTTTGTTGTGGACCTCATGGACACAAAGAAGAAAGCGCTCGTGG
GCGCAATCCTGCGGGCTTCTACCTACTACTTTTGTGATCTTGAAATTGCATGTGTGGGCGACCA
CACGAGGGCCTTCTGGCAGCCTAACGGGGCTCCACGAACCACCCAGCTTGGCGACAACCCCA
TGGTCTTTGCCAAGGGCGGTGTGACCCGCTTTGCCATCCCGTTTACAGCACCACACAGGTTAC
TGTCCACCGTCTACAATGGTGAGTGTGTATACAAGAAAACCCCCACCGCCATCCGCGGGGACC
GTGCGGTGCTCGCGGCAAAGTACGCTGGCGCCAACCACACTTTGCCGTCAACCTTCAATTTCG
GGTTCGTGACCGTCGACAAACCAGTCGATGTTTACTACCGGATGAAGAGAGCTGAGTTGTACT
GCCCACGCCCACTGTTGCCAGCTTATGAACACGCTGACAGAGACAGATTCGACGCACCCATCG
GCGTCGAAAGACAGACCCTG