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ISOLATION, MOLECULAR CHARACTERIZATION AND VACCINE MATCHING OF FOOT-AND-MOUTH DISEASE VIRUS CIRCULATING IN CENTRAL ETHIOPIA

MSc Thesis

By
Wondwossen Tolessa

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

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As member of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: **Wondwossen Tolessa Wario,** titled: **Isolation, Molecular Characterization and Vaccine matching 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.

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STATEMENT OF AUTHOR

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Behind the successes there were my families I thank you! God bless you.

LIST OF ABBREVIATIONS

BHK	Baby Hamster Kidney
cDNA	Complementary DNA
CPE	Cytopathic Effect
CSA	Central Statistics Agency
EA	East Africa
ELISA	Enzyme Linked Immunosorbent Assay
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GD	Genome Detected
Ig	Immunoglobulin
IRES	Internal Ribosome Entry Site
MEM	Minimum Essential Medium
NAHDIC	National Animal Health Diagnostic and Investigation Center
NSPs	Non Structural Proteins
Nt	Nucleotide
NVI	National Veterinary Institute
OD	Optical Density
OIE	Office International Des Epizooties
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAT	South Africa Territory
TCID	Tissue Culture Infectivity Dose
UTR	Untranslated Region
VNT	Virus Neutralization Test
VP	Viral Protein

WRLFMD	World Reference Laboratory for Foot and Mouth Disease

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ABSTRACT

The study was conducted Foot and Mouth Disease (FMD) outbreak reported in central part of Ethiopia namely Addis Ababa city council, Oromia Regional State of East Shoa zone and Amhara Regional State of North Shoa zone (Debre Berhan) from October 2016 to May 2017. It was designed with the objectives of identifying currently circulating serotypes and topotypes of foot and mouth disease viruses and to determine the antigenic relationship with FMD vaccine strains and newly isolated field isolate viruses. A total of 30 epithelial tissue samples were collected from cattle and used for virus isolation at National Veterinary Institute (NVI), Bishoftu. The samples were obtained from nine field FMD outbreaks, and 28 (93%) showed cytopathic effect (CPE) in BHK-21 cell culture. Of the total isolates, FMD viruses serotype O and SAT2 were identified using agarose gel based Reverse Transcription Polymerase Chain Reaction (RT- PCR) and 3 purified PCR products were send to international atomic energy for further molecular characterization for sequencing. The sequence results of serotype O FMDV isolates were used for phylogenetic tree reconstruction. The three isolates clustered with a single topotype, East Africa-4 (EA-4) with 99-100% similarity with each other. The five isolates from both serotypes, three O and two SAT2, were checked for their antigenic relatedness with the vaccine strain sera by vaccine matching test using one way testing (r1) by two-dimensional virus neutralization test. The finding discovered the entire tested field isolates have shown r1-value above the minimum requirement 0.3 compared to the vaccine strain which revealed antigenic match with vaccine strain. The mean r1- values were 0.79 and 0.82, respectively. Therefore, FMD vaccine containing serotype O and SAT2 produced by NVI still able to provide protection to the circulating field virus isolates in the study areas. Further work is recommended following the new outbreaks in other parts of the country.

Keywords: *Central Ethiopia, Cattle, FMDV, Molecular characterization, Virus isolation, Vaccine matching.*

1. INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most economically and socially devastating diseases affecting cloven-hoofed livestock worldwide. It is caused by a highly variable RNA virus with seven serotypes (O, A, C, SAT 1, SAT 2, SAT 3 and Asia1) and a large number of topotypes (Mort *et al.*, 2005). FMD is a vesicular disease and clinically, the disease is characterized by fever, salivation and vesicular eruptions on the feet and mouth (OIE, 2012). It is on the A list of infectious diseases of animals of the Office International des Epizooties (OIE). The clinical severity of which varies with the strain of foot-and -mouth disease virus (FMDV), the infecting dose, the species and individual susceptibility of the host. Direct or indirect contact with FMDV infected animals can result in susceptible animals becoming diseased or sub-clinically infected. In ruminants, but not pigs, complete clearance of virus from the pharynx may be delayed giving rise to persistently infected carriers. The main route of virus entry in natural infections is the respiratory tract through inhalation of airborne virus, although virus entry may gain through oral ingestion, or damaged epithelium (Alexanersen *et al.*, 2005).

FMD generally involves mortality rates below 5%, however, it is considered as the most important disease of animals since it causes huge losses in terms of livestock productivity and trade. Although FMDV rarely causes death in adult animals, the virus can cause severe lesion in the myocardium of young animals leading to high mortality rates (Woodbury *et al.*, 1995; Rufeal *et al*., 2008; Perry and Grace, 2009). The main constraints in controlling this disease and why it is considered as the most dreaded viral disease are its high contagiousness, wide geographical distribution, broad host range, and ability to establish carrier status, antigenic diversity leading to distribution, poor cross-immunity and relatively short duration of immunity. Poor surveillance and diagnostic facilities as well as inadequate control programs are major problems in control of FMD and a leading cause of loss of livestock economy. Outbreaks are still being reported from time to time around the year. Besides causing direct losses to livestock economy it also causes indirect losses in terms of severe trade restrictions impact which may be higher than direct losses (Tamilselvan *et al.*, 2009).

Among the seven serotypes of FMDV, O and A are the most widespread. FMD viruses frequently change at different antigenic sites. Within the serotypes, there is considerable antigenic variability (Barnet, 2001).There is no cross-immunity among the seven serotypes. This is evidenced in animals that have previously been infected with one serotype, but remain susceptible to the six other serotypes. Consequently, FMDV-specific antibodies protect only against homologous, but not heterologous FMD outbreaks. Thus, the vaccine selected must be highly specific to the strain involved and matched as closely as possible with the outbreak isolate. It has been indicated that lack of vaccine-induced protection may involve the use of an inadequately matched vaccine (Paton et al., 2005).

Effective control and prevention of FMD relies largely on the implementation of strategies such as physical separation of wildlife and livestock, repeated vaccination of cattle herds exposed to wildlife, control of animal movements and careful assessment of the risk of FMDV introduction into disease-free areas (Thomson *et., al* 2003; Jori *et al.,* 2009). Successful livestock disease control programs for example through mass vaccination depend not only on technical and economic feasibilities but also on the motivation of the farming community to fully participate in the implementation of the control programs (Roeder and Taylor, 2007). Farmers' motivation to implement a specific disease control measure is largely driven by their perceptions of the disease's risk and the effectiveness of available control measures (Garforth *et al.,* 2013). Perceptions about the effectiveness of farming technologies, including that of livestock disease control are, known to be important predictors of the eventual technology uptake (Fandamu *et al.,* 2006).

Ethiopia has the largest cattle population in Africa with 54 million heads of cattle (CSA, 2013). Within the cattle population, because FMD occurs endemic several outbreaks were reported per year (Ayelet *et al.,* 2012). These outbreaks affect a large part of the country causing significant economic losses in the affected herds (Jemberu *et al.,* 2014). The Ethiopian government is keen in to launching an official control program against FMD to reduce production losses and to improve the export trade of animals and animal products (Thomson, 2014).

The molecular epidemiology of FMDV has been studied in some detail in different countries of the world using nucleotide sequencing of the main antigenic determinant of the virus and phylogenetic analysis. In Ethiopia, however, records from the National Animal Health Diagnostic and Investigation Center (NAHDIC) and National Veterinary Institute (NVI) of Ethiopia indicated that serotypes O, A, C, SAT1 and SAT2 were responsible for FMD outbreaks during 1974 – 2008 (Sahle *et al.*, 2004; Gelaye et *al.*, 2005; Legess, 2008; Gelagay, 2009 and Haileleul et al., 2010). To initiate control measures for emergence of these viruses in Ethiopia, the following must be identified: origin of infection, links between outbreaks, extent of genetic variation of the causative viruses, and antigenic relationship of field isolates to the available vaccines.

Therefore, the main objectives of this study were:

- To isolate currently circulating FMD virus strains.
- To molecular characterization of the isolated FMD virus strain.
- To conduct *in-vitro* vaccine matching test against circulating virus.

2. LITERATURE REVIEW

2.1 Definition

Foot and mouth disease is a severe, highly contagious viral disease of livestock with significant economic impact. The disease affects cattle and swine as well as sheep, goat, and other cloven-hoofed ruminants. All species of deer and antelope as well as elephant and giraffe are susceptible to FMD. The disease is rarely fatal in adult animals but mortality rates are high in young animals and in susceptible populations morbidity approaches 100%. Those animals that recover are usually weakened and debilitated (OIE, 2012).

2.2 Etiology

Foot and mouth disease is a highly significant disease in the field of veterinary science. It is the first viral disease for which the causative agent was identified in 1897 by Loeffler and Frosch (Dupuis *et al.,* 2006). The etiological agent is classified within the genus *Aphthovirus* in the family *Picornaviridae* (Racaniello, 2001). The virus exists in the form of seven serologically and genetically distinguishable types, namely O, A, C, SAT 1, SAT 2, SAT 3 and Asia1, but a large number of subtypes have evolved within each serotype (Pereira, 1977; Radostits *et al*., 2006).

2.2.1 Taxonomy

The virus causing FMD was defined in 1963 by the International Committee of Taxonomy of viruses as belonging to the genus *Aphthovirus,* one of the genera of the family *Picornaviridae.* The name *Picornaviridae* is derived from the Latin word 'pico' (small) and 'ma' (RNA) which refers to the size and genome type while the genus name *aphthovirus* refers to the vesicular lesions produced in cloven hoofed animals (OIE, 2004).

2.2.2 Physicochemical properties

Picornaviruses are small RNA viruses that are 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 (Cooper *et al.,* 1978). FMDV can be inactivated by a number of chemical substances at the acidic and alkaline pH ranges, however, the virus is stable between pH 7 and 9 and at 4°C and -20°C. Two percent solution of NaOH or KOH and 4% Na_2C0_3 are effective disinfectants for FMDV contaminated objects but, the virus is resistant to alcohol, phenolic and quaternary ammonium disinfectants (Mann and Sellers, 1990).

2.2.3 Virus morphology

FMDV is a small nonenveloped, positive sense, single stranded RNA virus composed of an icosahedral protein coat (capsid) and the RNA core has a diameter of 22-30 nm (Cooper *et al.,* 1978). The capsid consists of 60 capsomers each consisting of four structural polypeptides, viral protein 1(VP1), viral protein 2 (VP2), viral protein 3 (VP3) and viral protein 4 (VP4). The VP1, VP2 and VP3 are exposed on the surface of the virus while VP4 is located internally (see figure 1). VP1 is the most antigenic protein and it is involved in cell attachment and carries an immunological important G-H loop which is one of the most important neutralizing sites of the virus (Logan *et al.,* 1993).

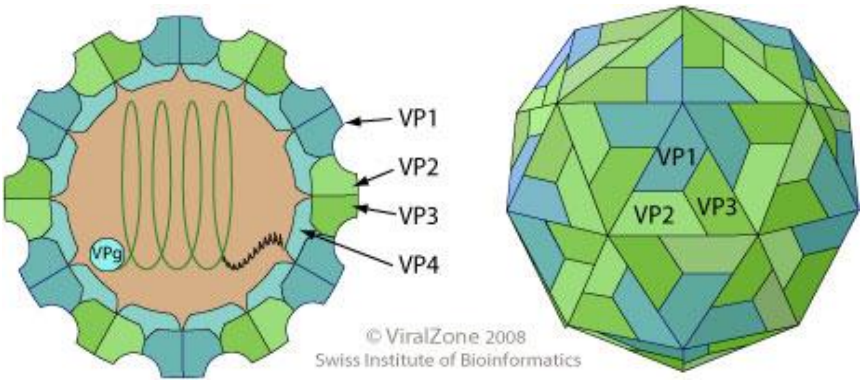


Figure 1. Morphology of FMD virus

Source: Adapeted from Viral Zone Swiss Institute of Bioinformatics (2008)

2.2.4 Genomic organization

The genome of FMDV is about 8.5 Kb in length enclosed within a protein capsid. It has four major parts: 5′ Untranslated region (5′UTR), Coding region, 3′ Untranslated region

(3′UTR), and a poly “A” tail. The 5′UTR is linked with Vpg (3B) which serves as primer in replication. 5′UTR contains an S fragment at its 5′ end which encompasses 360 bases, folds into long stem loop and plays a role in genome stability and the binding of protein involved in genome replication (Costa Giomi *et al.,* 1984). Following S fragment there is poly C tract comprising over 90% C residues. The length of this tract is extremely variable. There are some evidences that length of this tract is associated with virulence and hence persistence (Grubman and Baxt, 2004). After poly C tract, there is a series of RNA pseudo knot structures of unknown function (Mason *et al.,* 2003). Downstream to the pseudo knot there is cis-acting replicative element (cre). The cre has conserved AAA sequences and is essential for genome replication. Downstream to cre is internal ribosomal entering sites (IRES). The IRES has role in initiation of viral protein synthesis and shutting down of host cell protein synthesis (Mason *et al.,* 2002). Due to the presence of IRES, picornaviruses are able to bind to ribosomes to initiate the protein synthesis. The picornaviruses have no 5′cap (7-methyguanosine), so IRES serves as ribosomal entering site (Pilipenko *et al.,* 1992). The 3′UTR follows the ORF (open reading frame) termination codon and contains a short stretch of RNA which folds into specific stem loop structures followed by a poly A tract of variable length. This 3′UTR also serves as some functions in genome replication (Lopez *et al.,* 2002). 3′UTR is specific for each picornavirus. Poly A probably plays a role in FMDV translation and replication. The FMDV genome encodes a polyprotein from which four different structural and eight different non-structural proteins are formed by the viral proteases. After translation, the four primary cleavage products are formed: the amino terminal L protease which cleaves at its own carboxy terminus, P1- 2A, the precursor of the capsid proteins 2BC, and P3 which is cleaved to make the NSPs (Ryan *et al.,* 1991). FMDV has two proteinases. The NSP leader proteinses (Lpro) located in the N-terminal region of the polyprotein acts both intra and intermolecularly. This protease initiates cleavage by separating itself from P1, the precursor of the capsid protein and the remainder of the growing polypeptide chain (Newman *et al.,* 1994). The 3C protease is responsible for the cleavage of P1 into 1AB (VP0), 1C (VP3), and 1D (VP1). The 1A/1B (VP4/VP2) cleavage occurs at the late stage in virus morphogenesis and is associated with maturation of capsid. The 2C/3A primary cleavage is cis and subsequent cleavage is also mediated by the 3Cpro and producing processing intermediates and mature proteins (Capozzo *et al.,*

2002). In addition to viral protein processing, the 3Cpro cleave the host cell protein histone H3 and may be involved with the shuting down of host cell transcription. The cleavage between 2A/2B junctions is mediated by 2A polypeptide separating itself and P1 away from 2BC/P3. This change is independent of both L and 3C. The FMDV 2A region is very short (about 18 amino acids) and together with the N-terminal residues of protein 2B, represents an autonomous element capable of mediating cleavage at its own C terminus (Donnelly *et al.*, 1997).

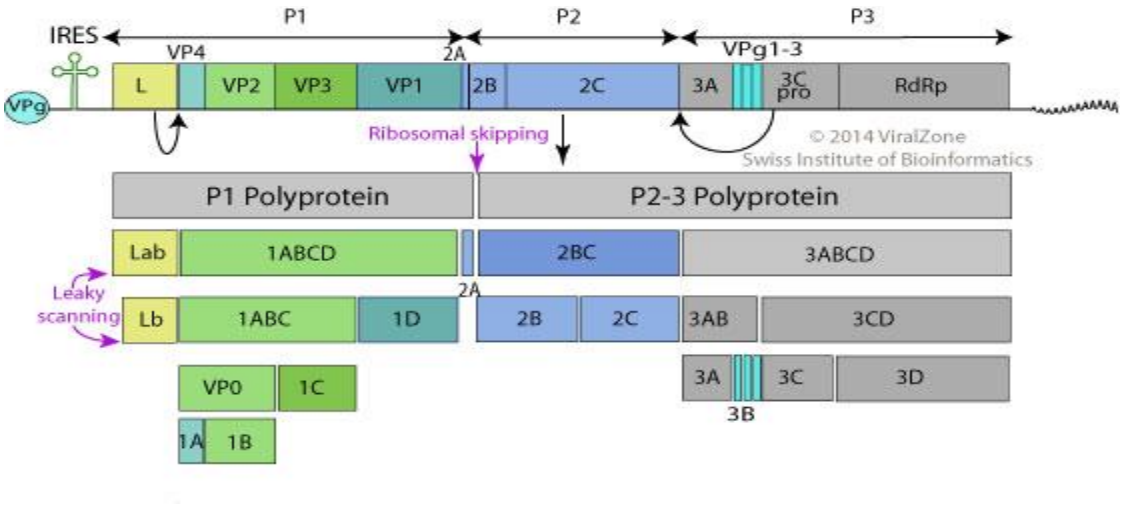


Figure 2. Foot and Mouth Disease Genome

Source: Adapeted from viral zone swiss institute of Bioinfomatics (2014)

2.2.5 Antigenic variation

The presence of seven serotypes and multiple subtypes and variants has added to the difficulty of laboratory diagnosis and control of FMD. The rise of new variants is inevitably caused by continued circulation of the virus in the field and the quasispecies nature of the RNA genome (Haydon *et al.*, 2001; Domingo *et al.*, 2003). RNA viruses in general and FMDV in particular, have very high mutation rates, due to the lack of error correction mechanisms during RNA replication (Drake and Holland, 1999**)**. Antigenic variation in the field increases with time and most probably results from immunologic pressure placed on the virus by either the infected or vaccinated host species (Haydon *et*

al., 2001). In addition, antigenic variation in FMDV has also been observed in tissue culture in the absence of immunologic pressure (Fares *et al.,* 2001; Haydon *et al.,* 2001), indicating that antigenic sites on the virion may also be involved in other virus functions. Regardless of the mechanism, analysis of both genome sequence and antigenic variation has been invaluable in epidemiological studies of outbreaks and analysis of virus within countries where the disease is enzootic, and, in the case of a possible deliberate introduction of virus (Hemadric *et al.,* 2000; Konig et al., 2001; Araujo et al., 2002; knoweles and Samuel, 2003).

2.2.6 Serotypes and subtype

FMD virus exists as seven different serologically distinct types. Serotypes O and A were initially discovered by Vallee and Carre in 1922. They showed that cattle that had recovered from clinical disease due to an FMD virus which originated in France became re-infected almost immediately when mixed with animals infected with FMD virus that originated in Germany. They named these serotypes after their place of origin; O for the department of Oise in France and A for Allemagne (the French word for Germany). Their work was extended by Waldmann and Trautwein in1926; with the discovery of a third serotype which was named serotype C. Later three additional serotypes were identified in samples originating from South Africa and they were named as Southern African Territories 1, 2 and 3 (SAT1, SAT2, SAT3) (Brooks, 1958). The seventh serotype, Asia-1, was initially detected in a sample collected from a water buffalo at Okara, Punjab, Pakistan in 1954. Examination of an extensive number of samples from across the world have failed to reveal the existence of another serotype although there are many different sub-types, some of which are quite distinct from other strains of the same serotype (Brooks and Rogers, 1957).

2.3 Epidemiology of the Disease

2.3.1 Geographical Distribution

The current global burden of FMDV infection is maintained within three continental reservoirs in Asia, Africa, and South America, which can be further subdivided into seven major virus pools of infection (see Figure 3). Each of these contains at least three serotypes

of virus, and because virus circulation is mainly within these regional reservoirs, strains have evolved which are specific to the region and which often (in the case of type A and SAT viruses) require tailored diagnostics and vaccines for control (Paton *et al.*, 2009).

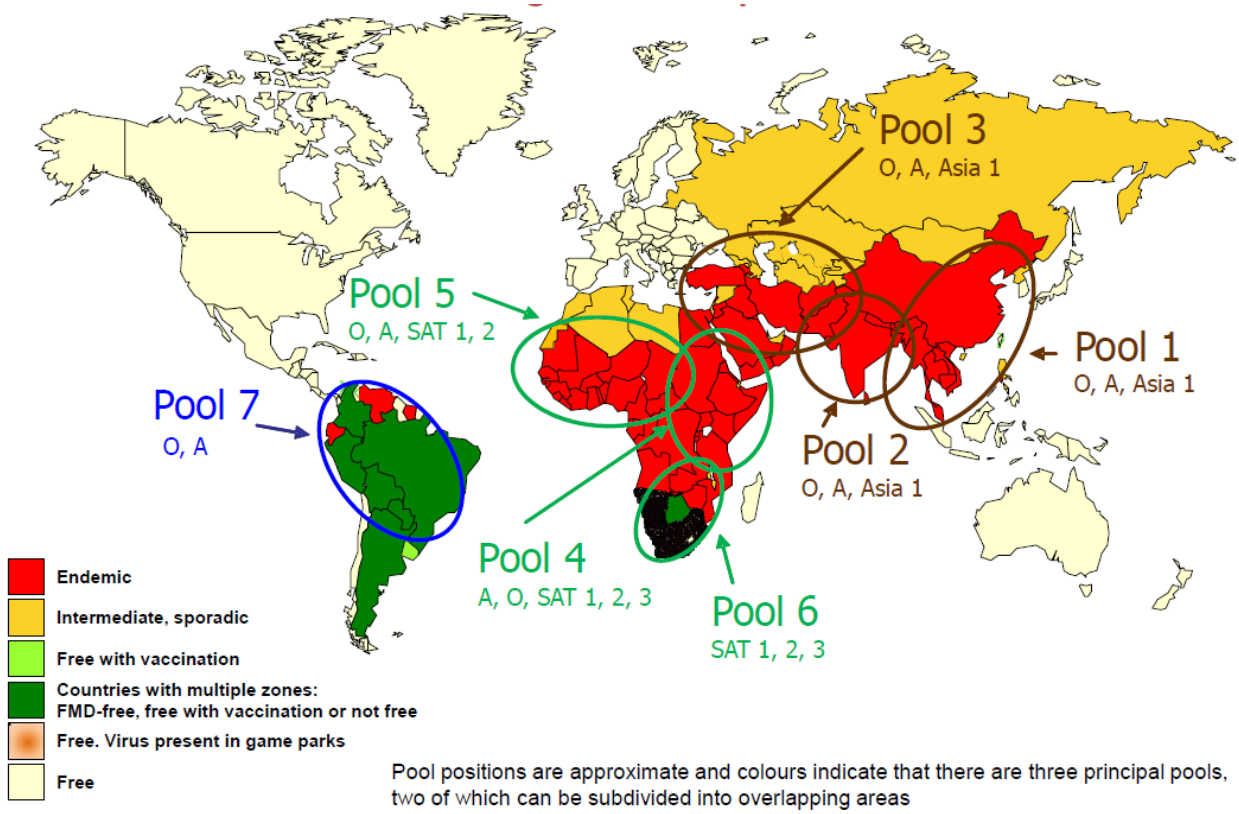


Figure 3. The conjectured status and distribution of FMD, showing regional virus pools (Hamond 2012).

2.3.2 Serotype distribution of the disease

The serotypes of FMDV are not distributed uniformly around the world. The serotype O, A and C viruses have had the widest distribution and have been responsible for outbreaks in Europe, America, Asia and Africa. However, the last reported outbreak due to serotype C FMDV was in Kenya during 2005 (Rweyemamu *et al.*, 2008b) and so serotype C viruses may no longer exist outside of laboratories. The SAT1-3 viruses are normally restricted to

sub-Saharan Africa. However, there have been some limited outbreaks due to SAT1 viruses in the Middle East between 1962–1965 and 1969–1970 and then in Greece in 1962 (Knowles and Samuel, 2003). Similarly, there have been reports of minor incursions of the serotype SAT2 in Yemen in 1990 and in Kuwait and Saudi Arabia in 2000. More recently, FMD outbreaks due to serotype SAT2 spread from sub-Saharan Africa through northern African countries (Egypt and Libya) and into Palestine (Valdazo *et al.,* 2012). This serotype was also detected in Bahrain. The serotype Asia-1 FMDVs are generally confined to Asia, except for two incursions into Greece, one in 1984 and a second in 2000.

2.3.3 Host range

Foot and mouth disease is highly contagious and affects over 70 domestic and wild Artiodactyls species (Hedger, 1981). It naturally infects and causes disease in cattle, pigs, sheep, goats and many wild ruminants and susceptibility of these animals can vary with breed of animal and strain of virus (Thomson, 1994; Kitching and Hughes, 2002; Kitching and Alexandersen, 2002).The disease is considerably less obvious or sub-clinical in breeds of cattle, sheep and goats indigenous to Africa and Asia, where FMD is endemic and these animals are believed to have been the source of infection for countries previously considered disease-free. Foot and mouth disease has been reported in several species of African antelope (Anderson *et al.,* 1975; Ferris *et al.,* 1989; Thomson *et al.,* 2003) and serological surveys showed that most species of animals in sub-Saharan Africa possessed antibodies to one or more serotype (Condy *et al*., 1969; Anderson, 1981).

2.3.4 Carrier animals

Following the acute phase of FMDV infection in ruminants, some animals may experience a long asymptomatic persistent infection. In addition, animals which have been successfully vaccinated may also become persistently infected if exposed to infectious virus. These animals are referred to as carrier animals, and the carrier state is a complication which can occur during outbreak situation (Alexandersen *et al.,* 2002**)**. Carrier animals are defined as those from which live viruses can be isolated at 28 days, or later, after infection **(**Sutmoller *et al.,* 1968**)**. In domestic cattle, the carrier state can last as long as 3.5 years, and it has also been identified in sheep and goats but not in pigs. African

buffalo have been reported to carry live virus for up to 5 years and other cloven-hoofed wildlife may become carriers. The number of carrier animals in a population depends on the species, the incidence of infection, and the immune state of the herd, i.e vaccinated or not vaccinated **(**Condy *et al.*, 1985).

2.3.5 Molecular epidemiology

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles and Samuel, 2003). Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks (Cottam *et al.*, 2008). RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed techniques for performing these studies, and reference laboratories hold databases containing over 3000 partial sequences (OIE, 2012).

2.3.6 Sources of infection and transmission

FMD is a highly contagious disease and can be transmitted in many ways leading to very rapid spread of the disease within farms and, to the surrounding farm areas. The primary transmission modes are: direct contact between infected animals and susceptible animals, mechanical transmission by indirect means e.g. contaminated human clothing and animal feeds, aerosol spread, including long distance wind-borne transmission under exceptional epidemiological and environmental conditions (Alexandersen *et al*., 2002).

Direct contact between infected and susceptible animals is the dominant mode of virus transmission and spread of the disease. Some ninety-five percent of outbreaks of FMD are the result of direct contact (Bannet and Cox, 1999). Infected animals release virus in exhaled air, all excretions, and secretions and from ruptured vesicles. As excretion may commence up to four days before the appearance of clinical signs, the movement of animals that are incubating the disease is of great epidemiological significance. The disease

may spread extremely rapidly in intensive farming areas because of high stocking density and the level of challenge both from infected animals and the environment conversely; disease spread in extensive grazing areas in hotter climates can be more insidious (Barnett and Cox, 1999).

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 rlater 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 peak in serum only 14-21 days after infection (Mulcahy, et al., 1990).

2.3.8 Pathological aspect

FMD is an acute systemic infection affecting even-toed ungulates, both domesticated and wild, including cattle, swine, sheep, and goats. Beside domesticated animals, other animals species affected with FMD include elephant, spotted deer, and wild buffalo (Vosloo *et al.,* 2002). FMDV produces an acute, systemic vesicular disease, which requires a differential diagnosis from other vesicular diseases (Sutmoller *et al.,* 1992). In natural infection, the main route of virus entry is the respiratory tract. The initial virus multiplication usually takes place in the pharynx epithelium, producing primary vesicles, (Burrows *et al.,* 1981). The vesicles produced by FMDV generally affect epithelial tissues. The clinical outcome of the disease may vary among the host species considered and the infecting virus strain. In

cattle and pigs, fever and viraemia usually start within 24–48 hr after epithelium infection, leading to viral spread into different organs and tissues and the production of secondary vesicles preferentially in the mouth and feet. The acute phase of disease lasts about 1 week and declines gradually coinciding with the emergence of a strong humoral response (Salt, 1993). The morbidity and mortality in FMD depends upon the breed and age of the animal where mortality in adult animals is very low in comparison to 20 per cent in young stock. The calves show prominent signs of myocarditis, whereas piglets manifest gastroenteritis. In sheep and goats, symptoms are frequently less severe and may make the detection of the disease difficult (Knowlers *et al.,* 2001). Asymptomatic, persistent infection can also be established in ruminants, during which infectious virus can be isolated from the esophagus and throat fluids of the animals from a few weeks up to several years of the initial infection. There is epidemiological evidence to support the hypothesis that carrier animals may be the origin of outbreaks of acute disease when brought into contact with susceptible animals. This mode of transmission has been experimentally reproduced for serotype SAT isolates (Vosloo *et al.,* 1996).

2.4 Diagnosis

Due to the rapidity of spread of FMD and the serious economic consequences that can arise from an outbreak, prompt, sensitive and specific laboratory diagnosis and identification of the serotype of the viruses involved in disease outbreaks is essential. The accurate diagnosis of infection with FMDV is of prime most importance for both control and eradication campaigns in FMD endemic areas and as a supportive measure to the stamping out policy in FMD-free areas (Remond *et al.,* 2002).

2.4.1 Clinical Sign

When susceptible animals are in contact with clinically infected ones, clinical signs usually develop in 3 to 5 days, although in natural infection, the incubation period may range from 2-14 days. The severity of clinical signs of the disease varies with the strain of the virus, the exposure dose, the age and breed of the animal, the host species and its degree of immunity. The signs can range from a mild or in apparent in sheep and goats to a severe disease occurring in cattle and pig (Rufael, 2006).The disease in cattle is characterized by

fever, depression, excessive salivation, lameness and formation of vesicular type lesions on the mucous membrane of the mouth (tongue, dental pad and gums) and the skin of the muzzle, interdigital spaces, udder, teats and coronary band (Blowey and weaver, 2003). Lesions on the tongue often heal within few days, but those on the feet and within the nasal cavities often become infected secondary with bacteria resulting in prolonged lameness and mucopurulent nasal discharge (Knipe and Howely, 2001). Young calves, lamps, kids and piglets may die before showing any vesicles because of necrotizing myocarditis, udders of lactating cows in which milk yield drops dramatically and resulting in mastitis.

The sudden onset of severe lameness is the commonest finding in affected pigs, the feet of which are obviously painful. Vesicles appear as raised white areas of 0.5-1cm in diameter on the dorsum of the tongue, on the snout and on the teats of the sow and rupture readily leave small ulcers (Sileshi *et al.,* 2006; Radostits *et al.,* 2007). In sheep and goats, if the clinical signs occur, it tends (Aitken, 2007) to be very mild and may include dullness, fever; and small vesicles or erosions on the dental pad, lips, gums and tongue. In most cases mild lameness is the only sign which occurs with vesicles and erosion of the interdigital space (Hughes *et al.,* 2002). The clinical signs can be confused with other diseases (e.g. vesicular stomatitis and swine vesicular disease) and thus laboratory based diagnosis is also necessary. Furthermore, there is no cross protection between the serotypes and the serotype of a virus involved in an outbreak cannot be ascertained on the basis of clinical signs. Thus determination of the serotype involved in field outbreaks has to be established within laboratories to permit proper control/vaccination programs to be followed. Various techniques have been used to diagnose the disease and to ascertain the serotype of the virus. The current methods are described below:

2.4.2 Identification of the agent

Existing diagnostic techniques for the detection of FMD are mainly based on the following principles, identification of the infectious agent by virus isolation involving propagation on susceptible cell cultures (Jamal and Belsham, 2013). The detection of viral antigen by ELISA systems using FMDV-specific antibody or capturing reagents (Ferris *et al*., 2005), Molecular detection of viral nucleic acid by reverse-transcription polymerase chain reaction (RT-PCR) and the genetic analysis of the nucleotide sequence, mostly of the VP1-

coding region (Nardo *et al*., 2011) Detection of FMDV-specific antibody in animals previously exposed to the virus. The VNT is usually used as a confirmatory test for sera found positive by ELISA (Paton *et al*., 2005).

2.5 Prevention and Control

FMD control is largely based on the FMD status of a geographical region. In endemic countries, it is based on regular (twice a year) vaccinations to reduce disease and transmission. On the other hand, the control policy for FMD-free countries usually includes the slaughter of animals in affected regions, as well as in neighboring regions, regardless of the disease status. However, large outbreaks in the United Kingdom and the Netherlands in 2001, as well as more recent outbreaks in Japan and Korea, where millions of animals that were mostly non infected were sacrificed and burned or buried, resulted in public outcry and questioning of these control measures. As a result, there is a need for emergency vaccination programs accompanied by vaccinate to live policies as an alternative to mass culling of infected animals (Parida, 2009).

2.5.1 Control using vaccination

The existing vaccines against FMD consist of complete, chemically inactivated virions combined with an adjuvant. The adjuvant used in the vaccine formulation has undeniably a huge effect on the efficacy and potency of the vaccine (Doel, 2003). Despite successful application in the developed world, the effective administration and optimal induction of protective immunity are hampered by several factors in developing countries. Vaccines used in the control of FMD in endemic regions are mostly used for mass prophylactic application. Such vaccines are multivalent to provide protection against multiple serotypes, and should have a potency of at least 3 PD_{50} per dose. Generally, prophylactic vaccines incorporate 146S particles combined with saponin-alhydrogel (Rweyemamu *et al.*, 2008a).

2.6 Foot and mouth disease situation in Ethiopia

2.6.1 Disease status

FMD is endemic to Ethiopia as it is in all the bordering countries like Eritrea in the northeast, Sudan in the west, Kenya in the south, and Somalia in the east and restriction of animal movement is limited. A large number of wildlife, including African buffalo (particularly in the Mago and Omo national parks), could act as FMDV reservoirs. The association of SAT serotypes with wildlife, particularly African buffalo, has been indicated (Bastos *et al.,* 2000). The disease is widely prevalent and previously used to occur frequently in the pastoral herds of the marginal lowland areas of the country. However, this trend has been changed and currently the disease is also frequently noted in the highlands of the country (Tefera, 2010).

2.6.2 FMD virus serotypes identified

FMD was first recorded in Ethiopia in 1957 when serotypes O and C were detected (Martel, 1994). Serotypes A and SAT 2 were not identified until 1969 and 1989, respectively (Roeder *et al.,* 1994). During 1988–1991, analysis of outbreak samples from Ethiopia at the National Veterinary Institute (NVI), Debre Zeit, Ethiopia, and at the Food and Agriculture Organization World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD), Institute for Animal Health, Pirbright, UK, identified serotype O and serotype SAT 2 FMDV as the causative agents (Roeder *et al.,* 1994). 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 (OIE, 2012).

2.6.3 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 the 58 FMD outbreaks which occurred in different regional states of the country showed that 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).

2.6.4 Economic impact

Foot and mouth disease is considered as the most economically important animal disease in the world. The economic impact of the disease and the incentive to invest on control depend, among other factors, on the type of farming system and the exporting potential of the country for animal and animal products. In Ethiopia, livestock production is dominantly traditional extensive system (crop livestock mixed farming system and pastoral system) and small portion of intensive dairy and feed lot farming. Foot and mouth disease occurs endemically in all production systems. Although it is generally considered that FMD has less impact at farm level in extensive production systems, there are evidences elsewhere in the world that show economic importance of the disease in these types of systems and a positive economic return from investment in control programs. (Barasa *et al.*, 2008; Bayissa *et al.*, 2011). Large numbers of ruminants are exported in the Ethiopian financial year (July 2010–July 2011), meat and livestock export revenue was $211.1 million, mostly from live animal trade with the Middle East (>472,041 heads of live animals, 70% of which were cattle) (SPS-LMM, 2011). Having an economy that is highly dependent on small holder and animal-based agriculture, including the widespread use of beasts of burden, the direct impacts of FMD are substantial in Ethiopia. In agro-pastoral areas, FMD infected oxen are unable to work for the entire season when affected at cropping time. Pastoralists are particularly vulnerable to FMD as their living depends entirely on their livestock (Bayissa *et al.*, 2011). By reducing the supply of milk FMD impacts on food security, particularly when outbreaks occur during times of the year when other food sources are limited and dependency upon milk is greatest (Barasa *et al.*, 2008). Many cattle were kept for draft power to cover for FMD affected cattle. Impacts largely occurred as reduced

household food production and farmer welfare and not income due to limited market participation (Jemberu *et al.*, 2014). Outbreaks in commercial dairy farms caused losses of almost US$2000 (Ashenafi, 2012). Milk constituted half of the daily diet of Borena pastoralists. About a quarter of cattle were infected within the last year or two. Infected cattle experienced milk reductions of >70% for about 1 month on average (Bayissa *et al.*, 2011).

2.6.5 Vaccine Type

Currently trivalent inactivated vaccine manufactured from locally isolated FMDV serotypes O, A and SAT2 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 from October 2016 to May 2017 in central part of Ethiopia in areas where outbreak of FMD occurred. Outbreaks have been reported at Addis Ababa city council (Akaki, Bole and Yeka sub city), Oromia regional state East Shoa zone (Mojo, Koka and Alemtena) and Amhara regional state Debre Berhan (Angolela, Birbersa and Godoberet). The study areas were selected based on frequent outbreak occurrence and accessibility. The specific district kebeles and farms were purposively selected following the report of FMD outbreak.

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°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 bordered by Oromia Special Zone Surrounding Finfinne (CSA, 2009).

East Shoa Zone, which is located in the central part of Oromia regional state of Ethiopia; with Adama city being the administrative center. Absolute location of East Shoa Zone extends from 70 33′0″ to 9008′56″N and 38024′10″E to 400 05′ 34″E which indicate that this zone is located in tropical climatic zone though the 31 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 9,633.52km2. 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 18ºC and 30ºC and its mean annual rainfall is 410mm-820mm (CSA, 2015).

Debre Berhan Located in the north Shoa Zone of the Amhara Region, about 120 kilometers north east of Addis Ababa, the town has a latitude and longitude of 9°41′N 39°32′E and

9°41′N 39°32′E and an elevation of 2,840 meters. is found at sub tropical zone of Ethiopia. The average annual temperature of the city during day and night hour is 17.8ºC and 8.83ºC respectively with precipitation 66.17mm (CSA, 2009).

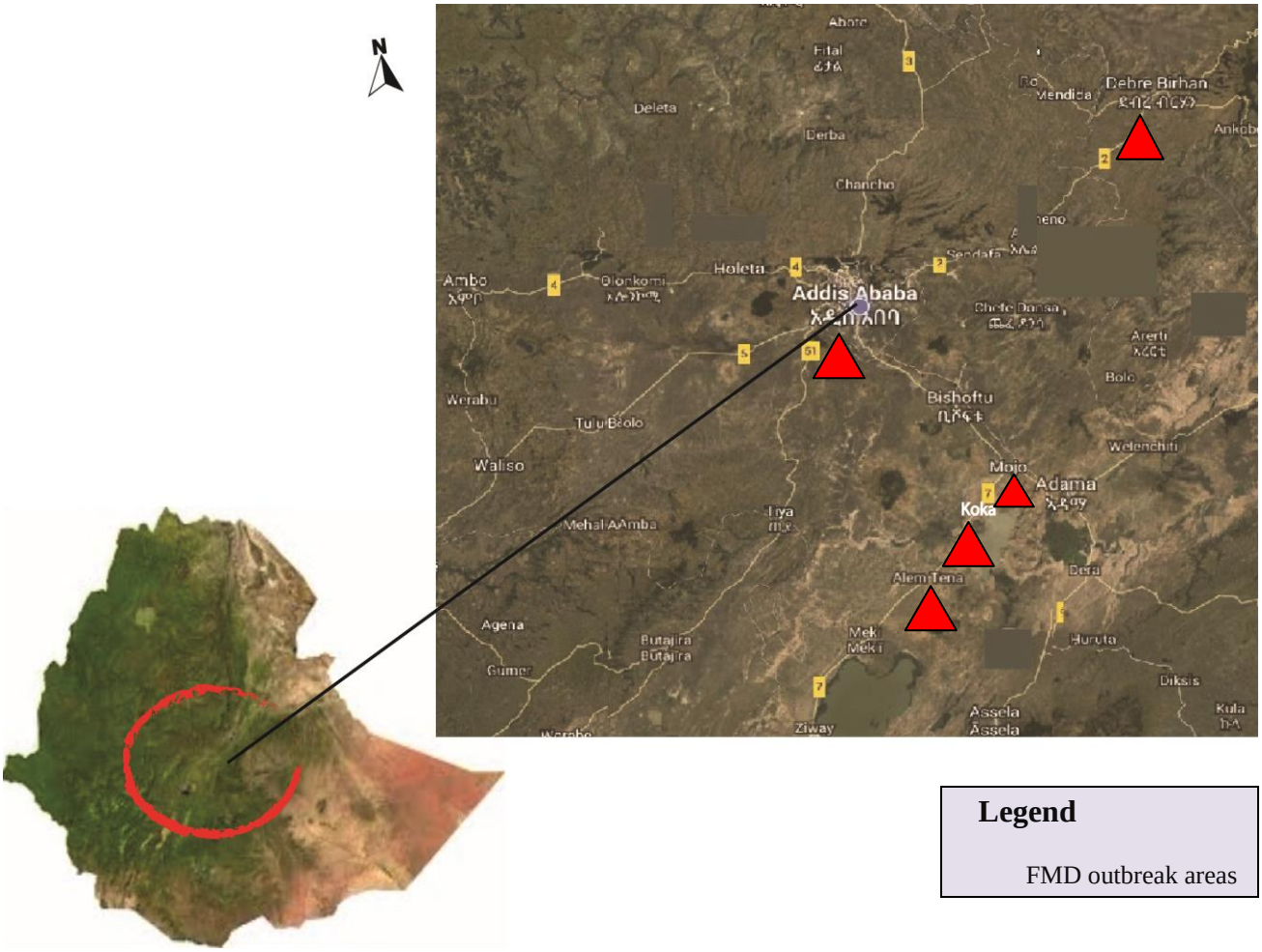


Figure 4. Map of Ethiopia showing the study areas.

3.2 Study population

The study population consists of cattle that manifest clinical signs of FMD in the outbreaks of the study areas. Cattle of all age group, sex, breeds and different management practices were purposively sampled. Production system of the selected areas were mixed crop farming for most zebu breeds while Holstein and Holstein-zebu cross cattle were kept under intensive dairy farming.

3.3 Study Design

Based on occurrence of FMD cross sectional study design was used for outbreak investigation so as to isolate and characterize the serotypes of FMD virus circulating in the study area. When an active outbreak was reported, a field investigation was conducted at specific site of an outbreak. Sick animals were clinically examined for the presence of FMD lesions on the mouth, feet, teat and udder. Specimens were collected from clinically sick animals with vesicular lesions suspected of FMDV infection. Infectious virus isolation, molecular characterization and sequencing were done. *In vitro* experimental study was also conducted to evaluate antigenic or serological relationship between FMDV vaccine strains and representative field isolates. For the purpose of vaccine matching the existing vaccine produced by NVI of Serotypes O-ETH/38/2005 and SAT2-ETH/64/2009 were included. FMDV specific antibody negative calves (n=5 per vaccine) were vaccinated and from each vaccine strain antiserum was collected. The titer of antibody to the vaccine strain is established for each serum collect for the intended vaccine matching.

3.4 Sample Collection

During the study period, at least 1gram of epithelial tissue samples which were un-ruptured or recently ruptured vesicle were collected from FMD suspect cattle usually from the tongue or buccal mucosa. The samples were transported from the collection site to the NVI, Bishoftu in 0.04 M Phosphate Buffer Saline solution (pH 7.2–7.6) with glycerol containing antibiotics and antifungal through maintaining the cold chain. The samples were immediately stored at –20ºC until processed (OIE, 2012).

3.5 Laboratory work

3.5.1 Preparation of working materials (glassware)

Glassware, reagents and media were prepared and sterilized according to the standard operating procedures prepared by the NVI

3.5.2 Preparation of Baby Hamster Kidney (BHK-21) cell monolayer

For sub-culturing of BHK-21 cell line; confluent monolayer BHK-21 cell line, complete media (base media plus 10% fetal calve serum), enzyme and tissue culture flask 25cm^2 are required. The old medium overlaying the cell monolayer was poured off in a sterile beaker under BSC class II. The confluent monolayer cell was washed twice with PBS and cover the whole cell surface with trypsin/versin and incubate for few minutes and decant the enzyme and place the flask upside down the cell to reduce the effect of remnant trypsin/versin for 3-5 minutes then the confluent monolayer cell detached from tissue culture flask and then repeatedly pipette to made single cell by adding 10 ml complete media and finally dispensed in to another new sterile tissue culture flasks.

3.5.3 Virus Isolation

Virus isolation was done under Bio Safety Cabinet class II using confluent monolayer of BHK-21 cell line. A tissue suspension was prepared by grinding the tissue sample with sterile pestle and mortal with small volume tissue culture medium and antibiotics, so as to be the final volume was 10x that of the epithelial tissue, producing 10% suspension. The suspension was centrifuged at 3500 rpm for 10 minutes. The supernatant was collected and filtered by Millipore filter that has 0.22 micro pore sizes. About 0.5 ml of filtered tissue suspension was inoculated on baby hamster kidney (BHK-21) monolayer cells grown on 25 cm^2 tissue culture flask and incubated at 37°C for 1 hour for adsorption of the virus and then hanks virus media 10 ml was added and incubated at 37°C for 24-48 hour. The inoculated cell line was harvested when 85- 100% of CPE was observed. Tissue-cultured FMD virus samples that showed CPE was labeled using the following format three-letter: country code / isolate number / year (e.g., ETH/01/2016) and stored at -20^{0}c. The three letter country codes were designated as outlined by the World Reference Laboratory for FMD. 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 (Buxton and Faser, 1977; Yoseph et al., 1991; OIE, 2012). Samples not exhibiting CPE by 72 hours post infection on the second passage were considered virus negative.

3.5.4 Polymerase chain reaction (PCR) assay

3.5.4.1 Extraction of Virus RNA

The total RNA was extracted from the cell culture isolates by using RNeasy® mini kit (Qiagen, USA) based on the manufacturer protocols. Briefly, 350µl of tissue culture sample was taken and put into a 1.5 ml eppendorf tube labeled with sample code and then 350µl volume of Lysis buffer RLT was added to the sample and mixed by vortexing, then centrifuged at 13400 rpm using mini spin centrifuge for three minuts. Next to centrifuged 350µl of 70% ethanol was added and mixed by vortexing. The mixture was transferred to RNeasy mini spin column (700µl maximum loading volume) placed in a 2ml collection tube under class II laminar air flow cabinet and spinned in a micro centrifuged for one minute at 12,500 rpm. The flow through was discarded from the collecting tube and repeated with remaining volume. The RNA was washed with 700µl washing buffer RW1and centrifuged for 1minuts at 12,500 rpm and again washed and centrifuge at 13400 rpm with 500µl RPE buffer subsequently. After the flow through was discarded the RNease mini spin column was centrifuged at 13,400 rpm speed for 2 min to dry the membrane. Then, the RNease mini spin column was transferred to new clean and labeled eppendorf tube, then RNA free water (elution buffer) 50µl was added and then centrifuged at 13400 rpm for 1 minutes, then the RNA was elute with RNA free water in to eppendorf tube and stored at -20oC until used.

3.5.4.2 *Complementary DNA Synthesis (cDNA)*

The complementary DNA was synthesized based on the manufacturers protocol(Invitrogen, Germany) in 20µl reaction volume. Primarily 1µl oligo(dT) primer, 1µl 10 mM dNTPs, 3µl RNase free water and 5 µl extracted RNA were added to 0.5ml PCR tube and incubated at 65°C for 5 minutes (PCR machine) and chilled on ice for 3 minutes. Then cDNA synthesis mix were prepared as 2µl 10x RT buffer, 4µl 25mM MgCL2, 2µl 0.1M DTT and 1µl RNase OUT and 1µl Superscript III(Reverse transcriptase).The10 µl cDNA synthesis mix were added to RNA/primer mixture mixed gently incubated at 50°C for 50 minutes, followed by heating at 85°C for 5 minutes chilled on ice for 2 minutes then added 1 µl

RNase-H and centrifuge and incubate at 37°C for 20 minutes and cDNA was stored at -20°C until used.

3.5.4.3 Polymerase Chain Reaction (PCR) and Primers Used

FMDV suspected RNA samples were screened after RT-PCR Master mix preparation by universal primer combinations of FMDV7 – forward 5'GCCTGGTCTTTC CAGGTCT3' and FMDV7–reverse 5'CCAGTCCCCTTCTCAGATC 3' for the RT-PCR of FMDV in NVI. The thermal profiles used for amplification of the 5' UTR to screen the FMDV were as follow: Initial denaturation at 95^{O}C for 5 min, 30 cycles of denature at 94^{O}C for 1 min, annealing at 54^{O}C for 1 min and extension at 72^{O}C for 1 min, followed by final extension 72^{O}C for 10 min to get the DNA of 328bp.

After screening FMD positive samples, a specific primer was used for amplification in serotype identity, for the serotypes O viruses: a primer (Oligoname) set Primer-FMDV Sero type O–lC244-Fow-5pm/µl5'-GCAGCAAAACACATGTCAAACACCTT-3' and primer-FMD serotype O-EUR-2B52-Reverse-5pm/µl-5'-GACATGTCCTCCTGCATCTG TTGAT-3' were used. For the serotype A viruses, Primer-FMDV serotype A-Fow-5pm/µl 5'-ACTGCAACGGGGGGAA TCT-3'Primer-FMDV serotype A-Reverse-5pm/µl 5'-AGTTGTTTGCGGGTGTGCAAT3'were used and for the serotype SAT-2 viruses Primer-FMDV Sero typeSAT2 -Fow-5pm/ µl 1C-445 5-TGGGACAMGGTYTGAACTC-3' Primer-FMDV Sero type SAT2-Reverse-5pm/ µl 5'- 2B 208 5'-ACAGCGGCCATGC ACGACAG-3' were used for RT-PCR amplification. The thermal profiles used for amplification of the VP1of 1165 bp for the serotype O was: Initial denaturation at 95^{O}C for 5 min, 35 cycles of denature at 95^{O}C for 1min, annealing at 58^{O}C for 1 min and extension at 72^{O}C for 1hour and 30 min, followed by final extension 72^{O}C for 7 min. The thermal profiles used for amplification of the 866 bp (VP3 and 2B Gen) for the serotype A was: Initial denaturation at 95^{O}C for 5 min, 35 cycles of denature at 95^{O}C for 1min, annealing at 55^{O}C for 1mni followed by final extension 72^{O}C for 7 min. The thermal profiles used for amplification of the VP3 of 1445bp for the serotype SAT-2 was: Initial denaturation at 95^{O}C for 5 min, first round 15 cycles of denature at 95^{O}C for 1min, annealing at 60^{O}C for 1 min and extension at 72^{O}C for 1 min, followed second round 20 cycles of denature at 95^{O}C

for 1min, annealing at 57^{O}C for 1 min and extension at 72^{O}C for 1hour and 30 min by final extension 72^{O}C for 7 min.

3.5.4.4 Agarose Gel Electrophoresis for Serotype Identification

The PCR products were analyzed on the prepared 1.5% agarose gel by adding 4µl Gel red with loading dye then the PCR product were loaded in the volume of 10µl in each well and 10µl molecular marker(Ladder) was added started 100bp plus. Electrophoresis was run for one hour at 120v then the DNA fragments was visualized by UV illumination, at the targeting bp and documented.

3.5.4.5 Sequence generation

Sequencing of the viruses was performed at the International Atomic Energy Agency laboratory in Vienna, Austria.

3.5.5 Titration of foot and mouth disease virus

Both FMDV vaccine strains and field isolates were titrated after being adapted on monolayer BHK-21 cell culture and showed 100% CPE in 24 hours, using tenfold serial dilution beginning with 10^{-1} by adding 0.5 ml of the virus suspension to 4.5 ml of the diluents minimum essential base medium (MEM). Using a sterile pipette tips, 0.5 ml from the first dilution was taken and transferred to the next tube after vortexing and continued serially to the end using different sterile pipette tips at each transfer. Fifty microliter of each virus dilution (10^{-1} to 10^{-8}) was distributed in the wells of respective rows on microtiter plates containing established monolayers cell of baby hamster kidney (BHK-21).Then 100 µl/well of MEM base medium was added and incubated at 37° C for 24 h and the titer of each virus was determined by the use of Spearmann Karber formula.

$$\text{Log10} = (Xo - (\ \frac{d}{2}\ ) + \frac{d(\sum ri)}{ni}$$

Where: X_o = Log10 of reciprocal of the lowest dilution at which all test monolayer's are Positive.

d = Log 10 of the dilution factor that is the difference between the log dilution intervals.

ni = Number of test monolayers use at each individual dilution

ri = Number of positive test monolayer's out of ni

$\sum$ (ri/ni) = $\sum$ (p) sum of proportion of the tests beginning at the lowest dilution showing 100% positive result. The summation is started at dilution X_o (Rweyemamu *et al*., 1994).

3.5.6 Vaccine preparation

Vaccine was prepared using the standard operating procedures prepared by the NVI. The virus strain was inoculated onto a monolayer cell culture of BHK-21 cells and the resulting preparation was clarified and inactivated. The safety of the inactivated clarified virus was tested by three serial blind passages in BHK 21 cell cultures (Iyer *et al*., 2001). Then after the prepared monovalent vaccine (O-ETH/38/2005 and SAT2-ETH/64/2009) checked to be free from residual live virus by subcutaneous administration of 2ml prepared vaccine in calves for each (n=5) separately, after being screened by 3ABC ELISA for the presence of antibody against FMDV and follow up for clinical signs up to 10-14 days. The prepared vaccines were kept at +4ºC until used.

3.5.7 Antiserum production in cattle

A total of ten young cattle (6-12 months old) were used for production of anti-FMD sera against two vaccines strains (O/ETH/38/2005 and SAT2/ETH/64/2009). Prior to vaccination, these animals were screened for the presence of FMD specific antibodies using IDEXX FMD 3ABC Bovine-Ovine antibody test kit. Briefly, micro titer plates were supplied precoated with recombinant FMDV 3ABC viral antigen. Dilutions of the samples to be tested were incubated in the wells of these plates. Any antibody specific for 3ABC binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbounded material was removed from the wells by washing. A peroxidase-labeled anti-IgG conjugate was added, that binds to the antibodies of sample complexed with the 3ABC antigen. Unbounded conjugate is removed by washing, and the TMB substrate was added to the wells. The degree of color that develops (Optical density measured at 450 nm) was directly proportional to the amount of antibody specific for 3ABC present in the sample. The diagnostics relevance of the result is obtained by

comparing the optical density (OD) that develops in wells containing the samples with the OD from the wells containing the positive control and the result expressed in percent (see annex 1).

Then FMDV sero-negative animals (n=5) were selected for each serotype, quarantined in a separate area, ear-tagged and vaccinated with 2ml dose subcutaneously in dewlap region with the monovalent vaccine prepared from each vaccine strain. Sera were collected 21 days post vaccination and then tested for antibody titer against known titer of the homologous strains. Sera with strong titer were selected and pooled for each vaccine serotype, excluding low responders and stored at -20^{O}C for the intended vaccine matching. The sera raised against the vaccine strains were inactivated at 56°C for 30 minutes before using for vaccine matching test (OIE, 2012).

3.5.8 Vaccine matching of FMD virus field isolate

The serological relationship between a field isolate and a vaccine virus ('r' value) can be determined by virus neutralization test (VNT) (Pereira, 1977, Kitching *et al.*, 1988; Mattion *et al.*, 2009). One way testing is recommended (r1) with a vaccine antiserum, rather than two way testing (r2), which also requires an antiserum against the field isolate to be matched according to the standard protocol of the (OIE, 2012) manual. This test uses antiserum raised against a vaccine strain. The titers of this serum against 100 $TCID_{50}$ of the homologous vaccine strain and the same dose of a field isolate were compared to determine how antigenically 'similar' the field viruses were to the vaccine strain. The field isolates were assessed for their serological relationships to the reference vaccine strain viruses sera of O-ETH/38/2005 and SAT2-ETH/64/2009, respectivel. Briefly, a VNT was performed using BHK-21 clone 13 cell line in flat-bottomed tissue culture-grade micro titer plates. Both field isolates and vaccine strains that were passaged on monolayer of BHK-21cell, which adapted to give 100% CPE in 24 hours with known titers, were used. The sera were inactivated at 56°C for 30 minutes before testing. The test was an equal volume test in 50 µl amounts, Minimum essential complete media (MEM) supplemented with 10% fetal calf serum (specific antibody negative) with 2% antibiotics used to dilute the sera and virus, also as a medium for growing the cells. Fifty micro liter of serum raised against the

reference vaccine strain was added into top wells of row A in micro titer plate containing 50µl/well of minimum essential media (MEM) and serially in two fold down the plate diluted by mixing sera and medium (A to H). Then constant amount (50µl) of pre-titrated vaccine and field isolate samples of similar serotype 100 $TCID_{50}$ dose which were diluted and made the standard log 2 titer in the range of log10 1.5–2.5 $TCID_{50}$ was added in each well using two columns for each antigen in all diluted serum and virus control medium to be the test valid (OIE, 2012), then sealed the plate and was incubated the mixture at 37°C for 1hr. After 1hr. of incubation, 50 µl BHK-21 cell suspension was added to each well including cell control then plate was sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Finally the plate test wells were fixed and stained on the third day in 0.05% methylene blue in 10% formalin for 30 minutes, and the cytopathic effect (CPE) was read macroscopically. The endpoint titers of the serum samples, tested against both vaccine and field virus, were expressed as the reciprocal of the highest dilution of serum neutralizing exactly 100$TCID_{50}$ virus particles in 50% of the wells was considered as antibody titre of the vaccine serum against the vaccine strain and field isolate for each virus dose used was calculated using the Spearman–Kärber method. All tests were repeated three times and average 'r' value was taken for each test virus on different days as per the recommendation of (Rweyemamu and Hingley, 1984; OIE, 2012) to increase the confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated. The titer of the vaccine serum against 100 $TCID_{50}$ of each virus was then be estimated by regression. The relationship between the field isolate and the vaccine strain was then expressed as an 'r' value as:

$$r1 = \frac{\text{Serum titer against heterologous field virus}}{\text{Serum titer against homologous vaccine virus}}$$

Interpretation of the results:

- It is generally accepted that in the case of neutralization, r1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate (Rweyemamu, 1984).
- Conversely, values less than 0.3 suggest that the field isolate is sufficiently different from the vaccine strains tested that a vaccine based on these strains is less likely to protect. In this case, either the field isolate should be examined against other vaccine strains or the field isolate could be tested against existing vaccines in a heterologous cross protection challenge test. Alternatively, a suitable field isolate could be adapted to become a new vaccine strain.

4. DATA MANAGEMENT AND ANALYSIS

Antibody titers of the vaccine serum against the field isolate for each virus dose used were calculated using the Spearman–Kärber method. The titer of the vaccine serum against 100 $TCID_{50}$ of each virus was estimated by regression (r1-value). The relationship between the field isolate and the vaccine strain is then expressed as an 'r' value as: r1= serum titre against heterologous virus / serum titer against homologous virus. Sequence raw data forward and reverse nucleotides obtained from international atomic energy agency was assembled by Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). Published sequences relevant to this study were collected from Genbank by BLAST searches from NCBI to see sequence similarity. The new and Genbank sequences were saved as Fasta format and bio edit new sequences by bio informatics software and pair wise alignment were done and trim in equal length using MEGA 6 (Koichiro *et al.,* 2013) then a phylogenetic tree of these sequences were constructed using the Neighbor-Joining method.

5. RESULTS

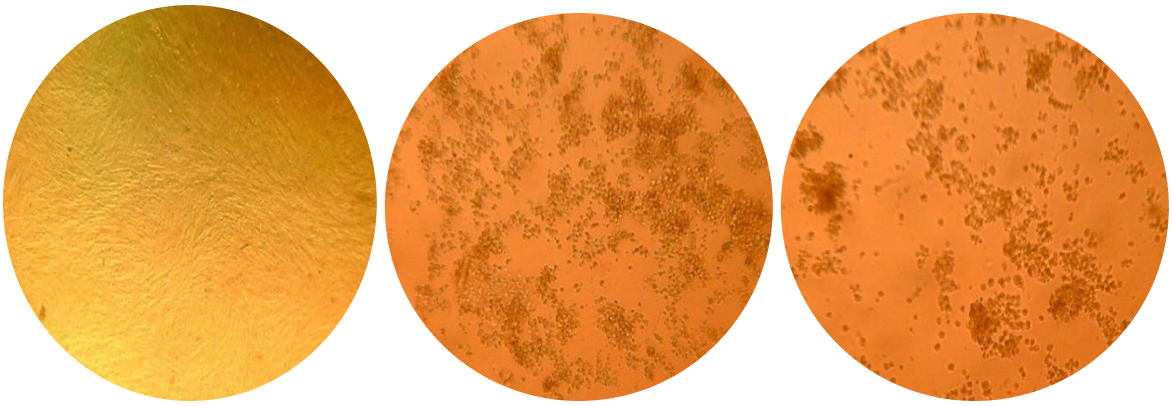
5.1 Virus Isolation

Out of the 30 epithelial tissue samples collected from Addis Ababa, Debre Berhan and East Shoa zone 28 cultured samples showed FMDV CPE on BHK-21 monolayer cell cultures (Table 1). The CPE was characterized by a fast destruction of the BHK-21 monolayer cells and infected cells were found singly and the cell was found rounded (clustered) in shape (Figure 4). Complete destruction of the cell sheet was mostly seen within 24- 48 hours of inoculation.

Table 1. FMD virus isolate from study area of outbreak

Site of outbreak	**Species**	**Date of sample collection**	**Sample type**	**No of sample**	**No of sample showing CPE**
Addis Ababa					
Bole subcity	Bovine	27/01/2016	TE	2	2
Yeka Subcity	Bovine	27/06/2016	TE	4	4
Akaki Subcity	Bovine	03/07/2016	TE	4	3
Debre Berhan					
Angolela	Bovine	09/11/2016	TE	4	4
Birbersa	Bovine	21/11/2016	TE	4	4
Godeberet	Bovine	20/12/2016	TE	4	4
East Shoa					
Moji	Bovine	08/8/2016	TE	2	2
Koka	Bovine	01/03/2016	TE	3	3
Alemtena	Bovine	16/03/2016	TE	3	2

TE-Tongue Epithelial



A B C

Figure 5. BHK21 cell line infected with FMD viruses (A control, B and C infected) cells showing CPE.

5.2 Molecular Characterization

Among the 28 isolates, 11 representative isolates were taken forward for molecular characterization by RNA extraction, RT-PCR amplification and gel electrophoresis and serotyping. The PCR product revealed 3 isolates of serotype O and 2 isolates of serotype SAT2 FMD viruses were the identified serotypes (Table 2). Purified PCR products of the 3 O FMDV serotypes were sent to International Atomic Energy Agency (IAEA) for sequencing.

Table 2. FMDV serotypes identified in different sites of outbreak.

Site of outbreak	No of sample	CPE positive	Agarose gel-based RT-PCR	
			GD	Serotype
Addis Ababa	10	9	2	SAT2
Debre Berhan	12	12	1	O
East Shoa	8	7	2	O

GD-Genome Detected

For genome detection, serotype identification and FMDV screening tests, specific primers were used in RT-PCR assay. The procedure consists of the three successive procedures of RT of the extracted RNA, PCR amplification of the RT product and detection of the PCR products by agarose gel electrophoresis. The detected PCR products were run on agarose gel electrophoresis and the results were shown in Figure (6, 7 and 8) below:

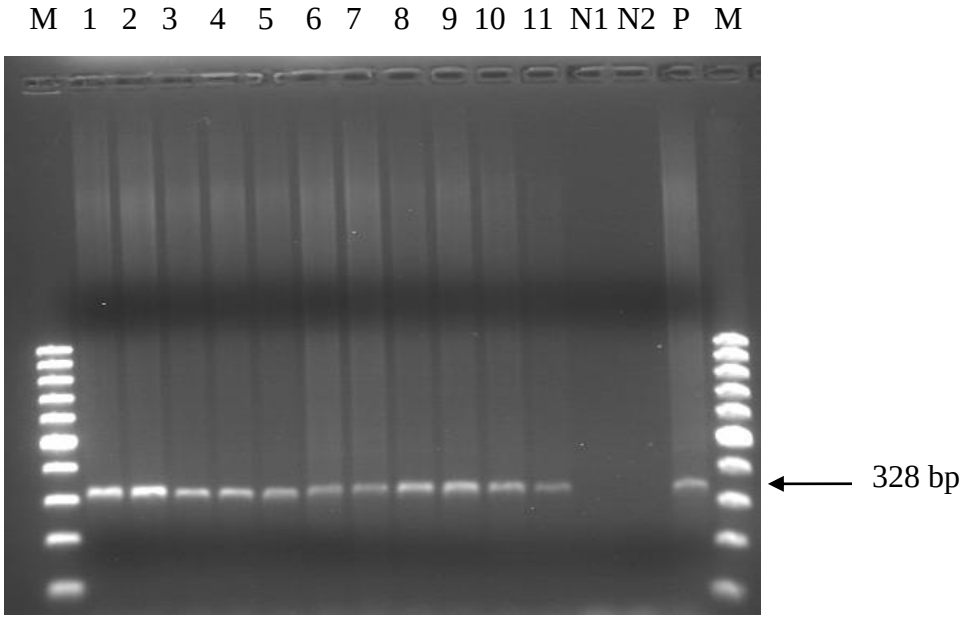


Figure 6. Gel electrophoresis result of FMDV genome detected by RT– PCR Primer mixture FMDV7 F/ FMDV7 R was targeting around 328 bp 5' UTR coding region of the virus.

M- Molecular ladder started 100 bp (Invetrogen) 1 KB.

N1 and N2- Negative controls.

P- positive control.

All 11 samples were positive for FMD virus.

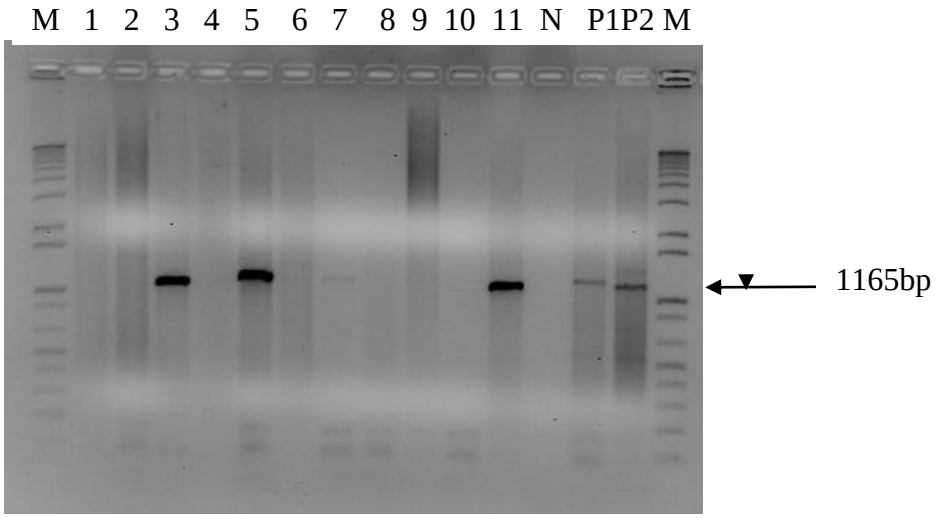


Figure 7. Result of FMDV genome by RT – PCR, Primer mixture FMDVO F / FMDVO R targeting around 1165 bp VP1 coding region of the virus.

M-Molecular ladder started 100 bp (Invetrogen) 1 KB plus

N- Negative control

P1 and P2- positive control

ETH/03/16, ETH/05/16 and 3-ETH/11/16 were positive for O serotype

ETH/01/16, ETH/02/16, ETH/04/16, ETH/06/16, ETH/07/16, ETH/08/16, ETH/09/16, and ETH/10/16 were no amplification.

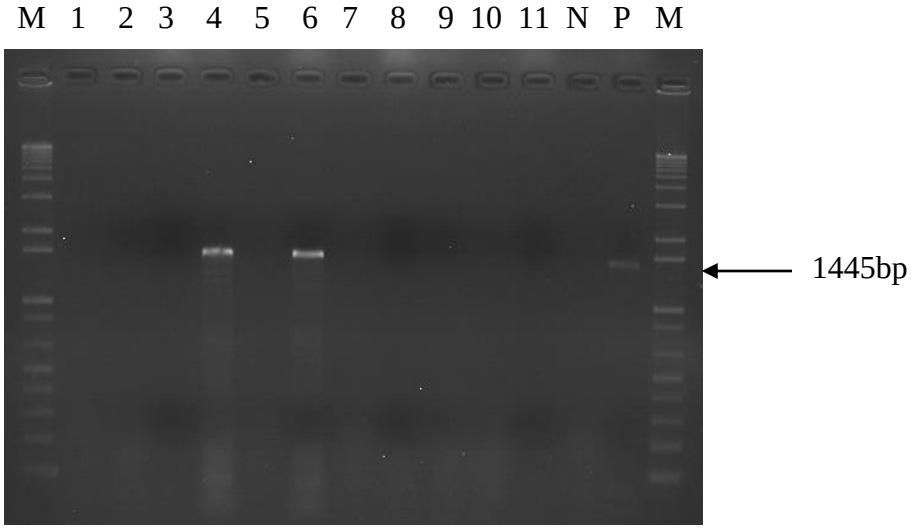


Figure 8. Result of FMDV genome by RT–PCR Primer mixture FMDV SAT2 F / FMDVSAT2 R was targeting around 1445bp VP3 coding region of the virus

M- Molecular ladder started 100 bp (Invetrogen) 1 KB plus

N- Negative control

P- Positive control

ETH/04/16 and ETH/06/16 were positive for SAT2 serotype

ETH/01/16, ETH/02/16, ETH/03/16, ETH/05/16, ETH/07/16, ETH/08/16, ETH/09/16, ETH/10/16 and ETH/11/16 were no amplification

5.3 Phylogenetic Analysis

Midpoint-rooted neighbor-joining tree based on the complete virus protein (VP) 1 coding sequence (639 nucleotides) showing the genetic relatedness between the sequences of FMDV serotype O isolated from the study areas of Ethiopia and the previously characterized Ethiopian isolates and other reference viruses of African origin were retrieved from the database. The current 3 isolates collected in 2016 clustered together with the previously characterized Ethiopia isolates in East African (EA) topotype-4.The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pair wise deletion option was computed using MEGA6. The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The analysis involved 56 nucleotides of the VP1 gene coding sequences. The three isolates sequenced in the current study are indicated in color rectangle.

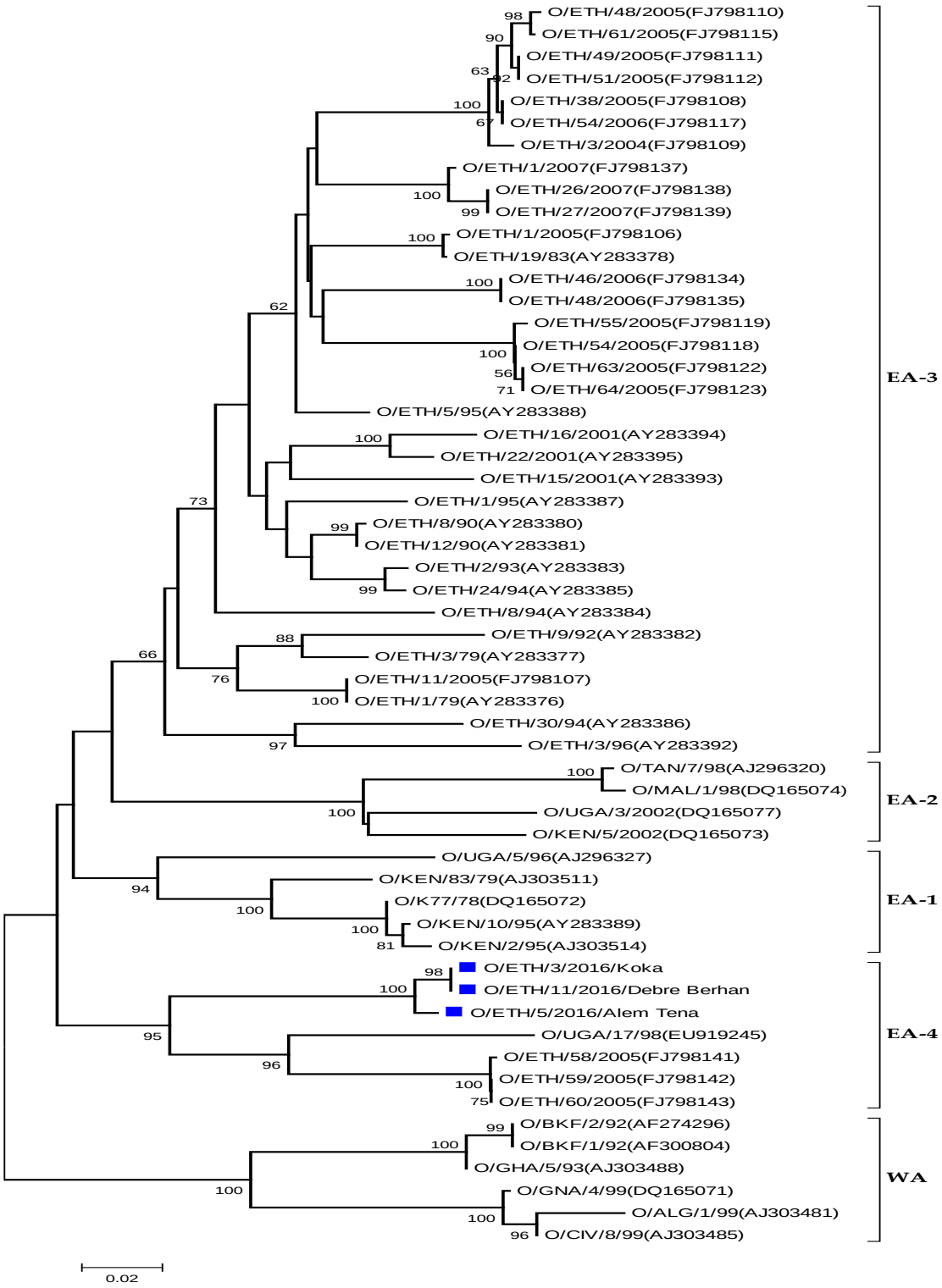


Figure 9. Neighbor –joining tree comparing the complete VPI coding sequence of type O FMD virus.

5.4 Vaccine matching of FMD virus field isolates

The field isolates were assessed for their serological relationships to the reference vaccine strain viruses of O-ETH/38/2005 and SAT2-ETH/64/2009, by VNT of one way testing with a vaccine antiserum respectively. The field isolate were selected for vaccine matching from different area of study sites based on their titer strength and distribution throughout the study areas (Table 3) and those identified serotypes in NVI. The antigenic relationships of serotype O and SAT2 field isolates to the currently corresponding used vaccine strain were shown in (Table 4 and 5). The test results were interpreted as per criteria set by Rweyemamu, (1984). A total of 5 isolates were subjected to vaccine matching using antiserum raised from bovine vaccinated by currently available vaccine in NVI. From the result, it can be seen that 100% of the tested isolates showed an r1 value of >0.3 with currently used vaccine strains O/ETH/38/2005 and SAT2-ETH/64/2009. This result revealed the vaccine virus strains has been in use at NVI still able to provide near optimal antigenic coverage to the currently circulating field isolates in the study areas.

Table 3. FMDV serotypes selected for vaccine matching.

Name of the virus	Site of isolation	Species	Date of isolation	serotype	Topotype
O/ETH/03/2016	Koka	Bovine	12/12/2016	O	EA-4
SAT2/ETH/04/2016	Addis Ababa	Bovine	14/12/2016	SAT2	
O/ETH/05/2016	Alemtena	Bovine	21/12/2016	O	EA-4
SAT2/ETH/06/2016	Addis Ababa	Bovine	29/12/2016	SAT2	
O/ETH/11/2016	Debre Berhan	Bovine	04/01/2017	O	EA-4

Table 4. Mean ‘r1’ values obtained between serotype O field isolates and vaccine strains

Field virus	Vaccine virus (O/ETH/38/2005)
O/ETH/03/2016	0.67
O/ETH/05/2016	0.80
O/ETH/11/2016	0.90
Mean ‘r1’ value	0.79

The results presented (Table 4.) revealed that there is a close antigenic relationship between the vaccine strain and tested serotypes O field Isolates and the calculated ‘r1’ value was considerably greater than the minimum requirement (>0.3). The mean ‘r1’ values of the serotype O field isolates to the vaccine strains O-ETH/38/2005 was 0.79

Table 5. Mean‘r1’ values obtained between serotype SAT2 field isolates and vaccine strains.

Field virus	Vaccine virus (SAT2/ETH/64/2009)
SAT2/ETH/04/2016	0.75
SAT2/ETH/06/2016	0.89
Mean ‘r1’ value	0.82

The antigenic relationship of the two serotype SAT2 field isolates with the currently used vaccine strain SAT2-ETH/64/2009 was determined. Both the isolates had an antigenic relationships of (>0.3) with the vaccine strain. The mean r1-value of the field isolates to the vaccine strain was 0.82. The serotype SAT2 vaccine strain, has been in use in NVI is still able to provide optimal antigenic coverage to the circulating field isolates in the study area.

6. DISCUSSIONS

6.1 FMD Virus Isolation and Serotype Identification

In this study from the total number of 30 epithelial tissue samples collected from bovine which were suggestive of FMD lesions 28 (93%) showed CPE characterized by a fast destruction of the confluent monolayer in cell culture and the result obtained was in agreement to previous work by (Huang *et al*. 2011; Negussie *et al*. 2011; Haileleul *et al*. 2013; Tesfaye, 2014; and Mishamo, 2016) 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 this study two serotypes (O and SAT2) FMD viruses were isolated. The isolated O serotype from tissue samples collected from East Shoa and Debre Berhan were in agreement with the previous work who reported the predominant circulation of serotype O causing an outbreak in Ethiopia (Gelaye *et al.,* 2005; Ayelet *et al.,* 2009; Nugussie *et al.,* 2010 and Menda *et al.,* 2014).

Serotype SAT2 was isolated from tissue sample collected from Addis Ababa. The results agree with the previous serotype SAT 2 isolated and reported from cattle in Addis Ababa (Dejene, 2004; Ayelet *et al*., 2013 and Mishamo, 2016) the virus was responsible for the occurrence of the investigated outbreak in these areas.

6.2 Phylogenetic Analysis

The isolated O serotype from Koka, Alemtena and Debre Berhan (O/ETH/03/2016, O/ETH/05/2016 and O/ETH/11/2016) FMD viruses were compared based on 639 nucleotide sequence of VP1. The viruses were closely related (99-100% nt. sequence similarity) with each other. This indicated that they are antigentically related. The nucleotide sequence analysis revealed that the nucleotide sequence differences of the two virus isolates (O/ETH/03/2016 and O/ETH/11/2016) were <2% and this indicated that outbreaks due to these isolates were from the same origin. These might be due to uncontrolled animal movement from one part of the country to the other which facilitated dissemination of the virus. These new isolates are homologous, geographically clustered and formed a single genetic lineage called topotype Africa (EA-4). The result agrees with the previous studies of Mizan Teferi area in 2005 samples collected from cattle, the isolated

O serotype falls in to topotype EA-4 (Ayelet *et al.,* 2009). Virus from Uganda O/UGA/17/98 with 95% nucleotide identity with the current isolated Ethiopia virus also belonged to this topotype EA-4.

6.3 Vaccine matching

The serological matching between three O serotype and two SAT2 field isolates of vaccine strain serotypes O-ETH/38/2005 and SAT-ETH/64/2009 in the VNT test described reliable protection level conferred for circulating O and SAT2 serotypes in the study areas. The r1-values obtained for both serotypes indicated that 100% of the tested viruses were above the cut-off point. The mean r1- values obtained for O-ETH/38/2005 was 0.79 and for SAT2-ETH/64/2009 was 0.82. Results agreed with the interpretation criteria of (Rweyemamu, 1984 and Paton et al., 2005) in which r1-values greater than 0.3 is an indicative of serological match between field isolates and vaccine strain viruses. This also supported by (Ayelet *et al*., 2013) finding that the O-ETH/38/2005 and SAT2-ETH/64/2009 vaccine strains assessed for their serological relationship by VNT revealed 0.84 and 0.76 serological match respectively.

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7. CONCLUSIONS AND RECOMMENDATIONS

FMD remains a major threat to the livestock industry in Ethiopia. Direct losses due to death and disease are easy to appreciate, however, the burden of FMD often manifests as widespread and ongoing losses that limit development of the livestock sector, in terms of restrictions on the trade of animals and livestock productivity. Ethiopia is among the countries that are endemic for FMD. Outbreak occurrence frequency increasing time to time. The circulation of multiple serotypes of the virus that are responsible for current infection in Ethiopia are O, A, SAT 1, and SAT 2 in different species of cloven hoofed animals that remain uncontrolled. During the study period among the circulating serotypes O and SAT 2 were identified from the collected tissue samples. Sequence result revealed the isolate O virus lies in topotype East Africa (EA-4). The determined r1 value of the serological relationship between the field isolates and vaccine strains (O/ETH/38/2005 and SAT2/ETH/64/2009) revealed the antigenic similarity of vaccine strain serotypes and field isolates, which indicated the vaccine strains still, have a capacity to protect the circulating field isolates. FMD is extremely challenging, as such regular monitoring and more detailed investigations are needed to formulate an efficient vaccine-based FMD control strategy for Ethiopia. Progressive control of FMD may be achieved if founded on sound epidemiological understanding of the disease which is ecosystem specific.

Therefore based on the above conclusions the following points are recommended:

- Regular monitoring and surveillance of the circulating FMD serotypes across the country in livestock may help with selection of appropriate vaccine strains for FMD control and vaccine quality control.
- Vaccine matching should be conducted at intervals covering since the virus serotype of subtype circulating in the country might change.
- Animals need to be covered under vaccination so as to control the outbreak of diseases. It can be made possible only through implementation of veterinary education for livestock owners about economics of the diseases and by the availability of vaccination services.

- Implementing strict animal movement control both across national and international boundaries to limit the spread of existing serotypes and introduction of new serotypes.

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9. ANNEXES

Annex 1: Test procedure of IDEXX FMD 3ABC Bo-Ov antibody test kit.

1. Predilute sample and positive and negative controls 1:100 in a tube using sample Diluents. (For example, add 5 µl of sample or control to 495µl of sample diluents).
2. Dispense 100µl of prediluted samples and positive and negative control in to the appropriate wells of the micro titer plate. (Final dilution =1:100).
3. Cover the micro titer plate with a lid and incubate for 60 minutes (±10 min.) at $+37^0$C (± 3^0C) in a humid chamber.
4. Wash each well with approximately 300µ l wash solution three times. Aspirate liquid contents of of all wells after each wash. Following the final aspiration, firmly tap residual wash fluid from each plate on to absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.
5. Dispense 100 µl of the conjugate in to each well.
6. Cover and incubate the micro titer plate for 60 minutes (±10 min.) at $+37^0$C (± 3^0C) in a humid chamber.
7. Repeat step 4.
8. Dispense 100 ul of TMB substrate N.12 into each well.
9. Incubate the TMB substrate N.12 at 18-26^0C for 15 minutes (±1 min.) .
10. Stop the color reaction by adding 100 µl stop solution N.3per well. The stop solution should be dispensed in the same order and the same speed as the substrate.
11. Read the results using a photometer at a wavelength of 450 nm.

Results

To validate the assay, the optical density (OD) of the positive control (PC A_{450}) should not exceed 2.00 and the OD of the Negative control (NC A_{450}) should not exceed 0.500. The difference between the positive and the negative control (PC A_{450}) should be $\geq$ 0.400. Make sure to read the plates within two hours after the addition of the stop solution.

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Calculation

The OD of duplicates must be averaged. The OD of the positive control ($PC\bar{X}$) and the OD of the samples (sample A450) are corrected by subtracting the OD of the negative control ($NC\bar{X}$):

Positive control Corrected OD:	**Sample corrected OD:**	**S/P ratio for each sample:**
$PC\bar{X} - NC\bar{X}$	Sample $A_{450} - NC\bar{X}$	$S/P\ \% = 100 \times \frac{\text{sample } A_{450} - NC\bar{X}}{PC - NC\bar{X}}$

Interpretation of results

S/P	<20%	≥20% and <30%	≥ 30%
Interpretatio	Negative	Suspect	Positive

Annex 2: Procedure for Two-Dimensional Virus Neutralization Test (VNT)

1. Virus dilution series

   The pre-determined virus titer (e.g. $10^{5.5}$) is assumed, and the half log dilution series for this example would be in the region of $10^{-2.5}$ to $10^{-4.5}$, which ensures the theoretical 2 log dose ($10^{-3.5}$) is midway between and therefore in the middle of the plate.

1.1. Using the above as a guideline, set out as many bijoux as needed for each virus to be tested, including the homologous reference virus, and label accordingly.

1.2. Make up the required media from the appropriate stock solution

1.3. Again using the previous theoretical virus titer as a guideline, aliquot media as follows:

Log dilution steps	Volume of media (ml)
10^{-1}	0.9
10^{-2}	1.8
$10^{-2.5}$	2.2
10^{-3}	2.2
$10^{-3.5}$	2.2
10^{-4}	2.2
$10^{-4.5}$	2.2

1.4. In the cabinet, wearing an approved virus gown, and with arms bare to the elbows,. transfer 0.1 ml of stock virus suspension into the first (10^{-1}) dilution bottle, mix, and discard the tip into freshly prepared disinfectant. Using a new tip, remove 0.2 ml of this dilution and transfer into the second (10^{-2}) bottle, mix, and again discard the tip. Remove 1.0 ml from this bottle and transfer to the third ($10^{-2.5}$). Continue transferring 1.0 ml of the previous virus dilution to the end of the series. Repeat for all the test viruses

1.5. Disinfect and place all virus dilution bottles at +4ºC until required.

2. Prepare plates as shown

One for each test virus neutralization (VN) + one test virus titration (VT) plate, and the same for the reference virus and it's titration plate.

VN plate layout

<------- half log pre-prepared virus dilutions ------->

Cell ct											Virus Ct
	A										
	B										
	C										
	D										
	E										
	F										
	G										
	H										

<----------well columns 1-12---------->

2.1. Add 100µl/well of media to well column 1 (cell control) and 50µl/well to columns 2-12.

2.2. Add 50µl/well of reference sera to the top well rows A2-A10, making an initial serum dilution of 1/2.

2.3. * Switch on the plate diluter, and set the tip switches to the required number (dependent upon the orientation of the test plate, and how many wells will be utilized).

2.4. * Sterilize the 50µl diluting tips by flaming and fill the larger section of the reservoir with sterile distilled water. Place a folded tissue in the smaller section, and put this receptacle onto the plate cradle with the water reservoir nearer the diluter arm.

2.5. * Place the VN plate on the cradle with the serum/medium wells nearest the diluter arm. Check the 'blot' switch is on, press 'run', and dilute the sera 2 fold (0.3 log) down the plate (rows A-H). Repeat for all the test plates.

NB* – steps 2.3 to 2.5 can be performed manually using a multichannel pipette with appropriate tips. Discard tips after each plate.

2.6. In the cabinet, add 50µl/well of test virus (starting with the weakest dilution) to each pair of well columns, i.e. $10^{-4.5}$ in well columns 10, 11 and 12 (virus control), $10^{-4.0}$ in well columns 8 and 9 and so on. Discard all tips and bottles into disinfectant. Repeat in similar plates for each virus in the test.

2.7. Stack the plates and cover the top one(s) and place in the incubator for 30 minutes or 1 hour in the cabinet at RT.

3. VT plate layout

<---------- 0.3 log virus dilutions ---------->

Cell											Virus
	A										
	B										
	C										
	D										
	E										
	F										

c t	G										C t
	H										

<----------well rows 1-12---------->

3.1. Add 50µl/well of media to well rows 2-12. Leave well row 1 empty.

3.2. In the cabinet, add 100µl/well of chosen virus dilution (e.g. 10-3.0) to each well in row 1(this is taken from the dilution bottle used in the test plate) and dilute 2 fold (0.3 log) down the plate (i.e. rows 2-12), using the multichannel pipette and tips.

3.3. Overlay every well with 50µl/well of media. Leave to incubate with the test plates.

4. After incubation, add 50µl/well of BHK21 cells at a seeded rate of between 0.7^{-1}x106 per ml to every plate. Seal each plate with a semi permeable sealer and incubate in a CO_2 incubator for 48-72 hrs.
5. Observe the plates microscopically for Cytopathic effect.
6. In the cabinet, prepare a reservoir with a suitable disinfectant. Treating one plate at a time, remove the plate sealer and discard into the disinfectant. Discard the spent medium from the plate in the same fashion. Repeat for all the test plates.
7. Dispense a volume of naphthalene black stain into the stain reservoir, and put 50µl of stain into every well in each plate. Leave for at least 30 minutes at room temperature.
8. Discard all the spent stain into the disinfectant, and wash each plate under cold running water.
9. After each series of procedures when the cabinet is no longer required, thoroughly disinfect the cabinet with an approved disinfectant, and leave to circulate for at least one hour before re-use. Log all actions in the cabinet log book.

Annex 3: Test procedure for Molecular characterization

Protocol 1: Extraction of RNA Using RNeasy® Mini Kit.

1. Put volume of 350µl of the sample in an eppendorf tube and adds equal Volume of lysis buffer RLT (Containing 2 – mercaptoethanol).
2. Vortex and Centrifuge the homogenate at 12500 rpm for 3 minutes.

3. Add 350 µl volume 70% ethanol to sample and lysate mixture mix well by pipetting (Vortexing). Do not centrifuge. Proceed immediately to the next step.
4. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.
5. Transfer up to 700µl of sample to the spin cartridge (with collecting tube).
6. Centrifuge in a Mini spine centrifuge at 12500rpm for 2 minutes at room temperature. Discard flow through, and reinsert the spin cartridge into the same collection tube and reuse collection tube. Repeat with remaining volume.
7. Add 700µl wash buffer I to the spin cartridge.
8. Centrifuge at 12500 rpm for 3 minutes at room temperature. Discard the flow – through and the collection tube. Place the spin cartridge into a new collection tube
9. Add 500µl wash buffer II with ethanol to the spine Cartridge.
10. Centrifuge at 12500 rpm for 3 minutes at room temperature. Discard the flow – through.
11. Centrifuge the spin cartridge at 13400 rpm for 3 minutes to dry the membrane with bound RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.
12. Add 40µl RNase – free water (DEPC-H2O) to the center of the spin cartridge.
13. Incubate at room temperature for 3 minutes.
14. Centrifuge the spin Cartridge for 3 minutes at 13400 rpm at room temperature to elute the RNA from the membrane into the recovery tube.
15. Label the tube and store at -200C until used.

Protocol 2: Reverse Transcription of extracted RNA into cDNA and cDNA into PCR with two tube reaction

1. On the first tube, first – Strand cDNA Synthesis

1. Mix and briefly centrifuge each component before use.

2. Combine the following in 0.2 – or 0.5-ml tube:

Component	Amount
Up to 5µg total RNA	*n* µl
Primer	1 µl
50µM oligo(dT) or	
2µM gene-specific primer(GSP) or	
50 ng/µl random hexamers	
10mM dNTP mix	1 µl
DEPC-treated water	To 10 µl

3. Distribute 5 µl of the above mix to PCR tube, add 5 µl templates (RNA extract) and incubate the tube at 65^{O}C for 5 minutes, then place on ice for at least 1 minute.

4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Rxn	10 Rxn
10x RT buffer	2 µl	20 µl
25mM $MgCl_2$	4 µl	40 µl
0.1 M DTT	2 µl	20 µl
RNaseOUT^{TM} (40 U/µl)	1 µl	10 µl
SuperScript^{TM}III RT (200 U/µl)	1 µl	10 µl

5. Add 10 µl cDNA Synthesis mix to each RNA/Primer mixture, mix gently and collect by brief centrifugation. Incubate as follow:
   Oligo$(dT)_{20}$ or GSP primed: 50 minutes at 50^{O}C
   Random hexamer primed: 10 minutes at 25^{O}C, followed by 50 minutes at 50^{O}C
6. Terminate the reaction at 85^{O}C for 5 minutes.Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate the tubes for 20 minutes at 37^{O}C.
8. cDNA synthesis reaction can be stored at – 20^{O}C to or used for PCR immediately.

2. On the Second tube, PCR run by universal primer and then specific primer

1. Add the following to eppendrof tube to prepare PCR Master Mix

Component	**Volume**
RNase free water	2µl
Primers*	2µl each
a. FMDV7 – For and Rev for screen the sample as positive or negative	
b. FMDV O – For and Rev for Serotye O	
c. FMDV SAT – 2 – For and Rev for Serotype SAT – 2	
d. FMDV A – For and Rev for Serotype A	
Taq DNA polymerase (1Q supermix) which contain 5 Unit/ µl	10 µl
PCR buffer minus Mg++,50mM MgCl2 and 10mM dNTP mix Template (cDNA)	4 µl

2. Mix 16 µl of PCR master Mix and 4 µl template of the contents of the tube. Centrifuge briefly to collect the reaction components.

3. For universal primer, Place reaction mixture in preheated ($94^{O}C$) thermal cycler.

Perform an initial denaturation step: $94^{O}C$ for 4 minutes,

Denaturation $94^{O}C$ for 1minute,

Annealing $54^{O}C$ for 1 minute 30 cycles

Extension $72^{O}C$ for 1 minute

Final Extension $72^{O}C$ for 10 minutes

4. Upon completion, maintain reactions at $4^{O}C$

Protocol 3: Agarose Gel Electrophoresis of PCR products

Note: use GelRedTM Nucleic Acid Gel Stain; in steady of Ethidium Bromide which is harmful, gloves should be worn at all times.

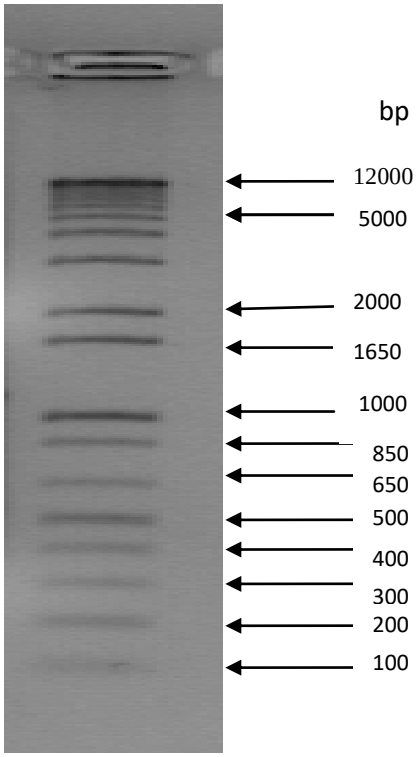
1. Prepare 100ml of 1.5 % agarose in 1x TAE buffer
2. Heat in microwave for 3min on full power 800 watt.
3. Add 5µl GelRedTM per 100ml 1.5% agarose gel (for post – staining Protocol: dilute the gelRedTM 10,000X stock solution 3,300 fold to make a 3X staining

solution in water. Generally 50ml staining solution is adequate volume for one mini gel. Note: including 0.1 M NaCl in the staining solution enhances sensitivity, but may promote dye precipitation if gel stain is reused. For precast protocol for agarose Gel: dilute the GelRedTM 10,000X stock reagent into the molten agarose gel solution at 1: 10,000 and mix thoroughly. GelRedTM can be added while the gel solution is still hot).

4. Pour gel and insert well former (Comb). Remove the air bubble and allow setting on a flat surface for about 30 min.
5. Pour buffer 1x TAE (50X TAE buffer diluted into 1X as add 20ml of 50X TAE buffer into 980ml distilled water to get 1X 1 liter TAE buffer) into the tank and remove comb from gel
6. Prepare Samples in tubes, multiwall plate or on parafilm
   1µl loading buffer
   5µl PCR Product
7. Prepare molecular weight marker 0.5µl molecular weight marker VI (Boehringer), 1µl loading buffer and 4µl H2O
8. Loading samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the 1st and last lanes.
9. Electrophorese at 120 volts for 1 hour and 20 minutes.
10. View and photograph the gel on an UV-transilluminator. Use UV-safety spectacles.

1 KB Plus DNA Ladder

The 1 KB Plus DNA ladder is suitable for sizing linear double-stranded DNA fragmentes from 100 bp to 12 KB. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000bp to 12000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp. The 1650-bp contains approximately 8% of the mass applied to the gel.



1 KB Plus DNA Ladder (Invitroge)