ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MOLECULAR EPIDEMIOLOGY OF FOOT AND MOUTH DISEASE VIRUS OUTBREAKS IN ETHIOPIA IN 2011/2012

MSc THESIS

By
SENTAYHU MINDA

JUNE 2012
DEBRE ZEIT, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Tropical Veterinary Epidemiology

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LIST OF ABBREVIATIONS

AGE	Agarose gel Electrophoresis
BHK	Baby Hamster Kidney
BHU	Bahrein
bp	Base Pair
cDNA	Complementary Deoxyribonucleic Acid
CF	Complement Fixation
cm	Centimeter
CO_2	Carbon dioxide
CPE	Cytopathic Effect
CSA	Central Statistical Authority
DEPC-H_2O	Diethylpyrocarbonate Treated Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside 5' Triphosphate
DTT	Dithiothreitol
e.g	For Example
EA	East Africa
EDTA	Ethyline Diamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assays
EMDTI	Ethiopian Meat and Dairy Technology Institute
ETH	Ethiopia
FBS	Fetal Bovine Serum
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
g	Gram
Ig	Immunoglobulin
IND	India
IRES	Internal Ribosome Entry Site
IRN	Iran
KEN	Kenya
km	Kilometre
km^2	Kilometre Square

M	Molar
m.a.s.l.	Meter above Sea Level
MAL	Malawi
MEM	Minimum Essential Media
ME-SA	Middle East South Asia
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mM	Millimolar
NAHDIC	National Animal Health Diagnostic and Investigation Centre
nm	Nanometer
nt	Neuclotide
NVI	National Veterinary Institute
^{O}C	Degrees Celsius
OIE	Office International des Epizooties
OP	Oesophageal-Pharyngeal
P	Protein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pmol	Pico Mole
RdRP	RNA dependent RNA Polymerase
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic Acid
RNase	Riboneuclase
rpm	Revolutions per Minute
RT-PCR	Reverse Transcriptase PCR
s/n/y	Substitutions per Nucleotide Site per year
SAT	South African Territories
SDS	Sodium Dodecyl Sulphate
sec	Second
SNNP	Southern Nation Nationalities and People's
SUD	Sudan
TAE	Tris Acetate EDTA
TAN	Tanzania

UGA	Uganda
UK	United Kingdom
US	United State
USDA	United State Department of Agriculture
UTR	Untranslated
UV	Ultra Violet
VP	Viral Protein
WRL	World Reference Laboratory
WRLFMD	World Reference Laboratory for FMD
µg	Microgram
µl	Microlitre
µm	Micrometer

ABSTRACT

The study was conducted in five regional states of Ethiopia: Amhara, Oromia, Southern Nation Nationalities and People's (SNNP), Tigray, and Addis Ababa city council from January 2011 to March 2012 with the objectives of identifying the serotypes and topotypes of foot and mouth disease viruses and to determine the genetic relationship of FMD viruses in Ethiopia. Epithelial tissue samples were collected from cattle and swine found in the FMD outbreak areas of the country and submitted to the National Veterinary Institute (NVI), Debre Zeit, Ethiopia and World Reference Laboratory for FMD (WRLFMD), Pirbright, UK. Thus, virus isolation, serotype identification, and phylogenetic analysis were performed. From the total of 59 samples, cytopathic effect (CPE) was observed in 43 (72.88%) samples in BHK-21 cell culture. Serotyping of FMD viruses were made by applying agarose gel-based RT-PCR at NVI, Debre Zeit, and by cell culture ELISA at WRL for FMD, Pirbright, UK and serotype O was recorded throughout the country where outbreaks occurred. A total of 36 samples were sent to WRLFMD, Pirbright, UK for further molecular characterization and phylogenetic analysis. Of this, 12 serotype O FMD viruses of Ethiopia were further characterized and phylogenetic relationships with each other and type O isolates from other countries of the world were determined. All serotype O isolates of Ethiopia falls into a single topotype, East Africa-3 (EA-3). More than 98% similarity with each other and approximately 14-15% different to members of the EA-1, EA-2, EA-4, and ME-SA topotypes of other countries of Africa and Asia were found. Serotype O isolated from Mekele University Farm was also 93-95% similarity with Sudan O isolates of 1999, 2004 and 2008. Generally regular investigation of FMD outbreaks to have more detailed information about the serotypes and topotypes circulating in Ethiopia is important for effective vaccine development.

Keywords: Ethiopia, FMDV, Phylogenetic Analysis, Serotype and Topotype.

1. INTRODUCTION

Foot and mouth disease (FMD) is a severe, highly contagious viral disease of livestock with significant economic impact. The main effect of the disease is its economic losses resulted from the loss of milk production, retarded growth, loss of draught power, abortion in pregnant animals, and deaths in calves, kids and lambs. In areas of the world where food and draft animals are essential for subsistence agriculture, FMD can affect nutrition. In countries that have highly developed animal industry and free trade, outbreaks cause economic devastation (OIE, 2007).

The disease affects cattle, swine, sheep, goats, and other cloven hoofed ruminants. Furthermore, elephant and giraffe are susceptible to FMD (Kitching, 2005; Mahy, 2005). Depending on weather conditions the virus can become aerosol and spread to susceptible animals. FMD virus is a notifiable disease because the exports of infected livestock and animal products could easily cause outbreaks in counties currently free from FMD. Recently, FMD has been endemic in several parts of the world, particularly in Asia, South Africa, the Middle East, and South America (Mahy, 2005).

Foot and mouth disease virus (FMDV) was identified by Loeffler and Frosch in 1898 as the first filterable viral agent to cause animal disease. The virus responsible for FMD is a member of the *Aphthovirus* genus in the *Picornaviridae* family (Alexandersen and Mowat, 2005). There are seven immunologically distinct serotypes: O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1 and over 60 strains within these serotypes. New strains occasionally develop spontaneously. The early indications of the disease are characterized by fever, excessive salivation, and vesicles on the tongue especially in small ruminants in which clinical signs often milder depending on the strain of the virus. The disease spreads rapidly among none immunized animals, because this disease has a very high morbidity rate, but a low mortality rate, except in young animals which have higher mortality rates (Cameron *et al.*, 1999; Geering and Lubroth, 2002; Ryan *et al.*, 2007).

Foot and mouth disease is probably the most important livestock disease in Ethiopia in terms of economic impact. Recently, the disease had become the major constraint hampering export of livestock and livestock products to the Middle East and African countries; the Egyptian

trade ban of 2005/2006, in which Ethiopia lost more than US$14 million, being a recent memory (Leforban, 2005). Livestock are at risk from endemic strains as well as from antigenic variants prevailing in neighbouring countries. The official data may not exhibit the reality of the disease due to the insidious nature of the disease, the unreported cases by farmers, as well as the few samples submitted to WRL for FMD, Pirbright, UK, for identification.

An understanding of the epidemiology of the disease is critical for the implementation of good control programmes and the eradication of the disease. An important part of combating FMD is virus characterization, where the relationships between field isolates using reference viruses are used to investigate the possible origins of the disease. The identification of the possible origin of the virus is one of the major factors contributing to control of the disease. In recent years the nucleotide sequences of viruses generated have much attention in this regard and sequence data has been instrumental to identify the origin of an outbreak (Beck and Strohmaier, 1987; Krebs and Marquardt, 1992). To facilitate studies on the genetic relationship of isolates in outbreaks conditions, the World Reference Laboratory for FMD virus has established a database of FMD virus sequences from isolates collected worldwide (Samuel *et al.*, 2001).

In recent years, a detailed knowledge of the molecular characteristics/epidemiology of the major antigenic sites of FMDV were helpful to define strains, to identify transmission events, to characterize biodiversity, to effective quarantine measures against reintroduction (Samuel and Knowles, 2001), and the development of specific diagnostic tests and protective vaccine. Most studies of FMDV have concentrated on the highly variable region of the capsid gene VP1, known to be an important antigenic site (Gonza´lez *et al.*, 1992; Pattnaik *et al.*, 1998; Gurumurthy *et al.*, 2002). Phylogenetic analysis of the VP1 region of FMDV has been used extensively to investigate the molecular epidemiology of the disease worldwide. The techniques have assisted in studies of the genetic relationships between different FMDV isolates, geographical distribution of lineages and genotypes, and the establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks (Knowles and Samuel, 2003; Sangare *et al.*, 2003)

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, 2004; Gelaye *et al*., 2005; Legess, 2008; Gelagay, 2009; Haileleul *et al*., 2010). Therefore, continuous research is needed to establish genetic relationships between different FMDV isolates, geographical distribution of lineages and genotypes, it is also important to identify the origin of infection.

Therefore, the objectives of this study were:

- ✓ To isolate and identify the serotypes and topotypes of foot and mouth disease viruses causing outbreaks in Ethiopia,

- ✓ To determine the genetic relationship of FMD viruses in Ethiopia

2. LITERATURE REVIEW

2.1. Definition

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven hoofed animals, especially in cattle, sheep, goats, swine, and wild ruminants. FMD infected animals may have fever and vesicular on the mouth, feet and udders. Although FMD has a low mortality rate in adult animals, the mortality is high in young animals (Quinn *et al.*, 2005).

2.2. Etiology

The virus responsible for FMD is a member of the *Aphthovirus* genus in the *Picornaviridae* family (Alexandersen and Mowat, 2005). There are seven immunologically distinct serotypes - O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 and over 60 strains within these serotypes. New strains occasionally develop spontaneously

2.3. Molecular Biology of FMDV

2.3.1. Morphology and biophysical characteristics of FMDV

The name picornavirus comes from the word "pico" which means very small and "rna" for the genome type. They are among the smallest known RNA viruses and have a naked, ether-resistant, icosahedral protein shell with a symmetry of 22-30 nm in diameter (Melnick *et al.*, 1975; Cooper *et al.*, 1978). In acidic conditions the FMDV particles are disrupted into pentameric subunits composed of five copies each of the virus structural capsid proteins (VP1-3) with the liberation of the internal capsid protein (VP4) and the RNA. FMDV is also unstable at pH > 11 and when treated by heat or by gamma radiation loose infectivity for susceptible cells (Acharya *et al.*, 1990).

2.3.2. Genome structure and polyprotein synthesis of FMDV

The picornavirus genome consists of a single molecule of linear, positive (+) sense, single strand RNA and is non-segmented. The 5'-terminus of the genome has a long untranslated (UTR) region 600-1200 bases in length, which is important in translation, virulence, and possibly encapsidation. There is a shorter untranslated (UTR) region (50-100 bases in length) on the 3'-terminus, which is important in (-) strand-synthesis during replication. The 5' terminus untranslated region also has a "clover leaf" secondary structure known as the Internal Ribosome Entry Site (IRES), which distinguishes picornaviruses from other RNA viruses; this structure is important in translation and replication (Figure 1). The 5'-terminus is modified by a covalently-attached VPg protein which may act as a primer for RNA synthesis during replication, while the 3'-terminus is modified by polyadenylation (Mason *et al.*, 2003).

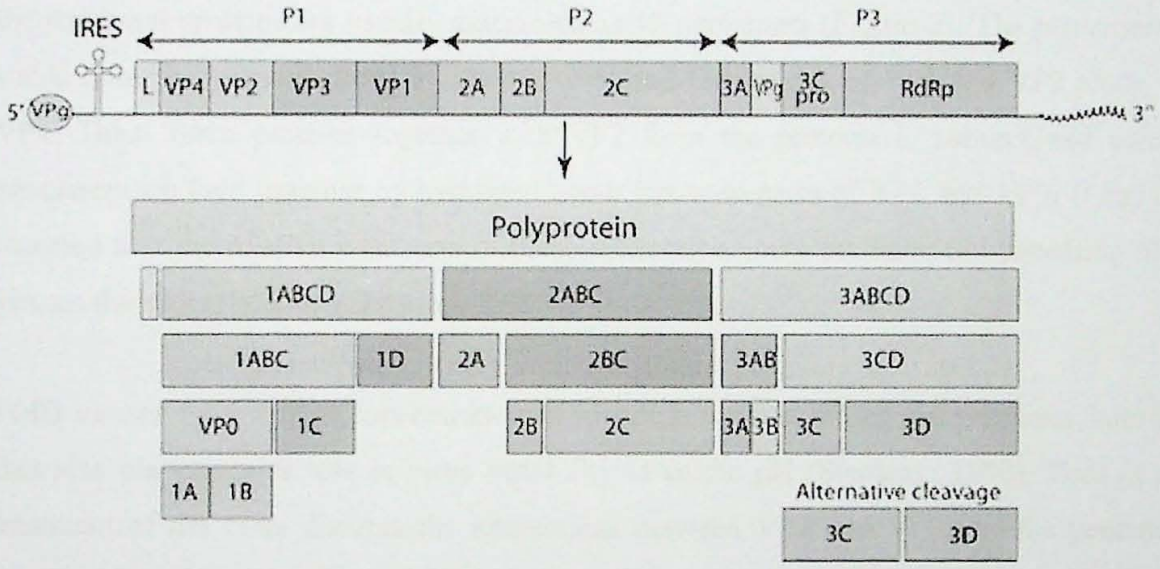


Figure 1: *Picornaviridae* genome and polyprotein synthesis. (Mason *et al.*, 2003).

The figure show the genome encodes a single polyprotein, which undergoes a series of cleavages to give rise to all the structural and non-structural proteins. The polyprotein is first cut to yield P1, P2 and P3. P1 becomes myristylated at the N- terminus before being cleaved to VP0, VP3 and VP1, the proteins that will form procapsids; VP0 will later be cleaved to produce VP2 and VP4. Other cleavage products include 3B (VPg), 2C (an ATPase) and 3D (the RNA polymerase) (Mason *et al.*, 2003).

2.3.3. Foot and mouth disease virus proteins

Structural proteins

The capsid is composed of the four major structural capsid proteins (VP1, VP2, VP3 and VP4) and contains 60 copies of each (Cooper *et al.,* 1978). Five copies of VP1 are clustered around the fivefold axis of symmetry; while VP2 and VP3 are positioned at the two and three-fold axis of symmetry (Acharya *et al.,* 1990) (Figure 2). These proteins are elements of identical four segmented protein subunits called protomers which are defined as the smallest identical subunit of an oligomeric protein (Cooper *et al.,* 1978).

The basic building block of the icosahedral capsid is a pentamer made up of five copies of VP1 to VP4 protomers. To establish the icosahedral symmetry structure in the virus capsid the structural proteins are usually assembled as 12 pentamers (Figure 2). The pentamers are stable through the interactions involving the N and C terminus of VP1 and VP3 along with VP4. These three proteins together with VP2 form the protomeric subunit and adjacent pentamers are held together by hydrogen bonds between parts of VP2 and VP3. It has been reported that the relative weakness of these interactions may facilitate the uncoating of the viruses during replication (Stanway, 1990).

FMD viruses have a high concentration of histidine residues lining the pentamer interfaces that also play a major role in virus instability at acidic pH (Stanway, 1990). Heat or acid treatment of the virus disrupts the interactions between VP2 and VP3 and the pentameric interfaces resulting in pentamer dissociation with release of the internal capsid protein VP4 and the RNA genome. VP1, VP2 and VP3 are situated at the surface of the FMD virus. The smallest capsid protein, VP4, is internal and can be thought of as an N-terminal extension of VP2 which is cleaved from the VP2/VP4 precursor at the final stage of maturation of the virus particle. The VP4 protein interacts with the viral RNA (Strohmaier and Adam, 1974; Chow *et al.,* 1987; Acharya *et al.,* 1990). VP1 is the most important protein for epidemiological studies of FMD viruses and VP3 is the most conserved surface exposed structural protein among different FMD viruses (Acharya *et al.*, 1990).

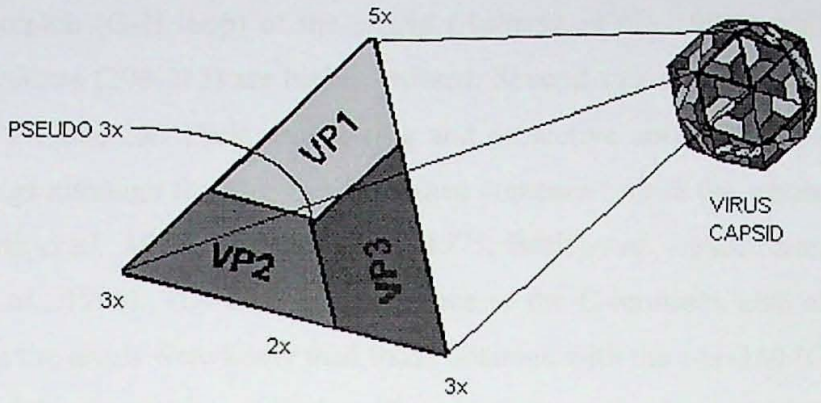


Figure 2: Protomer of VP1, VP2 and VP3 (VP4 lies on the inner side of the capsid) with the symmetry (Teurlings, 1998)

Non-structural proteins

The P2 and P3 precursors are processed into non-structural proteins which are involved in virus RNA replication and protein processing (Figure 1) (Sanger, 1979; Forss *et al.*, 1984; Acharya *et al.,* 1990; Belsham, 1993). The genome segments P2 and P3 encode the non-structural proteins 2A, 2B, 2C and 3A, 3B, 3C, 3D, respectively (Ryan *et al.*, 1989). The protein 2A is a protease and cleaves itself liberating the precursor of the capsid proteins whilst 3C carries out the majority of the processing of the polyprotein (Strebel and Beck, 1986; Stanway, 1990). The protein 3D is the RNA-dependent RNA polymerase. The role of the proteins 2B, 2C, and 3A still remains unclear.

2.3.4. Properties and functions of the VP1 protein

The antigenic diversity of FMD viruses has made the diagnosis and control of these viruses very difficult in countries where the disease is endemic. Several studies have shown that VP1 (encoded by 1D) is the most important protein in FMD virus both for its antigenic properties and as the virus-cell attachment site.

Two regions of VP1, amino acids 140-160 and 200-213, have been shown to induce antibodies involved in neutralization of viral infectivity (Bittle *et al.,* 1982; Pfaff *et al.,* 1982;

Strohmaier *et al.*, 1982). The electron density map has also revealed that the immunogenic site (140-154) is exposed on the surface of the virus particle and located at a highly disordered region (G-H loop) of the capsid (Acharya *et al.*, 1990), while the C- terminus antigenic residues (200-213) are highly ordered. Several subsequent studies have shown that purified VP1 alone can elicit neutralizing and protective antibodies in mice, guinea pigs, cattle and pigs although the titre was low when compared when the whole virus particle was used (Laporte *et al.*, 1973; Bachrach *et al.*, 1975; Bittle *et al.*, 1982; Strohmaier *et al.*, 1982; Acharya *et al.*, 1990). The 201- 213 sequence at the C-terminus also elicited neutralizing antibody but the levels were lower than those obtained with the 141-160 (G-H loop) sequence. Treatment of the intact virus particle with proteolytic enzymes, such as trypsin leads to the loss of infectivity and immunogenic activities (Wild *et al.*, 1969, Rowlands *et al.*, 1971). Since VP1 is the only protein cleaved by trypsin it is believed to be responsible for both immunological and infectivity functions (Laporte *et al.*, 1973; Bachrach *et al.*, 1975; Bittle *et al.*, 1982).

Several studies have reported that the RGD sequence within the G-H-loop of the VP1 is involved in attachment of the virus to susceptible cell receptors (Leippert *et al.*, 1997; Liebermann *et al.*, 1991). The cleavage of VP1 by trypsin highly reduces or abolishes the ability of the FMD virus to bind and infect susceptible cell cultures (Baxt *et al.*, 1989). Similar work conducted by Liebermann and co-workers (1991) on Type O1 Kaufbeuren reported that the highly conserved triplet, Arginine- Glycine-Aspartic acid (RGD) within the G-H-loop is responsible for binding of FMD virus to pig kidney cell receptors. FMD virus infection of susceptible cells is successfully blocked following the binding of antibodies directed against the RGD region as well as peptides representing part of its sequence. In addition, many studies have reported that the RGD binds to a large family of integrin receptors and many extracellular substrate proteins (Ruoslahti and Pierschbacher, 1987). Although, the RGD has been reported to play the main role in cell-virus attachment, it is not the sole element in the binding process since removal of the C-terminus of VP1 in the absence of cleavage within the FMD virus loop also affects the attachment (Fox *et al.*, 1989).

2.3.5. Antigenic variation

Early work on FMDV discovered the existence of multiple serotypes by demonstration that convalescent animals could not be reinfected by the same isolate but was susceptible to

diseases caused by isolates from a different location (Vall é e and Carr é, 1922). These studies were repeated in a second laboratory (Waldmann and Trautwein, 1926) that used similar techniques to show that there were three different serotypes of virus circulating in Europe in the early 1900s (originally designated A, B, and C, but later renamed A, O, and C to be consistent with the A and O strains identified by Vall é e and Carr é). The ramifications of these finding are very significant. Despite the ability of animals to be resistant to reinfection with the same serotype, they can be infected with a different serotype, so naive animals can suffer multiple episodes of FMD. Thus, countries with multiple serotypes of FMDV need multivalent vaccines, and countries that want vaccines to protect their animals from all strains of FMDV need to vaccinate with a heptavalent vaccine.

An additional problem with vaccination coverage is that FMDV can evolve rapidly in the field, and as a result, vaccines for individual serotypes, particularly type A, can rapidly become out-of-date, as field strains evolve (Doel, 2003). A portion of the reason for this rapid evolution appears to be the flexibility of the G–H loop of the capsid structure, which has also appeared to limit the utility of peptide-based vaccines targeted to this region of the virion surface (Domingo *et al.*, 2003).

Mutations

Antigenic variation can be caused by nucleotide mutations or recombinations in the RNA viral genome. Studies revealed that the rates of mutations of the European serotype FMDV RNA genome can reach 10-2 substitutions per nucleotide site per year (s/n/y) (Gebauer *et al.*, 1988). These mutations may give rise to variant viruses that can be a source of new outbreaks (De La Torre *et al.*, 1988; Domingo *et al.*, 1992; Vosloo *et al.*, 1996). Mutations that lead to conformational changes produce a population of neutralizing escape variants (Stave *et al.*, 1988; Domingo *et al.*, 1992; Sanyal *et al.*, 1997).

Recombination

The mechanism of recombination in FMD virus has been described as the modification of the surface proteins due to segment crossing over during co-infection of the animal cells by more than one FMD virus serotype (King *et al.*, 1982; Krebs and Marquardt, 1992). Methods such as electro focusing using radioactive labelled structural polypeptides or SDS-polyacrylamide

gel electrophoresis with molecular markers were also used by King and co-workers (1985) to determine recombination between different subtype strains in the unsegmented genome of FMD virus.

Crossing over or reciprocal recombination involves the even exchange of homologous sequences. Nonreciprocal recombination, on the other hand, involves the uneven replacement of a sequence which results in the loss of one of the variant sequences involved in the recombination event. This procedure has been reported to be responsible for severe types of mutational change that may affect the susceptibility of the natural hosts (King *et al.*, 1982).

Quasi-species concept

The quasi-species concept was proposed by Eigen in 1971. RNA viruses have genomes that replicate in the absence of repair mechanisms; they evolve very rapidly with a mutation frequency per nucleotide site of 10-3 to 10-5 substitutions per year (Van Regenmortel *et al.*, 1997). The high rate of error during RNA replication in the picornaviruses gives rise to a range of multiple co-circulating viral genomes within a host. A dominant virus genome sequence called a master or consensus sequence in regard to the specific environment emerges and competes with the others in the virus population. This is defined as the quasi-species principle of RNA viruses (Eigen, 1971; Holland *et al.*, 1982; Sobrino *et al.*, 1986; Domingo *et al.*, 1992; Van Regenmortel *et al.*, 1997).

2.4. Epidemiology

2.4.1. Geographic distribution

FMD has occurred in most areas of the world except Greenland, New Zealand, and the smaller islands of Oceania. Australia has not experienced an outbreak since 1870; the United States of America since 1929; Canada since 1952 and Mexico since 1954 (Samuel *et al.*, 2001). Europe has reported a number of sporadic outbreaks since the cessation of vaccination in 1990-1991. These outbreaks occurred in Bulgaria (1991, 1993 and 1996), Italy (1993), Greece (1994, 1996 and 2000), Turkish Thrace (1995 and 1996), Albania (1996), the former Yugoslav Republic of Macedonia (1996), and the Former Soviet Republics of Georgia and

2.4.4. Susceptible hosts

Aphthovirus can infect more than 70 species of mammal belonging to more than 20 families (Murphy *et al.*, 1995) particularly cloven-hoofed animals domestic and wild such as; cattle, swine, sheep, goats, camels, deer, moose, llama, chamois, alpaca, vicuna, giraffe and others. The most sensitive hosts of FMDV are cattle and swine, because of their extreme sensitivity to respiratory infection, these animals due to their sensitivities develop sever clinical sings. The clinical signs in sheep, goats, and wild ruminants are milder than in cattle (Geering and Lubroth, 2002). Horses, dogs and cats are not susceptible hosts but they can inevitably spread the virus via their fur if contaminated (USDA, 2002). FMDV is not a human pathogen (OIE, 2009), although there have been reports of infection in human but they are rare.

2.4.5. Carrier animals

A carrier status is applied to an animal from which infectious FMD virus could be recovered 28 days after infection (Woodbury, 1995). Cattle were first identified as carriers by Van Bekkum *et al.* (1959) while Hedger (1968), later reported that cattle can carry FMD virus in the oropharyngeal areas for up to 2 and half years. The development of the carrier state in cattle occurs in both clinical and sub-clinical infections with FMD virus (Salt *et al.,* 1996). Studies have shown that there is a high incidence of sub-clinical infection in cattle in endemic areas (Malirat *et al.,* 1994). The carrier state has been attributed to a defective immune response in the animal or incomplete virus replication due to poor enzyme production. Cross species infection is also believed to play a role in carrier status (Rina and Martin, 1976; De La Torre *et al.,* 1988; Salt, 1996; Woodbury, 1995). Among livestock, cattle and pigs are regarded as the primary source for FMD virus transmission to other susceptible animals, followed by sheep in which a carrier status of up to 9 months has been recorded (McVicar and Sutmoller, 1968).

African buffalo can carry FMD virus without disease up to 5 years (Sutmoller and Gaggero, 1965) and experimentally infected buffalo developed disease with or without clinical lesions (Young *et al.*, 1972; Anderson *et al.*, 1979; Gainaru *et al.*, 1986). This contributes to the buffalo being important amplifiers and a big threat in spreading the virus to domestic livestock populations (Sutmoller and Gaggero, 1965; Gainaru *et al.*, 1986; Vosloo *et al.,* 1996). Studies have reported the natural transmission of FMD virus between buffalo and

impala *(Aepyceros melampus)* (Bastos *et al.*, 2001) and have proposed that impala can act as intermediaries in disease transmission between buffalo and cattle.

2.4.6. Transmission

The virus is transmitted by direct contact, from contaminated secretions, body fluids, contaminated fomites, animal products (e.g. meat, milk and wool), people, vehicles and inhalation of airborne virus (Cooper *et al.*, 1978; Brooksby, 1982; Woodbury, 1995). Animals can shed FMDV for up to four days before the onset of symptoms. This virus is also found in large quantities in vesicle fluid, and peak transmission usually occurs when vesicles rupture. Some animals carry FMDV for prolonged periods after recovering from acute disease. Animals with natural or vaccine-induced immunity can also become carriers if they are later exposed to virus; these animals can remain asymptomatic. FMDV can persist for up to nine months in sheep and up to four months in goats. Most cattle carry this virus for six months or less, but some animals remain persistently infected for up to 3.5 years (Woodbury, 1995).

2.5. Pathogenesis

The replication of the infectious particles is extremely rapid after entry through the upper respiratory tract or lung through inhalation, with viraemia seeding infection into the epithelium where secondary virus multiplication results in vesicles and shedding from the udder in milk (Hyslop, 1965; Sellars, 1971). The incubation period, from infection to clinical signs, may be as short as 2/3 days or as long as 14 days (Garland and Donaldson, 1990) and infected animals may become infectious before showing clinical signs (Burrows, 1968a). The virus is excreted during viraemia for some days; thereafter as serum antibody develops viraemia decreases, and the animal ceases to be infectious as the lesions heal.

The lesions on the dental pad and tongue appear as reddened areas and progress within a few hours into vesicles. The vesicles are easily ruptured within 24 hours leaving a raw surface and healing occurs within one to two weeks of rupture. Lesions at interdigital areas occur and animals can loss their hooves in severe cases (Geering, 1967; Donaldson *et al.*, 1984). There

has also been supportive evidence that FMD virus replicates in the bovine mammary gland and mastitis may occur due to secondary bacterial infection.

2.6. Diagnosis

2.6.1. Clinical signs of the disease

The disease is highly contagious and known as one of the most economically devastating diseases of livestock (Brooksby, 1982). The incubation period 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 sows (Garland and Donaldson, 1990). The incubation time may be even shorter in experimental infections. A rise in temperature (41^0C - 42^0C) occurs a day before the first appearance of mouth and feet lesions and viraemia in cattle lasts for 3-5 days (Cottral and Bachrach, 1968). The lesions develop 2-14 days post infection. The common clinical signs of the disease are vesicle formation on the mucous membranes of the tongue, lips, interdigital spaces and on the coronary bands which make animals reluctant to eat or move (Geering, 1967; Brooksby, 1982; Woodbury, 1995). However, mouth lesions are less common and less pronounced in other species such as sheep and pigs. Commonly in sheep and other small ruminants lesions occur on the dental pad, where they may be difficult to detect (Thomson, 1994). Mortality in adult animals is rare, while in young animals, death can occur due to myocarditis and mortality may exceed 50% in calves (Donaldson *et al.*, 1984; Mckercher *et al.*, 1985; Woodbury, 1995). Morbidity can approach 100% (Woodbury, 1995; Salt *et al.*, 1996). A chronic panting syndrome characterized by dyspnoea, anaemia, hair overgrowth, and lack of heat tolerance has been reported as a sequel in cattle (Geering, 1967).

2.6.2. Laboratory Diagnosis

Beside clinical diagnosis based on lesion identification, in the early stage of infection, FMD virus or viral antigen can be detected using several diagnostic techniques.

Specimens for antigen detection

Vesicular fluid usually contains the highest quantity of virus. Epitheliums from early vesicles and from recently ruptured vesicles are tissues of choice for virus isolation. When epithelium tissue is not available from ruminant animals e.g. in advanced or convalescent cases and infection is suspected in the absence of clinical signs, samples of oesophageal-pharyngeal (OP) fluid is collected by means of a probing and used for virus isolation(OIE, 2009)

Virus isolation

The isolation and characterization of the virus is the "golden rule" for the diagnosis of a viral disease. The suspensions obtained from the specimens are inoculated onto susceptible cells (e.g. baby hamster kidney) incubated at 37°C and examined for CPE 24 to 48 hours post infection. CPE can be seen as early as 12 hours as cell detachment and destruction with high virus concentration. If no CPE is observed the cells are frozen and thawed followed by 2 blind passages on cell culture (Kahrs, 1981; Westbury *et al.,* 1988).

Complement fixation test

Since it was first described by Traub and Mohlmann in 1946 the complement fixation (CF) test was commonly used in the diagnosis of FMD. It has been an important tool in the early studies for the comparison of the antigenic variation of two viruses. The CF test is based on the principle that complement (a series of serum proteins) serves as a mediator of many antigen-antibody reactions in which it is fixed in the formation of immune complexes. The presence of complement (usually provided by addition of guinea-pig serum) is revealed by its ability to mediate lysis of sensitized sheep red blood cells.

Enzyme-linked immunosorbent assays (ELISA)

Because the complement fixation (CF) test lacks sensitivity and cell culture isolation takes up to 2-7 days, a more sensitive, rapid and practical alternative to traditional assays was needed for an efficient diagnosis of FMD. The enzyme-linked immunosorbent assay (ELISA) was developed with a number of applications which include the detection of antigen, and antibody (Crowther and Abu-Elzein, 1979; Hamblin *et al.*, 1984).

Currently, two procedures are used, the sandwich ELISA for antigen detection (Roeder and Le Blanc Smith, 1987) and the liquid phase blocking ELISA for antibody detection (Hamblin *et al.,* 1984, Esterhuysen *et al.*, 1985); both tests are routinely used in the diagnosis of FMD.

Polymerase chain reaction (PCR)

A reverse transcriptase polymerase chain reaction (RT-PCR) and nucleotide sequencing analysis have been developed for diagnostic purpose (Kitching *et al.*, 1989), but has also evolved into useful tools for molecular epidemiological studies. The PCR provides an extremely sensitive and rapid assay for the identification of the viral genome. This technique allows the detection of genetic material even in the absence of infectivity for tissue culture or laboratory animals. This was demonstrated from samples of oesophageal- pharyngeal scrapings of carrier cattle taken at 180 and 560 days post infection (Laor *et al.*, 1992). The detection of genetic material by PCR was also demonstrated in aerosols (Suryanarayana *et al.*, 1999), clinical samples and cell culture isolates (Laor *et al.*, 1992), nasal swabs of asymptomatic cattle (Callens and De Clercq, 1997), oesophageal – pharyngeal samples, skin and tongue epithelium (Laor *et al.*, 1992) and blood (Bastos *et al.*, 2000). Serotype specific PCRs have been developed using serotype specific primers derived from sequences coding for the structural protein VP1 (Suryanarayana *et al.*, 1999). The differences in VP1 sequences are the basis for developing RT-PCR assays to identify FMD viruses (Rodriguez and Carrasco, 1993; Stram *et al.*, 1995)

Agarose gel electrophoresis (AGE)

The PCR products are analysed with 1.5% Agarose gel containing ethidium bromide. The electrophoretic mobility of the proteins is dependent on both their size and charge.

2.7. Immunity

Recovery from clinical foot and mouth disease is correlated with the development of antibody. The early IgM antibodies neutralize the homologous type of virus and may also be effective against heterologous types. In contrast, the IgG produced during convalescence is type specific and, to varying degrees, subtype specific. Little information is available on the

role of cell-mediated immunity in recovery from foot and mouth disease, but as in other picornavirus infections, it has been assumed to be of minor importance. Cattle that have recovered from foot and mouth disease are usually immune to infection with the same virus type for a year or more, but immunity is not considered lifelong. Recovered animals, however, can be infected immediately with one of the other types of foot and mouth disease virus and develop clinical disease (Woodbury, 1995; Salt *et al.*, 1996).

2.8. Control and prevention in endemic and disease free countries

Vaccination against FMD virus has been used successfully to help in the eradication of the disease in some parts of the world. However, FMD is still endemic in many countries in Africa and Asia. In 1998, (Freiberg *et al.*, 1999) have reported about 2000 type A and O outbreaks from 44 countries. The first aim of disease control is the local reduction of incidence of the infection by vaccination, movement restriction of animals and regular inspection of cloven-hoofed animals (Brown, 1999; Woodbury, 1995). In endemic areas, vaccination is very costly because the duration of immunity induced by the FMD vaccine is short and two to three vaccinations are required annually to maintain high levels of neutralizing antibodies in susceptible animals (Garland, 1999).

In countries free from FMD, stamping-out of infected and in contact animals is favoured in the case of outbreaks. Severe restriction on the import of animals and animal products from countries where FMD virus infections occur are also implemented (Brown, 1999; Woodbury, 1995). To contain the disease in outbreak conditions in countries from the disease with regard to the recent UK conditions, free ring vaccination around infected areas is required and precautions should also be taken to destroy the vaccinated animals (Samuel and Knowles, 2001). Unlike parts of Europe and South America, eradication of the disease in Africa is presently impossible because domestic and wild animals are sharing the grazing areas in many African countries (Thomson, 1994).

2.9. Economic importance

Although mortality is rare in adult cattle, FMD virus causes an economically devastating disease of cloven-hoofed animals. Studies have estimated 25% loss of productivity of animals following infection e.g. reduction or loss of milk production in lactating animals and the deterioration in body conditions (Bachrach, 1968). The loss in animal production and international trade restrictions imposed following an outbreak make FMD of major concern for livestock owners. The control of an outbreak (slaughter of infected and in contact animals, disposal of carcasses in disease free zones) and the loss due to the ban on livestock exports cost several million US dollars for a single outbreak (Sellers and Daggupaty, 1990). The cost of the UK epidemic in 2001 was estimated to be more than US $29 billion (Samuel and Knowles, 2001).

3. MATERIALS AND METHODS

3.1. General description of study areas

The study was conducted from January 2011 to March 2012 in five national regional states of Ethiopia: Amhara, Oromia, Southern Nation Nationalities and People's (SNNP), Tigray, and Addis Ababa. A total of thirteen FMD outbreaks; one in Amhara, seven in Oromia, two in SNNP, two in Tigray, and one in Addis Ababa city council were investigated as shown in figure 3.

The Amhara regional state is located in North-western and North central part of Ethiopia. It covers an estimated area of 170,752 km^2 (Central Statistical Authority, CSA, 2012), and FMD outbreaks occurred in Debre Berehan Zone. In Addis Ababa, which lies an altitude ranging from 2,000 - 2,800 m.a.s.l. There are about 5,200 dairy farms with some 58,500 cattle, and almost 50% are cross breed (CSA, 2012). FMD outbreak is investigated in Akaki-Kality District. In SNNP region, FMD outbreak was occurred in Sidama Zone, Malga woreda (Ketena District) and Alaba woreda (Negele Odisha district). Tigray regional State is located in Northern Ethiopia. The region has common boundaries with Afar and Amhara regional States at the Eastern and Southern parts, respectively, and international boundaries with Sudan and Eritrea at the Western and Northern parts, respectively. It covers 54,548.32 km^2. The FMD outbreaks were occurred at Mekele University Dairy Farm and Mekele (Enderta District). FMD outbreaks were also investigated in the Oromia regional state. It covers 366,000 km^2, accounting for 31.17% of the total area of Ethiopia. FMD outbreaks were investigated in Eastern Shewa, Alage Dairy Farm (Alage College, Zeway), Adamitulu (Jido Kombolcha woreda), Debre Zeit Swine Farm (Debre Zeit), Behylu Dairy Farm (Debre Zeit), Tigest Dairy Farm (Debre Zeit), Ethiopian Meat and Dairy Technology Institute (EMDTI) (Debre Zeit), and Adama administrative Zone of Oromia regional State (Figure 3).

Figure 3: Map of Ethiopia showing the distribution of FMD outbreaks in Ethiopia during the study period

3.2. Study population and sampling method

The study population consists of cattle and swine that manifest clinical signs of FMD in the outbreaks (Table 1). The outbreaks were occurred in five regions, eight administrative Zones, and thirteen areas were included for the occurrence of FMD outbreaks. Sampling was purposive sampling and based on temporal feasibility to investigate. Cattle and swine of all age groups, sex, breeds, and different management practices were recorded. Accordingly, a total of 59 epithelial tissue samples were collected.

3.3. Study design

At the beginning of an investigation proper information channel to Veterinary professionals, District Veterinary officers, Regional and Federal Animal and Plant Health Regulatory Authority, Regional laboratories, and NAHDIC were organized. All these were informed to report FMD outbreaks to the NVI. When an active outbreak was reported, a field investigation was conducted at specific site of an outbreak (Table 1). Sample of animals were

clinically examined for presence of FMD lesions on the mouth and feet, teat, and udder and specimens were collect for diagnostic testing.

Table 1: Summery of FMD Outbreaks in Ethiopia 2011/2012

No	Location (Area)	Date of Outbreak	Species
1	Alage Dairy Farm (Alage College)	1/1/2011	Bovine
2	Alaba (SNNP)	6/1/2011	Bovine
3	Adamitulu Jido kombolcha (Oromia)	7/1/2011	Bovine
4	Debre Zeit Swine Farm (Oromia)	15/7/2011	Swine
5	Behylu Dairy Farm (Debre Zeit) (Oromia)	3/9/2011	Bovine
6	Tigest Dairy Farm (Debre Zeit) (Oromia)	19/9/2011	Bovine
7	Malga (Sidam Zone, SNNP)	8/10/2011	Bovine
8	EMDTI (Debre Zeit) (Oromia)	14/10/2011	Bovine
9	Adama (Oromia)	26/10/2011	Bovine
10	Akaki-Kality (Addis Ababa)	25/11/2011	Bovine
11	Mekele Universty Farm (Tigray)	29/11/2011	Bovine
12	Enderta (Tigray)	1/12/2011	Bovine
13	Debre Berehan (Amhara)	30/3/2012	Bovine

3.3.1. Clinical examination

Cattle and swine were carefully examined for presence of characteristic clinical signs of FMD. In each outbreak, animals manifesting the characteristic signs of FMD like vesicular lesions (ruptured vesicles) in oral cavity and on the feet and teats, salivation, lameness, and rise in temperature were considered as clinically affected by FMD. Other animals in the herd without these signs were similarly examined, but sampling of epithelial tissue in such instance was done only when lesions were suggestive of FMD.

3.4. Sample collection

During the study period, epithelial tissue samples were collected from FMD suspected animals in different areas of Ethiopia (veterinary clinics, Institutes, and Farms) and submitted to the NVI, Debre Zeit, Ethiopia. Bovine and swine epithelial tissue samples were collected from where outbreaks occurred. The samples were transported from the collection site to the NVI in 0.04 M phosphate buffer saline solution (pH 7.2–7.6) with glycerol and antibiotics at $4^{O}C$ and stored at $-20^{O}C$ until processed (OIE, 2007). For those samples which are tested at the NVI, were also submitted to the WRL for FMD, Pirbright, UK for additional studies. A total of 59 epithelial tissue samples were collected from 13 outbreaks during the study periods.

3.5. Virus isolation and serotype identification

Virus isolation was established under laminar air flow hood class II on cell layers of baby hamster kidney (BHK-21) inoculated with 1 ml of filtered tissue suspension and incubated at 37°C for 1 hour for adsorption of the virus and then flashed with growth media (2% MEM media) and incubated at 37°C and 5% CO_2 in a humidified incubator for 24 - 48 hours. 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 (Buxton and Faser, 1977; OIE, 1990; Yoseph *et al.,* 1991). Samples not exhibiting CPE by 72 hours post-infection on the second pass were considered virus negative. Serotyping of FMDV were made by applying Agarose gel-based RT-PCR at NVI, Debre Zeit (Vangrysperre and De Clercq, 1996; Mehran *et al.*, 2006) or/and by cell culture ELISA at WRL for FMD, Pirbright, UK (Buxton and Faser, 1977).

Tissue-cultured FMD virus samples that showed CPE were submitted to World Reference Laboratory for FMD, Pirbright, UK for further serotyping, topotyping, and phylogenetic analysis (Table 3). According to Kitching and Donaldson (1987), specimens were submitted to the WRL for FMD using the following recommended international standards format of three letter country code / isolate number /year (e.g, ETH/02/2012).

3.6. Extraction of virus RNA

The total RNA was extracted from the cell culture isolated by using RNeasy® mini kit (Qiagen, USA) based on the manufacturer protocols. Briefly, 500µl of tissue culture sample was taken and put into a 1.5 ml eppendorff tube, centrifuge at 5000 rpm for 15 sec, and add 460µl volume of Lysis buffer RLT (Containing 1% 2-mercaptoethanol) was added to the sample and mixed by vortexing, and then 460µl of 70% ethanol was added and mixed by vortexing. The mixture was transferred to RNeasy spin column (700µl maximum loading volume) and spinned in a micro centrifuge for 15 sec at 13,000 rpm. The flow through was discarded and repeated with remaining volume. The RNA was washed with 700µl washing buffer RW1 (centrifuge for 15 sec at 13,000 rpm) and with 500µl RPE buffer subsequently. After the flow through was discarded the column was centrifuged at 13,000 rpm speed for 2 min to dry the membrane. Then, the RNA was eluted with 50µl DEPC-H_2O into a new clean collection tube and stored at -20^{O}C.

3.7. Complementary DNA synthesis (cDNA)

The complementary DNA was synthesised based on the manufacturer protocol (Invitrogen, Germany) in 20µl reaction volume. Primarily 1µl oligodT 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^{O}C for 3 minutes (PCR machine) and chilled on ice for 3 minutes. Then 10x RT buffer, 25mM $MgCL_2$, 0.1M DTT and RNase out were added with the rate of 2µl, 4µl, 2µl, and 1µl, respectively, and incubated at 42^{O}C for 2 minutes. Finally, 1.5µl Superscript III (Reverse transcriptase) was added and incubated at 42^{O}C for 50 minutes, followed by heating at 85^{O}C for 5 minutes and chilled on ice for 2 minutes then add 1 µl RNase-H and centrifuge and incubate at 37°C for 20 minutes and cDNA was stored at -20^{O}C until needed.

3.8. Polymerase chain reaction (PCR) and primers used

The PCR was performed using (Advantage® cDNA PCR, Novagen, USA) Kit. Primarily master mix containing (5µl 10x PCR buffer, 1.5µl 25mM $MgCl_2$, 1µl 10mM each dNTPs,

0.5µl Taq DNA polymerase and 35µl DEPC-H_2O) was prepared for each reaction. Then, 12µl of the master mix, 1.5µl of each of specific forward and reverse primer (10pmol) and 5µl of the cDNA were added to each tube. Final run on a 2720 Thermal cycler (Applied Biosystem, USA) program cycle of initial denaturation at 95^{O}C for 5 minutes and then 40 cycles of 94^{O}C for 45 seconds, 65^{O}C for 45 seconds, and 72^{O}C for 2 minutes, ending with incubation at 72^{O}C for 7 minutes.

Three alternative primer combinations were used for RT-PCR of FMDV serotype O viruses: O-1C244F/EUR-2B52R, O-1C272F/EUR-2B52R and O-1C283F/EUR-2B52R and two primer sets for each of the other serotypes: serotype A (A-1C562F/EUR-2B52R and A-1C612F/EUR-2B52R), serotype C (C-1C536F/EUR-2B52R and C-1C616F/EUR-2B52R) (Invitrogen, Germany) (Table 2).

3.9. Agarose Gel electrophoresis for serotype identification

The PCR products were analyzed on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide (Sigma, USA). Briefly, 10µl PCR product mixed with 2 µl loading buffer (Invitrogen, Germany) and loaded to wells in pre prepared gel and run at 100 volt for about 1 hour in parallel with DNA molecular weight marker in electrophoresis apparatus. The DNA band was visualized by UV illumination, documented and the size was determined against the DNA molecular weight marker and serotype was identified. For DNA quantification GeneRuler^{TM} 100bp plus DNA ladder (Fermentas, Germany) was used and run at 100 volt for one hour and DNA concentration is determined.

3.10. Sequencing reaction

Selected virus isolates were further characterised by sequencing of the VP1 gene between serotype O FMD viruses in Ethiopia as well as with other O type isolates from other countries of the world (Table 3). The sequencing reaction was performed at World Reference Laboratory for FMD, Pirbright, UK.

Table 2: Primers used for the amplification of FMDV genome

Oligo name	Sequence (5' – 3')	Sense	Location	Serotype	Use PCR/ Sequencing
EUR-2B52R	GACATGTCCTCCTGCATCTGGTTGAT	-	2B	O, A, C	PCR & Sequencing
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	+	VP3	O	PCR
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	+	VP3	O	PCR & Sequencing
O-1C283F	GCCCAGTACTACACACAGTACAG	+	VP3	O	PCR
A-1C562F	TACCAAATTACACACGGGAA	+	VP3	A	PCR
A-1C612F	TAGCGCCGGCAAAGACTTTGA	+	VP3	A	PCR & Sequencing
C-1C536F	TACAGGGATGGGTCTGTGTGTACC	+	VP3	C	PCR
C-1C616F	AAAGACTTTGAGCTCCGGCTACC	+	VP3	C	PCR & Sequencing

Key to symbol: R=A/G, Y=C/T, B=G/T/C, N=A/C/G/T

Table 3: Source of FMD Type O viruses used for genotyping comparisons (Sequencing reaction)

Pos	Virus name	File name	Topotype	Strain
1	O/ETH/9/2011	ETH11-09	EA-3	Unnamed
2	O/ETH/7/2011	ETH11-07	EA-3	Unnamed
3	O/ETH/7/2010	ETH10-07	EA-3	Unnamed
4	O/ETH/59/2011**	ETH11-59	EA-3	Unnamed
5	O/ETH/58/2005 (FJ798141)	ETH05-58	EA-4	Unnamed
6	O/ETH/51/2011**	ETH11-51	EA-3	Unnamed
7	O/ETH/50/2011**	ETH11-50	EA-3	Unnamed
8	O/ETH/5/2011	ETH11-05	EA-3	Unnamed
9	O/ETH/49/2011**	ETH11-49	EA-3	Unnamed
10	O/ETH/48/2011**	ETH11-48	EA-3	Unnamed
11	O/ETH/46/2011**	ETH11-46	EA-3	Unnamed
12	O/ETH/45/2011**	ETH11-45	EA-3	Unnamed
13	O/ETH/45/2009	ETH09-45	EA-3	Unnamed
14	O/ETH/42/2011**	ETH11-42	EA-3	Unnamed
15	O/ETH/4/2011	ETH11-04	EA-3	Unnamed
16	O/ETH/38/2011**	ETH11-38	EA-3	Unnamed
17	O/ETH/34/2011**	ETH11-34	EA-3	Unnamed
18	O/ETH/32/2011**	ETH11-32	EA-3	Unnamed
19	O/ETH/3/2011	ETH11-03	EA-3	Unnamed
20	O/ETH/3/2004 (FJ798109)	ETH04-03	EA-3	Unnamed
21	O/ETH/29/2011**	ETH11-29	EA-3	Unnamed
22	O/ETH/28/2011	ETH11-28	EA-3	Unnamed
23	O/ETH/26/2011	ETH11-26	EA-3	Unnamed
24	O/ETH/2/2011	ETH11-02	EA-3	Unnamed
25	O/ETH/2/2006 (FJ798127)	ETH06-02	EA-3	Unnamed
26	O/ETH/18/2011	ETH11-18	EA-3	Unnamed
27	O/ETH/13/2011	ETH11-13	EA-3	Unnamed
28	O/ETH/11/2011	ETH11-11	EA-3	Unnamed
29	O/ETH/1/2011	ETH11-01	EA-3	Unnamed

30	O/ETH/1/2007 (FJ798137)	ETH07-01	EA-3	Unnamed
31	O/BHU/3/2009	BHU09-03	ME-SA	Ind-2001d
32	O/UGA/5/96 (AJ296327)	UGA96-05	EA-1	Unnamed
33	O/UGA/3/2002 (DQ165077)	UGA02-03	EA-2	Unnamed
34	O/TAN/2/2004	TAN04-02	EA-2	Unnamed
35	O/SUD/6/2008 (GU566062)	SUD08-06	EA-3	Unnamed
36	O/SUD/5/2008 (GU566061)	SUD08-05	EA-3	Unnamed
37	O/SUD/4/99 (GU566044)	SUD99-04	EA-3	Unnamed
38	O/SUD/4/2008 (GU566060)	SUD08-04	EA-3	Unnamed
39	O/SUD/3/99 (GU566043)	SUD99-03	EA-3	Unnamed
40	O/SUD/3/2008 (GU566059)	SUD08-03	EA-3	Unnamed
41	O/SUD/2/86 (DQ165075)	SUD86-02	EA-3	Unnamed
42	O/SUD/14/2004 (GU566050)	SUD04-14	EA-3	Unnamed
43	O/SUD/1/99 (DQ165076)	SUD99-01	EA-3	Unnamed
44	O/MAL/1/98 (DQ165074)	MAL98-01	EA-2	Unnamed
45	O/K83/79* (AJ303511)	KEN79B83	EA-1	Unnamed
46	O/K40/84*	KEN84-40	EA-1	Unnamed
47	O/IRN/61/2001 (DQ164896)	IRN01-61	ME-SA	Irn-2001
48	O/IND/R2/75* (AF204276)	IND75--A	ME-SA	Unnamed
49	O/IND/53/79 (AF292107)	IND79A53	ME-SA	Unnamed

* Not a WRL for FMD reference number

** Samples submitted to WRL for FMD, Pirbright, UK.

3.11. Data analysis

A homologous region of 639 nucleotides corresponding to the whole VP1 gene was used for all phylogenetic analysis. Nucleotide sequences of serotype O isolates from other African countries were included to deduce the phylogeny of this serotype on the African continent as well as isolates from the Middle East and South Asia to ensure that all previously identified lineages and genotypes were represented (Knowles and Samuel, 2003). Phylogenetic tree were constructed using methods of analysis included in MEGA version 5.0 (Tamura *et al.*, 2007) and confidence levels were assessed by 1000 bootstrap replications. Serotypes were

distinguished on the basis of nucleotide sequence differences of 30-50% and high bootstrap support (> 70%) while a divergence of 15% to 20% distinguishes topotypes (Knowles and Samuel, 2003).

The result was analyzed according to Samuel *et al.* (2001), where ≤ 1% nt. sequence difference indicated that virus isolated from the same epizootic, < 7% nt. sequence difference indicated that virus belonging to the same epizootics (common origin), virus of the same genotype differ up to 15% and viruses from different genetic lineage differ by ≥ 20% nt. sequence.

4. RESULTS

4.1. Virus isolation

Out of the total 59 bovine and swine epithelial tissue cultured samples, 43 (72.88%) samples were showed FMDV CPE on BHK-21 monolayer cell cultures (Table 4). 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 round in shape. Complete destruction of the cell sheet was mostly seen within 48 hours of inoculation. Of 43 samples that showed CPE, 36 samples were sent to WRL for FMD, Pirbright, UK, for further serotyping, topotyping, and phylogenetic analysis.

Table 4: Summary of cytopathic effects observed on tissue cultures

Animal species	No of samples tested	No of CPE positives	% of CPE
Bovine	54	38	70.37%
Swine	5	5	100%
Total	**59**	**43**	**72.88%**

4.2. FMDV serotype identification

On those samples that showed CPE, further examination were done to identify the type of the virus with Agarose gel-based RT-PCR at NVI, Ethiopia and cell culture ELISA at WRL for FMD, Pirbright, UK. Thus, only serotype O was recorded from all samples collected from outbreaks (Table 5).

Table 5: FMDV serotype identified in different outbreaks of Ethiopia

No	Site of outbreak	No of sample	CPE Positive	Serotype by		Final Result
				Agarose gel-based RT-PCR	Cell culture ELISA	
1	Alage Dairy Farm (Alage College)*	7	1	O	-	O
2	Alaba (SNNP)*	3	1	O	-	O
3	Adamitulu Jido kombolcha (Oromia)*	1	1	O	-	O
4	Debre Zeit Swine Farm (Oromia)	5	5	O	O	O
5	Behylu Dairy Farm (Debre Zeit) (Oromia)	2	1	O	O	O
6	Tigest Dairy Farm (Debre Zeit) (Oromia)	4	3	O	O	O
7	Malga (Sidam Zone, SNNP)	7	7	O	O	O
8	EMDTI (Debre Zeit) (Oromia)	5	5	O	O	O
9	Adama (Oromia)	4	2	O	O	O
10	Akaki-Kaliti (Addis Ababa)	2	2	O	O	O
11	Mekele Universty Farm (Tigray)	8	8	O	O	O
12	Enderta (Tigray)	3	3	O	O	O
13	Debre Berehan (Amhara)*	8	4	O	-	O
Total		**59**	**43**			

* Not sent to World Reference Laboratory for FMD, Pirbright, UK.

4.3. Phylogenetic analysis

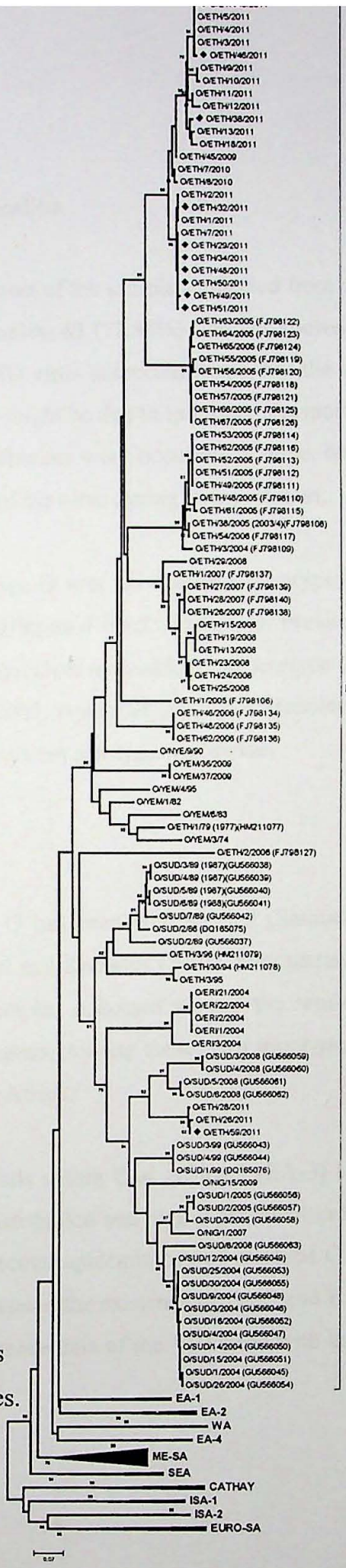
The VP1 gene characterization was used to study phylogenetic relationships between 12 serotype O FMD viruses in Ethiopia as well as with other O type isolates from other countries of the world. All serotype O isolates of Ethiopia falls it to a single topotype, East Africa-3 (EA-3) as shown in figure 4.

Serotype O isolated in different districts of Ethiopia during the study periods were also compared with each other and with other countries type O isolates. Isolates from Debre Zeit, Adama, and Sidama were showed 98.75-100% similarity to each other, and approximately 14–15% different to members of the EA-1, EA-2, EA-4 and ME-SA topotypes of other countries of Africa and Asia. Isolate from Mekele University Farm O/ETH/59/2011 was 99.84% similarity with Ethiopian isolates of 2011 (O/ETH/26/2011 and O/ETH/28/2011) which were collected by other person. This isolate (O/ETH/59/2011) was also 94.05–95.31%similarity with Sudan isolates of 2008 (O/SUD/3/2008(GU566059), O/SUD/4/2008(GU566060), O/SUD/5/2008(GU566061), O/SUD/6/2008(GU566062)), 93.11% similarity with Sudan isolates of 1999 (O/SUD/1/99(DQ165076), O/SUD/3/99(GU566043), O/SUD/4/99(GU566044)) and 92.96% similarity with Sudan isolates of 2004 (O/SUD/14/2004(GU566050)), respectively which are genetically homologues at 639nt. sequence level, but it differ 13.62-14.42% with topotypes of EA-1, EA-2, EA-4 and ME-SA of other countries of Africa and Asia.

◆ indicates viruses in this batch

Software: MEGA 5.0
Analysis
  Analysis ---------- Phylogeny Reconstruction
  Scope ---------- All Selected Taxa
  Statistical Method ---------- Neighbor-joining
Phylogeny Test
  Test of Phylogeny ---------- Bootstrap method
  No. of Bootstrap Replications ------- 1000
Substitution Model
  Substitutions Type ---------- Nucleotide
  Model/Method ---------- Kimura 2-parameter model
  Substitutions to Include ---------- d: Transitions + Transversions
Rates and Patterns
  Rates among Sites ---------- Uniform rates
  Pattern among Lineages ---------- Same (Homogeneous)
Data Subset to Use
  Gaps/Missing Data Treatment -------- Pairwise deletion
  Codons Included ---------- 1st+2nd+3rd+Non-Coding
No. of Sites : 642
No Of Bootstrap Reps = 1000
Only bootstrap values of 70% and above are shown

*, not a WRLFMD Ref. No.



ure 4: Phylogenetic tree showing the relationships
ween VP1 sequences of serotype O FMDV isolates.

5. DISCUSSION

FMD Virus isolation and serotype identification

In this study FMD virus was isolated from most of the samples collected from outbreaks. Out of the total 59 epithelial tissue-cultured samples, 43 (72.88%) samples showed FMDV CPE on BHK-21 monolayer cell cultures for FMD virus suspected tissue, but the other 16 tissue cultured samples had not yet any CPE. This might be due to improper transportation from the field to the NVI laboratory since some outbreaks were occurred in areas where vehicle is inaccessible and some may be due to death of the virus during transportation.

Serotyping of the virus revealed that serotype O was the dominant serotype identified from bovine and swine samples collected from different district of Ethiopia. Previous studies have also indicated that serotype O was highly prevalent and a dominant serotype causing most of the outbreaks in Ethiopia (Gelaye *et al.*, 2005; Ayelet *et al.*, 2009; Haileleul *et al.*, 2010). Klein (2009) indicated that it is the most prevalent serotype worldwide.

Phylogenetic analysis

The molecular epidemiology of serotype O has been well studied (Samuel and Knowles, 2001; Knowles and Samuel, 2003). Samuel and Knowles (2001) demonstrated the existence of eight serotype O topotypes within samples collected around the world based on the comparison of sequence data of the VP1 gene. Among those, two topotypes were found in Africa, one in East Africa and one in West Africa.

In this study all serotype O strains had falls within East Africa-3 (EA-3) topotypes. These indicated that EA-3 topotype has wider distribution and highly prevalent in Ethiopia. This is in agreement with previous study on molecular epidemiology of serotype O by Ayelet *et al.* (2009) and Haileleul *et al.* (2010) demonstrated the existence of EA-3 and EA-4 topotypes in Ethiopia based on the comparison of sequence data of the VP1 gene with the highest rate of EA-3 topotype.

Serotype O isolates from Debre Zeit, Adama, and Sidama were closely related to each other (<2% nt. sequence difference) were observed at 639 nt. sequence. This indicated that outbreaks due to these isolates were the same origin. Furthermore, serotype O isolated from Mekele University Farm O/ETH/59/2011 and Ethiopian isolates of 2011 (O/ETH/26/2011 and O/ETH/28/2011) collected by other person were closely related (< 1% nt. difference) which indicated that these outbreaks have the same origin. This might be due to free movement of livestock and livestock products among various markets in different regions and states which play an important role in the dissemination of the virus. This isolate (Mekele University Farm isolate (O/ETH/59/2011) were also genetically most closely related (~ 4-7% nt. sequence differences) from Sudan isolates in 2008 (O/SUD/3/2008(GU566059), O/SUD/4/2008(GU566060), O/SUD/5/2008(GU566061), O/SUD/6/2008(GU566062)), Sudan isolates in 1999 (O/SUD/1/99(DQ165076), O/SUD/3/99(GU566043), O/SUD/4/99(GU566044)) and Sudan isolates in 2004 (O/SUD/14/2004(GU566050)). These indicated that outbreaks due to these isolates were the same epizootics (common origin). This might be due to free movements of livestock and livestock products among Ethiopian and Sudan borders.

6. CONCLUSION AND RECOMMENDATIONS

Foot and mouth disease is endemic in Ethiopia due to the factors such as the presence of high numbers of susceptible domestic animals, free movement of livestock and livestock products in different regions and states across the country, free cross borders between neighbouring countries, lack of control of animal movements, and lack of effective vaccination contributed to the occurrence of FMD and to the difficulty in controlling the outbreaks. During the study periods only serotype O were identified throughout the Ethiopia where outbreaks occurred. Out the total samples collected, most samples showed CPE in BHK-21 cell culture. Samples were also sent to WRL for FMD, Pirbright, UK for further molecular characterization and phylogenetic relationship with other O type isolates form other countries of the world thus, all O serotype isolates of Ethiopia falls in to a single topotype East Africa-3 (EA-3) and they are related with each other but differ from other O topotypes; whereas isolate from Mekele University Farm was also have similarity with Sudan O isolates.

Therefore in light of the above conclusions the following points are forwarded:

- Restriction of animal movement across the regions and serious attention should be given during importation/movement of livestock and livestock products across the border areas.
- Regular investigation of FMD outbreaks should be done to have more detailed information about the serotypes and topotypes circulating in Ethiopia and for effective vaccine development.
- Efficient vaccine-based FMD control strategy program should be designed in the country.

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8. ANNEXES

Annex I: Procedure for FMDV Cell Culture Preparation

Protocol 1: BHK-21 Cell Line Preparation

Materials:

- 5, 10, and 25 ml sterile pipettes
- PBS
- αMEM medium (10%) Growth medium
- Previous BHK-21 cell line
- 75 cm2 flasks, 175 cm2 flasks and/or 35 mm plates (other flasks and plates may be used)
- Trypsin/versene (EDTA) solution or other trypsin solution

Procedure:

1. When cells are ~80-90% confluent, remove all medium from the flask.
2. Wash cells with 10 ml PBS (2x) to remove medium. Serum contains inhibitors of trypsin.
3. Add 5 ml of trypsin/versene (EDTA) solution to the monolayer and incubate 1 to 5 minutes at room temperature until cells detach. Check the cells under a microscope and confirm that most of the cells have detached. If cells are still attached, incubate a little longer until most of the cells have detached.
4. Once the cells have detached, briefly pipet the solution up and down to break up clumps of cells.
5. Add 10 ml of complete αMEM to stop trypsinization. Check the viability of the cells. Cells should be > 90% viable.
6. To maintain cells in 75 cm^2 flasks, transfer 2 ml of the 10 ml cell suspension from Step 5 to a new 75 cm^2 flask and add 10 ml fresh, complete αMEM medium.
7. To expand cells, transfer 3 to 4 ml of the cell suspension to a 175 cm^2 flask (3 flasks total) and add fresh, complete αMEM medium to a final volume of 30 ml.
8. Incubate flasks in a humidified, 37°C, 5% CO2 incubator.

Repeat Steps 1-8 as necessary to maintain or expand cells.

Protocol 2: FMDV Isolation on BHK-21 Cell Culture

Materais:

- Sample tissue
- Sterile mortar and pustule
- Scalpel and blade
- Scissors
- PBS
- αMEM medium (2%) Maintenance medium
- 5, 10, and 25 ml sterile pipettes
- Test Tubes
- BHK-21 cell Line (80-90% confluent)
- Centrifuge
- Millipore filter (0.22μm pore size)

Procedure:

1. The epithelial tissue samples is thawed at room temperature
2. washed three times using sterile phosphate-buffered saline (PBS) at a pH of 7.2–7.6 under laminar air flow hood class II.
3. Grounded 1 g of epithelial tissue sample using sterile mortar and pestle by adding 10 ml of sterile PBS containing antibiotic.
4. Centrifuge the tissue suspension at 3,500 rpm for 10 min.
5. Collect the supernatant and filtered by Millipore filter of 0.22μm pore size.
6. Inoculate 1 ml of filtered tissue suspension 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
7. Flashed with growth media (2% MEM media) and incubated at 37°C and 5% CO_2 in a humidified incubator for 24-48 hours.
8. Monitor the Cells for cytopathic effects (CPE) daily and frozen when CPE was developed or after 72 hours post-infection.

If no CPE was detected, the cells were frozen and thawed, and a second pass was performed with same procedure as the first pass.

Annex II: Procedure for RT-PCR for FMD Virus

Protocol 1: Extraction of Virus RNA using RNeasy Spin-Columns

1. Put 500 µl of your sample into a 1.5 ml tube and centrifuge at 5000 rpm for 15 sec. remove all supernatant by aspiration and add 460 µl volume of Lysis buffer RLT (containing 1% 2-mercaptoethanol) to your sample and mix by vortexing.
2. Add 460 µl 70% ethanol and mix by vortexing.
3. Apply to RNeasy spin column (700 µl maximum loading volume). Spin in a microfuge for 15 sec at 10,000 rpm. Discard flow through and reuse collection tube. Repeat with remaining volume.
4. Wash with 700 µl wash buffer RW1. Centrifuge as before.
5. Wash with 500 µl wash buffer RPE. Centrifuge as above.
6. Repeat wash with 500 µl wash buffer RPE. Centrifuge at max speed for 2 min to dry membrane.
7. Elute RNA with 50 µl DEPC-H_2 O into a new clean collection tube. Spin in a microfuge for 60 sec at 10,000 rpm.
8. Store in clean tube at -20°C. Label the tube clearly with "RNA", the virus name, today's date and your name.

Protocol 2: Reverse Transcription of Virus RNA (cDNA synthesis)

1. In a sterile 0.5 ml eppendorf tube prepare the following mixture (depending on the number of RNAs to be reverse transcribed).

Reagent	No. of RNA preps				
	1	2	3	4	5
10 mM dNTPs	1*	2	3	4	5
DEPC-H $_2$O	3	6	9	12	15
Oligo dT (25 pmol/µl)	1	2	3	4	5
Mix-1 (Total)	5	10	15	20	25
10× RT Buffer	2	4	6	8	10
0.1M DTT	2	4	6	8	10
25mM $MgCl_2$	4	8	12	16	20
RNase Out (3.3 U/µl)	1	2	3	4	5
Mix-2 (Total)	9	18	27	36	45

* amount in µl

2. Add 5 µl of the Mix-1 to 5 µl of each RNA template on PCR tubes giving at total volume of 10 µl.

3. Spin briefly in microfuge. Then incubate at 65°C for 3 mins and chilled on ice for 3 mins

4. Add 9 µl of Mix-2 to each tubes then Heat at 42°C for 2 mins and then add 1.5 µl Supermix III and incubate at 42°C for 50 mins and then at 85°C for 5mins.(either in a waterbath or in the thermocycler).

5. Chilled on ice for 2 mins. Then add 1 µl RNase-H on each tube and centrifuge at 13,000 rpm for 15 secs and finally incubate at 37°C for 20 mins.

5. Store at -20°C. Label the tube with "RT product", the virus name, today's date and your name.

Protocol 3: PCR Amplification of Reverse Transcribed RNA (cDNA)

1. Into a sterile 0.5 ml tube add the following. Take care to use sterile tips and avoid cross contamination. Use new tips for each reagent and template.

Reagent	Concentration	Amount (µl)
25 mM MgCl 2	1.5 mM	1.5
10 mM dNTPs	200 µM	1
10× buffer	1x	5
Taq DNA polymerase (5 U/µl)	2.5 U	0.5
DEPC-H O_2		35
Primer 1	10 pmol	1.5
Primer 2	10 pmol	1.5
Master Mix		46

2. Add 15 µl Master Mix and 5 µl template in PCR tubes and spin on microfuge

3. Run on Thermocycler as follows:

Temperature	Time	No. of cycles
95°C	5 min	1
94°C	45 secs	
65°C	45 secs	40
72°C	2 min	
72°C	7 min	1

4. Take 5-10 µl of PCR product and check it on an agarose gel.

Protocol 4: Agarose Gel Electrophoresis of PCR Products

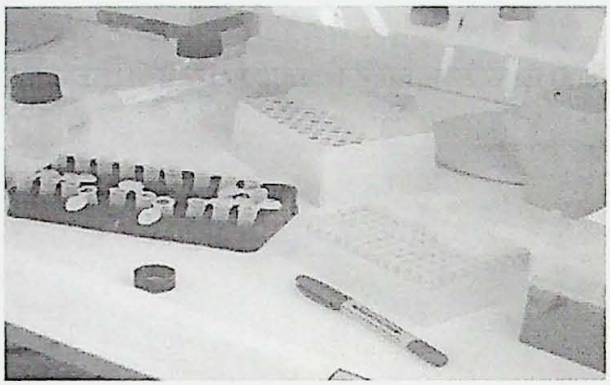
Note: Take care ethidium bromide is harmful; gloves should be worn at all times.

1. Prepare 120 ml of 1.5% agarose in 1x TAE buffer.
2. Either heat in microwave for ~2 min on full power or place in beaker of boiling water until melted.
3. Allow to cool to about 45°C and add 5-10 µl ethidium bromide (stock=5 mg/µl) per 10 ml, giving a final concentration of 0.5 µg/ml.
4. Pour gel and insert well former (comb). Allow to set on a flat surface for about 15 min.
5. Pour buffer 1x TAE (containing 0.5 µg/ml ethidium bromide, i.e. 1 µl of 5 mg/µl stock to every 10 ml of buffer) into tank and remove comb from gel.
6. Prepare samples in tubes, a multiwell plate or on parafilm. 2 µl loading buffer 5-10 µl PCR product
7. Prepare molecular weight marker. 8-10 µl molecular weight marker GeneRuler™ 100bp loading buffer
8. Load samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the first and last lanes.
9. Electrophoresis at 100 volts for 1 hour or 10 volts overnight.
10. The DNA band was visualized by UV illumination, documented and the size was determined against the DNA molecular weight marker and serotype was identified. Use UV-safety spectacles.

Annex III: FMD Clinical Sample Collection and RT-PCR for serotyping

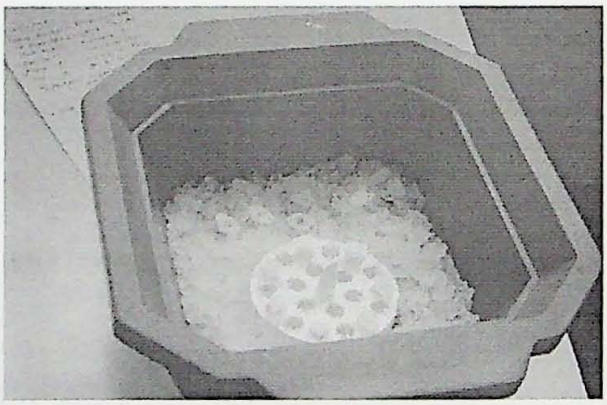
A. FMD Mouth Lesion

B. FMD Leg Lesion



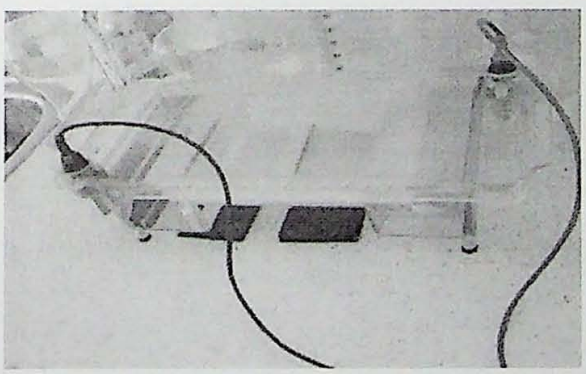
C. FMDV RNA extraction

D. cDNA Synthesis



E. Master Mix Preparation

F. RT-PCR for amplification

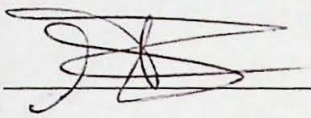


G. Agarose gel electrophoresis

9. SIGNED DECLARATION SHEET

"I, the signed, declare that this thesis is my original work and has not been presented for a degree in any university and that all sources of material used for the thesis have been duly acknowledged"

Name: Sentayhu Minda Tamirate

Signature 

Date of submission 2/7/2012

This thesis has been submitted for examination with our approval as university advisors.

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