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MOLECULAR CHARACTERIZATION AND VACCINE MATCHING TEST OF FOOT AND MOUTH DISEASE VIRUSES ISOLATED FROM OUTBREAK CASES IN CATTLE IN SELECTED SITES OF CENTRAL PART OF OROMIA, ETHIOPIA

MSc Thesis

By: Motuma Debelo Dibaba

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

JUNE, 2017

BISHOFTU, ETHIOPIA

MOLECULAR CHARACTERIZATION AND VACCINE MATCHING TEST OF FOOT AND MOUTH DISEASE VIRUSES ISOLATED FROM OUTBREAK CASES IN CATTLE IN SELECTED SITES OF CENTRAL PART OF OROMIA, ETHIOPIA

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

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Department of Microbiology, Immunology and Veterinary Public Health

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As member of the Examining Board of the final MSc open defence, we certify that we have read and evaluated the thesis prepared by: **Motuma Debelo Dibaba,** titled: **Molecular characterization and Vaccine matching test of foot and mouth disease viruses isolated from outbreak cases in cattle in selected sites of central part of Oromia, Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the degree of master of Science in Veterinary Microbiology.

	Signature	Date
Dr. Gezahegne Mamo		
	----------	----------
Chairman		
Dr. Belayneh Getachew	----------	----------
External Examiner		
Dr. Kebede Amenu	----------	----------
Internal Examiner		
1. Dr. Fufa Dawo	----------	----------
Major advisors		
1. Dr. Esayas Gelaye	----------	----------
Co- advisor		

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"Our knowledge of God is perfected by gratitude: we are thankful and rejoice."

~ Thomas Merton

I have had the pleasure of meeting many people in the course of my career to whom I am indebted for my achievements. Firstly, I would like to dedicate my thesis to my Brother Mekonnen Debelo and thank him for the unconditional love and support throughout my career. He has always helped me be better than I think I am. I am also grateful to all my families, relatives, friends and people of study areas for being my support structure both professionally and personally during this MSc.

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LIST OF ABBREVIATIONS

AU-IBAR	African Union Interafrican Bureau for Animal Resources
BHK21	Baby Hamster Kidney 21 day
Blastn	Basic Local Alignment Search Tool
Co2	Carbon Dioxide
CPE	Cytopathic Effect
CSA	Central Statistical Authority
eIF4G	Eukaryotic Translation Initiation Factor 4 G
ELISA	Enzyme Linked Immunosorbant Assay
FAO	Food and Agriculture Organization
FMDV	Foot and Mouth Disease Virus
GDP	Gross Domestic Production
IB-RS-2	Porcine Kidney Cell Line
ICTV	International Committee on Taxonomy of Viruses
IU	International Unit
MAbs	Monoclonal Antibodies
MEGA	Molecular Evolutionary Genetic Analysis software
MEM	Minimum Essential Media
NAHDIC	National Animal Health Diagnostic and Investigation Centre
NBS	Normal Bovine Serum
NCBI	National Center for Biotechnology Information
NCR	Non Coding Region
NCR	Non-Coding Region
Nt	Nucleotides
OIE	World Animal Health Organization
OP	Oro-Pharyngeal
Pas	Peasant Association
PBS	Phosphate Buffer Saline
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PH	*Potential of Hydrogen*
RGD	*Arginine-Glycine-Aspartic acid*
RNA	Ribonucleic Acid
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
SAT	Southern Africa Territory
SP-C ELISA	Solid Phase Competitive Enzyme Linked Immunosorbent
SPS-LMM	Sanitary and Phytosanitary Standards and Livestock and Meat Marketing
SVD	Swine Vesicular Disease
SVDV	Swine Vesicular Disease Virus
UK	United Kingdom
USA	United State of America
UTR	Untranslated Region
VNT	Virus Neutralization Test
VP1	Viral Capsid Protein one
Vgp	Viral Genomic Protein
VSV	Vesicular Stomatitis Virus
WRL	World Reference Laboratory
WRLFMD	World Reference Laboratory for Foot and Mouth Disease
α	Alpha
β	Beta

ABSTRACT

Foot-and-mouth disease (FMD) is the most contagious, acute viral disease of all cloven-hoofed animals/ungulates or its host range is extremely wide being capable of infecting nearly 70 species within 20 families of mammals and pigs. Based on the occurrence of FMD outbreak and active cases, outbreak specimens were collected, to isolate viruses and to determine molecular characteriza tion and vaccine matching test. Foot-and-mouth disease (FMD) investigation was conducted purposively in three closely located areas namely central Oromia; Addis Ababa and its surrounding Oromia zones, Bishoftu, Adama and its surroundings. A total of 41 samples comprising of tongue, inter digital tissue (n=20) and oropharyngeal fluid (n=21) were collected from suspected cattle. Sixteen representative samples were propagated in BHK21 cells. After 3 subsequent passages, progressive cytopathic effects (CPE) i.e., rounding and flattening of cells, breaking down of cells and finally cell death (almost 100%) were observed. Viral RNA was extracted from the grown viruses, and Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed using four sets of primers corresponding to the serotype 'O', 'A' SAT2 and 'SAT1', respectively. Out of the 16 samples, 10 of them were diagnosed by polymerase chain reaction (PCR) and all of them were found to be positive for FMDV by universal primer, also sequenced using specific primers and all of them were found to be serotype 'O'. Out of the 10 specimens 3 of them were East Africa topotype 4 and 7 of them were not sequenced because they had not good sequence. Some FMDV isolates were characterized by virus neutralization test vaccine matching test. It is concluded that FMDV serotype 'O' is circulating among the cattle of central part of Oromia, Ethiopia. Generally regular investigation of foot and mouth disease outbreaks is important to have more detailed information on the serotypes and topotypes circulating in Ethiopia and for effective vaccine development and control of the disease.

Keywords: *Cattle, FMDV, molecular characterisation, Oromia, serotype O, topotypes, vaccine matching test*

1. INTRODUCTION

Ethiopia is one of few countries in Africa with huge livestock resources. The different livestock species play a crucial role in the livelihoods of majority of Ethiopians via direct provision of draught power (95%) for crop production besides provision of meat, milk, and transport service. The country is believed to have the largest livestock population in Africa comprising approximately 57.83 million cattle, 28.89 million sheep and 29.70 goats (CSA, 2015/16).

Diseases have numerous negative impacts on productivity of herds causing death of animals, loss of weights, slow down growth, poor fertility performance and decrease in physical power (CSA, 2015/16). Animal diseases are currently widespread in all agro-ecological zone of the country and annual mortality rates due to diseases is estimated at 8-10% for cattle herd and 15% and 12% for sheep and goats flocks, respectively. It is estimated that animal diseases reduce the production and productivity of livestock by 50 to 60% per year. Among the livestock diseases hampering productivity of the sector foot and mouth disease (FMD) is considered as a major bottleneck to the livestock production and export (Ganesh, 2012).

FMD is a highly infectious disease of cloven hoofed animals and pigs characterized by the formation of vesicles in and around the mouth and on the feet (Alexandersen *et al*., 2003). The disease is caused by foot-and-mouth disease virus (FMDV) which a single stranded positive sense RNA virus that belongs to the genus *Aphthovirus* and family *Picornaviridae*. FMDV species has seven antigenically diverse serotypes (O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3) (Knowles and Samuel, 2003; Stanway *et al*., 2005) that cause indistinguishable clinical disease (Vosloo *et al*., 2002). The seven serotypes contain more than 65 strains. New strains occasionally develop spontaneously. Serotype A and the SAT viruses are highly variable, but the Asia-1 viruses have tended to remain relatively stable in their antigenic types. FMDV carriers are animals in which virus or viral RNA can be detected for more than 28 days after infection. FMDV persists mainly in the pharyngeal region, and is detected by testing esophago-pharyngeal fluid. The incubation period for FMD can be as short as 18-24 hours, or as long as 14 days in some species. FMDV can be found in all secretions and excretions from acutely infected animals, and shedding can occur for up to 4 days

before the onset of clinical signs. Shedding usually peaks at or near the time when the vesicles rupture and most clinical signs appear. Airborne transmission is more important for some topotypes and strains of FMDV than others. Transmission seems to occur less readily between sheep than between cattle or pigs. Even if sheep are not vaccinated, only a proportion of them within a flock may become infected. FMDV might be carried mechanically in the nares of uninfected humans for short periods. The disease spreads rapidly among non-immunized animals, because of very high morbidity rates, whilst mortality is low except in young animals (Ryan *et al*., 2007). In countries with highly developed animal industry and free trade, outbreaks are responsible for economic devastation.

There is limited information on the survival of FMDV in the environment, but most studies suggest that it remains viable, on average, for three months or less. Virus stability increases at lower temperatures, and in very cold climates, survival up to six months or more may be possible. FMDV can also persist in meat and other animal products, depending on the pH (OIE, 2012, 2013). Among other control measures, vaccination is one that should be considered. The genetic variability and antigenic diversity of FMD virus has implications for vaccine design and disease control (Sobrino *et al*., 2012). Nearly all currently licensed FMD vaccines are killed vaccines containing chemically inactivated virus. Effective vaccination can decrease transmission between animals by decreasing th e susceptibility of animals to infection, and reducing virus shedding, if a vaccinated animal becomes infected. Genetic characterization can suggest that a new strain has emerged and needs to be matched with a vaccine, or that the field virus is genetically close to one that already has vaccine matching information (Kitching *et al.*, 1988). It may fail to accurately predict the presence or absence of in vivo cross-protection between some viruses. Vaccine matching is used to determine whether a given vaccine is likely to provide good protection against a field strain. Vaccine matching and potency testing are used in concert, as more potent vaccines are more likely to be more effective against less closely related strains. The selection of potential vaccine strains to match should be based on the serotype of the field virus, its region of origin and any other information on its characteristics. In vitro serological tests can also be used for vaccine matching, and generate results rapidly. Matching by ELISA has also been described; however, the OIE currently recommends its use only for screening. The ‘r’ value indicates the closeness of the match in serological tests, with $r_1 > 0.3$ in the VNT suggesting that a potent vaccine is likely to be protective (Mattion *et al.*, 2009).

Serotype identification of FMDV in Ethiopia were done by 3ABC ELISA, but the recent (Yeneneh, 2014 and Mishamo, 2016) detailed knowledge of the molecular characteristics of FMDV major anti genic sites have been helpful to identify serotype, strains and transmission events, to characterize bi odiversity and effective quarantine measures against reintroduction and to develop specific diagnostic tests and protective vaccine (Samuel and Knowles, 2001).

Molecular techniques to identify FMDV have been studied in details in different countries of the world. In Ethiopia, however, the National Animal Health Diagnostic and Investigation Center (NAHDIC) and the National Veterinary Institute (NVI) indicated that serotypes O, A, C, SAT-1 and SAT-2 were responsible for FMD outbreaks during 1974-2008 (Gelaye *et al*., 2005; Legess, 2008; Gelagay, 2009; Haileleul *et al*., 2010).

According to Vosloo *et al*. (2002), all FMDV serotypes, other than Asia 1, have been detected in East Africa; however, serotype C has not been isolated since 2004. Thus, molecular characterization, identifying as non-structural (NST) and structural (ST) and vaccine matching of serotype 'O' and Identifying serotype of FMDV other than serotype 'O' that cause outbreak help to control of this disease and holds the potential to enhance food security, poverty alleviation and national development (Rutagwenda, 2003; Perry and Rich, 2007).

O and A serotypes cause outbreak dominantly at central part and border of Ethiopia, but SAT2 which is one of FMD serotype that cause negative impacts on livestock production was mostly exist at border of Ethiopia even though now days there is report at central part of Oromia (Mishamo 2016); even though, in this current finding no SAT2 serotype had been found; only Serotype O was detected.

The protective capacity of FMD vaccine could be evaluated through vaccine matching using on the calculation of the relatedness between the field isolate and available vaccine strains using in vivo challenge tests (Goris *et al*., 2008).The study areas were known with the abundance of dairy farms and are essential to generate important baseline information about the study disease. Currently, in Ethiopia there is no government strategy in FMD control through vaccination and movement

control. For the development of adequate FMD control and prevention; determining the status of FMD through virus isolation and characterization of the serotype is needed.

Therefore the objective of this paper was;

- To identify the serotypes responsible for suspected FMD outbreaks in the selected areas.
- To isolate and characterize serotype O FMD virus circulating in the study area by using molecular techniques.
- To determine vaccine matching for serotype O FMD viruses.

2. LITRETURE REVIEW

FMD is the most contagious, acute viral disease of all cloven-hoofed animals/ungulates or its host range is extremely wide being capable of infecting nearly 70 species within 20 families of mammals and pigs (Hedger, 1981). The disease is characterized by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet and teats and sudden death of young stock (Quinn *et al.*, 2005; Thomson, 1994). It is one of the most globally important notifiable diseases of livestock due to its high infectious and trans-boundary distribution nature of the disease. It is a list A disease according to OIE disease classifications (OIE, 2004).

2.1. Aetiology

2.1.1. Taxonomy

FMDV was defined in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as belonging to the genus *Aphthovirus*, family *Picornaviridae*. The name *Picornaviridae* is derived from the Latin word 'Pico' meaning small and 'rna' meaning RNA (ribonucleic acid), which refers to the size and genome type of the virus while the genus name '*Aphthovirus*' refers to the vesicular lesions produced in cloven-hoofed animals (OIE, 2004).

2.2. The Genome of FMDV

The genome of FMDV is a single-stranded ribonucleic acid (RNA) and it is roughly spherical in shape and about 25–30 nm in diameter. It consists of the RNA genome surrounded by a protein shell or capsid. The capsid is composed of 60 copies of the capsomers. Each capsomer consists of four structural polypeptides: VP1, VP2, VP3 and VP4. The VP1, VP2 and VP3 are exposed on the surface of the virus while VP4 is located internally. The protein coat surrounds the genome of about 8400 nucleotides (nt) in length. The RNA includes three separate parts i.e. the 5′ untranslated region

(5′ UTR)/ 5' non-coding region (NCR), a long coding region/ a single open reading frame and the 3′ untranslated region (3′ UTR)/ short 3' NCR (Belsham and Bøtner, 2015).

2.2.1. Non-structural / non-coding region (NCR) Proteins of FMDV

There are about seven to nine non-structural (non-capsid) proteins, viz., L (Lab and Lb) and 2A proteins; 2B, 2C and their precursor (2BC); 3A, 3AB, and 3D (VPg), 3ABC. Both Lab and Lb of FMDV are proteases with multiple activities. 3Cpro is a protease and an RNA-binding protein, whereas 3Dpro is an RNA polymerase and is involved in proteolysis in the form of 3CD. 2A pro and B^{Cpro} not only participate in the virus replication but also appear to inhibit essential host function (RNA transcription and protein synthesis) and VPg (3B) plays a role in the initiation of viral RNA synthesis (Lawson and Semler, 1991).

The L Protease; The first component of the FMDV polyprotein is the Leader (L) protein. FMDV is unique in having a protease as the Leader protein. The L protein is a papain-like cysteine protease and it has at least two distinct activities. It cleaves itself from the rest of the viral polyprotein at the L/P1 junction (Strebel and Beck, 1986), and it also induces the cleavage of the translation initiation factor eIF4G (Devaney *et al*., 1988; Medina *et al*., 1993).

The coding region follows the 5′ UTR. It is the major portion of the viral genome and is about 7000 nt in length. It encodes a large polyprotein which is then cleaved by viral proteases to form four different structural and eleven different non-structural proteins. After translation, initially four primary products are formed, namely, L pro, P1-2A, P2 and P3. The L pro is responsible for the inhibition of host cell protein synthesis by inducing the cleavage of the host protein, eIF4G, which is a translation initiation factor (Belsham, 2005) that is required for the translation of the capped cellular mRNAs. As a result, FMDV RNA can freely use the host cell's protein synthesis machinery for its own protein synthesis. The P1-2A capsid precursor is cleaved by the 3C protease (3Cpro) to produce 1AB (VP0), 1C (VP3) and 1D (VP1) (plus 2A) and during encapsidation of the genome the VP0 is cleaved to make VP4 and VP2. The VP4 is entirely internal within the virus particle whereas VP1, VP2 and VP3 are surface exposed and contribute to the antigenic properties of the virus (Thomas *et al*., 1988). The VP1 contains at least two important immunogenic sites, the G-H loop

and the C-terminus. The G-H loop includes an arginine, glycine and aspartic acid (RGD) motif, which is required for attachment of the virus to the host cell via an integrin receptor. Integrins are a group of α-β heterodimeric glycoproteins which are located on the cell surface. The αvβ6 heterodimer is a receptor for the extracellular matrix proteins whose expression is restricted to epithelial cells. The virus, however, can also infect cells in an RGD-independent manner using alternative molecules example, heparan sulphate proteoglycans receptors (Mason *et al*., 1994).

The nucleotide sequences of the VP1 coding region have been used for genetic characterization of FMDV strains because of their significance for virus attachment and entry, protective immunity and serotype specificity. VP1 sequence based phylogenetic analyses have been used widely to deduce evolutionary dynamics, epidemiological relationships among the genetic lineages and in the tracing of the origin and movement of outbreak strains (Jamal *et al*., 2012; Valdazo *et al*., 2011).

The P2 and P3 regions of the polyprotein are processed to the non-structural proteins (NSPs). The P2 region generates the proteins 2B and 2C while the P3 region is cleaved to form the proteins 3A. The P2 and P3 encoded proteins are involved in protein processing and genome replication (Belsham, 2005).

2.2.2. Antigenic structure/Coding region of FMDV

Antigenic site on the surface of the FMD virion have been identified. Serological studies showed that different serotypes of FMDV shared a highly variable region of VP1, comprising residue 135 to 155, as one of the major antigenic sites of the virus. Several overlapping Beta- cell epitopes are located within this region and are able to induce both neutralizing and non-neutralizing antibody responses. The high sequence variability found in this region accounts for the low cross-reactivity observed among different serotypes (Cheung *et al*., 1983).

This immunodominant region was seen to correspond to the loop which connects Beta-sheet G and H of the VP1 Beta-barrel, named the GH loop (Acharya *et al*., 1989). Within this loop antigenic site A is present and very likely to face substitution because of high mutation rates during RNA

replication resulting in antigenic variants. This site also contains the RGD (Arg-Gly-Asp) motif receptor binding recognition sequence and it is believed to be the major antigenic site.

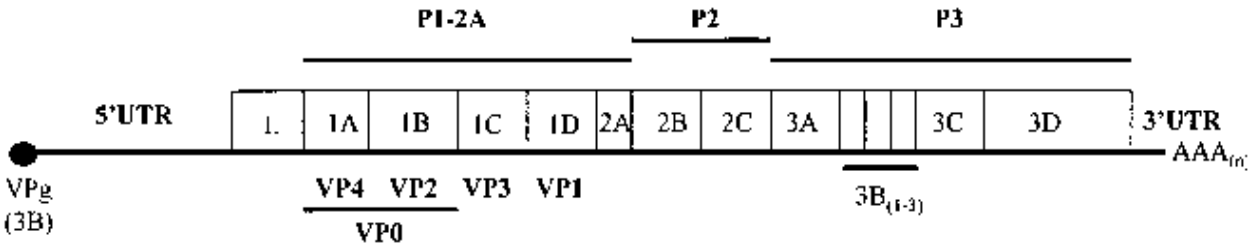


Figure 1: Genome organisation of FMDV. The non-coding and coding regions of FMDV RNA is indicated. The single large open reading frame encodes a polyprotein which is never observed. It is processed by virus-encoded proteases during synthesis. Many different precursors can be generated during this processing of the polyprotein; some of the major precursors are indicated.

2.2.3. Structure and function of FMDV RNA

FMDV genomic RNA is about 8.3 kb in length (Figure. 1). All of the viral RNA is of the same length; no sub-genomic mRNAs are produced by picornaviruses. The genome has some similarities to a eukaryotic cell mRNA in that it contains a single long open reading frame. A feature of the FMDV RNA is the presence of a very long 5′-UTR of about 1300 nt. The RNAs from other picornaviruses also have long 5′-UTRs, but the FMDV 5′-UTR is much larger than most. All cytoplasmic eukaryotic mRNAs have a cap structure. This structure is required for the efficient recognition of the mRNA by the translation initiation complex. The viral RNA lacks this cap structure; termed VPg (or 3B), is covalently attached to the 5′ terminus. Much of the viral RNA within infected cells has a free 5′ end (Gingras *et al*., 1999).

2.2.4. Antigenic variation

Antigenic variation is the process by which an infectious organism alters its surface proteins in order to evade a host immune response and is associated with mutation leading to amino acid replacement. This change in antigenic profile may occur as the pathogen passes through a host

population (also called antigenic diversity) or may take place in the originally infected host. The strategy is particularly important for organism that target long-lived host, repeatedly infect a single host, and are easily transmitted. Pathogens that express these characteristics and undergo antigenic variation have a selective advantage over their more genetically stable counterparts (Novella *et al*., 1996).

RNA viruses are characterized by an error-prone RNA replication, which gives them great potential for variation. Antigenic variants result from the high mutation rates during RNA replication which allow FMD viruses to continuously evolve and adapt to new environments. Although most mutations will be detrimental and eliminated by natural selection (negative selection), others can be of value under the particular conditions where the virus is replicating and are therefore selected (positive selection) (Novella *et al*., 1996). Antigenic variants have been isolated under variable conditions, such as in partially immune animals, persistently infected cattle and in cell culture, in the latter case both in the presence or the absence of immune pressure. This antigenic diversity has serious implications in vaccine design since synthetic vaccines should include multiple independent epitopes in order to decrease the probability of selection of FMD viruses resistant to the immune response (Piatti *et al*., 1995).

2.2.5. Causes of antigenic variation

High rate of mutation; FMD virus have very high mutation rate in the range of 10^{-3} to 10^{-5} per nucleotide site per genome replication, due to the lack of error correction mechanism, that is, proof reading during RNA replication. This high error rate leads to differences of FMD virus replicated genome from the original parental genome and thus resulting in antigenic variation (drake and Holland.1999).

Genetic recombination; Compare to DNA viruses, the RNA viruses are uncommon to recombination (there are probably no host enzymes for RNA recombination). But, picorna viruses show a form of very low efficiency recombination and study has shown that recombination was more likely to occur within the 3 half region of the genome coding for the NSP, and has not been demonstrated in the capsid-coding genes of FMDV (Wilson *et al*., 1988). However, a more recent

study has suggested, that RNA recombination within the capsid-coding region (PI) of the genome may contribute to the genetic diversity in FMDV isolated from the field. Wilson *et al*. (1988) also demonstrated that the recombination events increased much more steeply with increasing secondary structure in the region encoding non-structural proteins compared with the structural protein-coding region of the genome.

Continuous circulation of the virus in the field; the rise of new variants is inevitably caused by continued circulation of the virus in the field and the quasi species nature of the RNA genome. As the virus continues to circulate in the field, continues to cause infection of susceptible host and remained persistence in their host, then with due course of time or probably due to the immunological pressure placed on the virus either by the infected or vaccinated host or either by the challenge in the environment and also to the selective pressure exerted in the field by virus replication in animals having inadequate level of neutralizing antibodies had somehow lead to the antigenic variation giving rise to virus population different from those that initiated the infection (Domingo *et al*., 2003; Haydon *et al*., 2001).

2.3. Physicochemical Properties or Disinfection

2.3.1. Chemical toxicities / Disinfectants

There is limited information on the survival of FMDV in the environment, but most studies suggest that it remains viable, on average, for three months or less. In very cold climates, survival up to six months may be possible. Virus stability increases at lower temperatures; in cell culture medium at 4°C (39°F), this virus can remain viable for up to a year. The presence of organic material, as well as protection from sunlight, also promotes longer survival (Donaldson and Alexandersen, 2002).

FMDV is highly resistant to most disinfectants. Iodophores, quaternary ammonium compounds, hypochlorite and phenols are reported to be less effective, especially in the presence of organic matter. As the virus is sensitive to extremes of pH, both acids (example, acids such as citric acid (0.2% solution), bases (example, caustic soda or sodium hydroxide), alkalis such as washing soda

(sodium carbonate, 4% solution) disinfectants are effective at destroying virus. Formaldehyde may also be used. Their action is enhanced if physical cleaning and detergents are used in combination with the disinfectant as organic material must be penetrated. It is important not to mix acid and alkali disinfectants as their activity against the virus depend on their pH and they will neutralise one another if mixed. Higher temperatures may also enhance the cleaning effect (OIE, 2012).

2.3.2. Physical susceptibility (Inactivation)

FMD virus tends to be insensitive to cold and sensitive to heat. It is susceptible to pH changes away from neutral. At relative humidity over 60%, virus may survive for at least several hours in airborne droplets (OIE, 2012).

2.3.3. Environments - External habitats (Biogeographical / Climate type)

FMDV has been recorded in a wide variety of temperate, sub-tropical and tropical climates. The virus may rapidly multiply and spread (causing a disease outbreak) wherever susceptible animals in close contact are exposed to the virus. Cloven-hoofed mammals (the main host species) exist in virtually all biomes and so occasional outbreaks caused by introduction of the virus through import may occur in almost any habitat type. However, for a virus to become freely circulating it must be able to exist in the environment, either inside or outside the host, for long enough to infect a susceptible host. Many viruses survive for only a short time outside their hosts; however FMDV is one which may show a longer survival in the external environment (Murphy *et al*., 1999).

FMDV is killed by prolonged exposure to sunlight (due to drying and heating) and rapidly by acids and strong alkalis. However it has been reported to remain infective for over 2.5 years in carrier cattle, 1 year in infected premises and in hides, 2 months in carrier deer, 15 weeks on wood, hay and straw, 10-12 weeks on infected feed or clothing, 8 weeks in fragments of infected skin in winter, and 4 weeks on hair and soil particles (OIE, 2012, 2013).

2.4. Serotypes and Strains of FMDV

FMD virus exists as seven different serologically distinct types. Within these serotypes, more than 65 strains have been identified (OIE, 2016). Older strains have names such as O1 Manisa or A24 Cruzeiro, but the names of recently isolated strains are more standardized and include the date and location of isolation (example, O/UK/35/2001). Some serotypes have been divided into topotypes, genetically and geographically distinct units that contain closely related strains of the virus (Jamal and Belsham, 2013); and new subtypes occasionally arise spontaneously. However, at a specific time, there are only a few subtypes causing disease throughout FMD endemic areas. The importance of subtypes is that a vaccine may have to be tailored to the subtype present in the area in which the vaccine is being used (OIE, 2004). Multiple serotypes (O, A, C, SAT 1, and SAT 2) of FMDV have been reported from Ethiopia (Gelagay *et al*., 2009).

Serotypes A, C, and O are European serotypes and discovered in Europe in 1920's. 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, and SAT3) FMDV (Brooksby1958).

2.4.1. Susceptible species

FMDV infects cloven-hooved mammals (order *Artiodactyla*), as well as a few species in other orders. Livestock susceptible to FMD include cattle, pigs, sheep, and goats. In addition, deer, bison, and elk are also susceptible to the virus. Wild pigs, antelope, African buffalo, Bactrian camels, and giraffe are also all susceptible species. Llamas and alpacas have been infected experimentally, but do not seem to be highly susceptible to natural infection. Other animals like rats, mice, guinea pigs, and armadillos have all been experimentally infected (Musser 2004; OIE 2013).

2.4.2. Carrier state

A carrier is defined as an animal in which there is persistence of FMDV or the viral genome in the pharyngeal region for at least 28 days post-infection (Sutmoller and Casas-Olascoaga, 2002). Generally speaking, a "carrier" is defined in epidemiological terms as an animal that is infected and

can disseminate the infection in the absence of symptoms. However, with FMD, carrier animals may or may not be able to transmit infection (Sutmoller and Casas-Olascoaga, 2002). African buffalo are carrier animals that have been demonstrated to be able to transmit SAT FMDV to other buffalo and potentially to cattle (Sutmoller and Casas-Olascoaga, 2002).

In addition to African buffalo, cattle, sheep, goats, and water buffalo can also become carriers; however, there is not sufficient evidence to suggest a carrier state exists in pigs. In comparison to cattle and buffalo, sheep and goats are carriers for a shorter period of time—typically between 1–5 months (Sutmoller and Casas-Olascoaga, 2002). On the other hand, while most cattle carry FMDV for 6 months or less, there is evidence of cattle carrying FMDV for more than 3 years (Mushayabasa and Tapedzesa, 2015). Persistent infections have also been identified in some experimentally infected wildlife species, including white-tailed deer and kudu (Sutmoller and Casas-Olascoaga, 2002). The epidemiological significance of carrier animals in FMD outbreaks of cattle is uncertain (Tenzin *et al*., 2008).

2.4. 3. Humans as vectors for FMDV

People can act as mechanical vectors for FMDV, by carrying the virus on clothing or skin. The virus might also be carried for a time in the nasal passages, although several studies suggest prolonged carriage is unlikely. In one early study, nasal carriage was reported for up to 28 hours but less than 48 hours after contact with animals. In two recent studies, people did not transmit serotype O viruses to pigs or sheep when personal hygiene and biosecurity protocols were followed, and no virus could be detected in nasal secretions 12 hours after contact with the animals. In another recent study, FMDV nucleic acids (serotypes O or Asia 1) were found in only one person tested 16-22 hours after exposure to infected animals, and live virus could not be isolated from this sample. Because factors such as sub-optimal facility sanitation or poor compliance with personal hygiene and biosecurity protocols could also influence transmission to animals, these studies might not apply directly to the situation in the field (Bauer, 1997; Prempeh *et al*., 2001).

2.4.4. Introduction and transmission of FMDV

FMDV is highly contagious and spreads by direct contact between animals, by animal products (milk, meat and semen), by mechanical transfer on people or fomites and by the airborne route, with the relative importance of each mechanism depending on the particular outbreak characteristics.
FMDV can be found in all secretions and excretions from acutely infected animals, including expired air, saliva, milk, urine, faeces and semen, as well as in the fluid from FMD-associated vesicles, and in amniotic fluid and aborted fetuses in sheep. FMDV is typically introduced via contact with infected animals, their secretions, excretions, or fomites, or products contaminated with FMDV. FMD can also be introduced into a naïve animal population by feeding contaminated meat, milk, or garbage (Musser, 2004; Donaldson and Alexandersen, 2002). Conveyances may be responsible for transmitting the disease between an infected and an uninfected premise (Carolina *et al*., 2016).

The virus can enter the body by inhalation or ingestion, and through skin abrasions and mucous membranes. Cattle are particularly susceptible to aerosolized virus, while pigs require much higher doses to be infected by this route. Sexual transmission could be a significant route of spread for the SAT type viruses in African buffalo populations. In sheep, FMDV has been shown to cross the placenta and infect the fetus (Donaldson and Alexandersen, 2002).

Cattle typically become infected through aerosolized virus (Kitching and Hughes, 2002). Pigs usually become infected by eating virus-contaminated food, or through direct contact with the vesicular lesions of other animals (Kitching and Alexandersen, 2002: Alexandersen *et al*., 2003). Pigs also can excrete large quantities of the virus through respiration, infecting susceptible animals (Alexandersen *et al*., 2003). As such, pigs are considered key amplifiers of the virus (Amass *et al*., 2004).

2.4. 5. Live animals and virus shedding

Pigs produce nearly 3,000-fold more of FMDV in respiratory secretions than either cattle or sheep (Thomson, 1996). The amount of virus excreted by various species will vary based on the serotype

and strain of the FMDV (Donaldson *et al*., 1970). Because cattle are more likely to be infected through inhalation, and pigs shed a significant amount of FMDV via respiration, highly concentrated herds of infected pigs in close proximity to naïve cattle herds poses a significant risk of transmission from pigs to cattle (Donaldson and Alexandersen, 2002).

2.4.6. Air/Windborne transmission

Airborne FMDV can result from a large number of infected pigs, resulting in plumes of aerosolized virus in the atmosphere. Cattle, because they inhale more air and are easily infected through respiration, are the species frequently infected when FMDV via airborne (Alexandersen *et al*., 2003). Under specific climate conditions (particularly downwind), aerosolized FMDV produced by infected pigs can travel a significant distance infecting cattle from 20 kilometers ([km] up to 300 km away and infecting sheep from 10–100 km away) (Donaldson and Alexandersen, 2002: Alexandersen *et al*., 2003).

In addition, the amount of virus emitted into the air will be impacted by the stage of the disease in the infected animal, as well as the number and concentration of virus in infected animals, and the strain of the virus (Donaldson and Alexandersen, 2002).

2.4.7. Fomite transmission

FMDV is readily transmitted through vehicles, equipment, boots, clothing, and other fomites. In an FMD outbreak, the movement of fomites is a critical transmission pathway which must be addressed, particularly because FMDV can persist on fomites for an extended period of time with persistence based on many factors, including decreased temperatures (Cottral, 1969).

2.4.10. Incubation period

The incubation period for FMD is typically 2–14 days (OIE, 2013; OIE, 2016). During the beginning phases in the prevalence of FMDV, the incubation period may be as short as 24 hours (Yang *et al*., 1999). How fast clinical signs appear depends on the strain of virus and the host

susceptibility, the dose of the virus, species of the animal, as well as the route of infection (Kitching and Hughes, 2002; Kitching and Alexandersen, 2002). Animals may shed FMDV before the appearance of clinical signs (Orsel *et al*., 2009). Young ruminants may die without any clinical signs, and yearling cattle may fail to mature properly because of damage to glandular organs such as thymus and thyroid. Abortion is not usually seen (Donaldson, 1987). The course of the clinical disease in sheep rarely exceeds three days in uncomplicated cases, and the infection is frequently subclinical.

2.4.12. Disease duration (to recovery) in individual animals

The time course in individual animals depends largely on the degree of tissue damage caused by the virus. In uncomplicated cases, without secondary infection (usually bacterial) the disease course is considered to be two to three weeks. Convalescence may take over six months and in some cases damage may be permanent where there has been more extensive damage to the gut, feet, heart or hormonal system. Mechanical damage and secondary infection may slow down recovery considerably (Musser, 2004).

Among domesticated animals, deaths usually occur mainly in the young, as the result of multifocal myocarditis (vesicles are not always found in these cases) or starvation (Musser, 2004). In some outbreaks, the mortality in young animals can be very high. Although severe FMD may also cause deaths among older animals, the mortality rate is usually 1-5% among adult livestock after natural infections with most strains (Torres, 2008). High fatality rates have occasionally been reported in some species of wildlife or zoo animals (Thomson *et al*., 2003).

2. 5. Clinical Signs

FMDV is typically characterized by high morbidity, evidenced by characteristic vesicles on the oral and nasal mucosa, teats, coronary bands, and interdigital spaces. However, before vesicles appear, a decreased appetite and progresses fever develops (Musser, 2004). The clinical signs can vary based on the serotype and strain of the FMDV. Generally speaking, sheep and goats typically have milder

clinical signs than cattle (Musser, 2004). Anorexia is caused by painful oral lesions, and lameness is due to painful digits. Death is uncommon except in bovine neonates where cardiac and skeletal muscle necrosis may be evidenced upon necropsy.

In ruminants (example, cattle, sheep, goats), clinical signs include excess salivation and nasal discharge due to the virus infecting epithelial cells in the mouth and nose and causing painful vesicular lesions. Vesicles also appear on the epithelia of coronary bands, inter-digital spaces and teats in lactating animals. After rupturing, vesicles form into painful ulcerous erosions. The clinical disease can cause weight loss and drop in milk production and lameness. After resolution of clinical signs, ruminants can become persistent carriers, which is defined as having virus present (usually detected in their oesophageal-pharyngeal fluid) beyond 28 days post-infection (Zhang and Alexandersen, 2004).

Although pigs are major producers of virus aerosols, cattle produce several magnitudes more of the virus in the epithelium of the tongue, which often sloughs off and is shed during clinical disease, as well as in saliva. The virus is also shed in urine, feces and milk. The 10–30 g of tongue blister epithelium, which a cow with FMD can discharge, may represent not less than one billion infectious units (IU).These enormous quantities of shed virus can contaminate the environment (boots, clothes, tires etc.) and, therefore, cattle are considered the main source of environmental contamination. In cattle the vesicles will rupture in approximately 1 day, and recovery occurs in 8–15 days. The peak of infectivity is just prior to or during the development of lesions. It is then greatly reduced 3–4 days after the lesions develop (Sutmoller *et al*., 2003).

2.5.2. Clinical sign in pigs

In pigs, severe lesions typically are observed on the feet, as well as on the snout, udder, as well as hock and elbow. Excessive salivation is less likely in pigs than in cattle, and lesions in the mouth are milder than those observed elsewhere (Musser, 2004; Kitching, and Alexandersen, 2002).

2.5.3. Clinical sign Sheep and Goats

Fewer clinical signs are seen in sheep and goats. Mouth lesions are often not obvious, though lesions can develop on heel bulbs and on the coronary band (Kitching, and Alexandersen, 2002). Sheep and goats may be important in transmission, as infection presents with mild clinical signs and may not be as immediately recognized (Alexandersen *et al*., 2003: Kitching and Hughes, 2002).

2.6. Significance to Public Health

2.6.1. Zoonosis and food safety

FMD is not considered to be a public health threat. FMDV can be zoonotic, but infections in humans are thought to be very rare (Bauer, 1997). Exposure to extremely large amounts of virus or a predisposing condition may be necessary for infection with this virus. Between 1921 and 1969, more than 40 laboratory-confirmed cases of FMD in humans were published (Bauer, 1997). In three laboratories, 15% to 54% of workers were seropositive, but no clinical cases were reported (Bauer, 1997). One FMD laboratory reported only 2 cases in more than 50 years, and a large FMD vaccine manufacturer documented 3 human cases among its workers (Bauer, 1997).

Infected humans may be subclinically infected or develop vesicular lesions and influenza-like symptoms (Bauer, 1997). If clinical signs are present, the disease is generally mild, short-lived and self-limiting. Broken skin is a recognized route of entry for the virus, with the initial lesions developing at the inoculation site. In 1834, three veterinarians apparently infected themselves by deliberately drinking raw (unpasteurized) milk from infected cattle for three days (Bauer, 1997; Prempeh *et al*., 2001). No cases of FMD have been reported after eating meat from infected animals. Person-to-person transmission has never been reported (Prempeh *et al*., 2001); however, vesicles from affected people do contain virus (Bauer, 1997). Reports of FMD in humans have become even more rare since vaccination reduced the incidence of this disease (Bauer, 1997). The virus type most often isolated in humans with FMD is type O followed by type C and rarely A. The incubation period in humans ranges from 2-6 days. Symptoms displayed have mostly been mild and self-

limiting, mainly uncomfortable tingling blisters on the hands but also fever, sore throat, and blisters developing on the feet, in the mouth, as well as on the tongue (Bauer, 1997).

2.7. Economic Importance

FMD threatens the livelihoods of farmers, large sophisticated farming practices, the national and the international economies of the countries (OIE, 2004). The direct effects of the disease are loss of milk production, loss of draught power, retardation of growth, abortion in pregnant animals, death in calves and lambs, while indirect losses can be attributed to the disruption in trade of animals and derivative products (Paton *et al*., 2005; Bayissa *et al*., 2011). In therefore, FMD causes substantial economic loss to farmers and to the nation from embargoes of livestock and livestock product trade (Megersa *et al.,* 2009) as evidenced by hampering the export of livestock and livestock products to the Middle East and some African country (FAO, 2005). For example, the Egyptian ban of 2003 on Ethiopia livestock alone cause market loss of 14.36 million USD (MoARD, 2007). The disease threated Ethiopia's live animal export trade to Middle East and North African (MENA) countries which accounts for about 140 thousand heads that worth about 23.9 million USD annually (SPS-LMM, 2008).

2.9. Ecology

2.9.1. Geographic distribution

FMD is globally distributed some years ago but now its distribution is limited to South American, Asian, and African countries (Table 1)

Table 1: Serotypes of FMD isolated from certain geographical regions

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

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

FMD is endemic in parts of Asia, Africa, the Middle East and South America. While serotypes O and A are widely distributed, SAT viruses occur mainly in Africa (with periodic incursions into the Middle East) and Asia 1 is currently found only in Asia. North and Central America, New Zealand, Australia, Greenland, Iceland and Western Europe are free of FMDV. Western Europe was affected by some recent outbreaks (eradication was successful), but FMD has not been reported in North America for more than 60 years. The last U.S. outbreak occurred in 1929, while Canada and Mexico have been FMD-free since 1952-1953 (OIE. 2013).Analysis of outbreak data over a number of years has demonstrated the global clustering of FMD viruses and identified 7 virus pools, where multiple serotypes occur but within which are topotypes that remain mostly confined to that pool (Hammond *et al*., 2011).

The World Reference Laboratory for FMD (WRLFMD®) have defined 3 pools covering Europe, the Middle-East and Asia containing serotypes O, A and Asia 1, 3 pools covering Africa containing serotypes O, A, and SATs 1, 2 & 3 and 1 pool covering the Americas containing serotypes O and A. This distribution enables a regional approach to be taken to assist global control of FMD. An increased regional knowledge of FMD outbreaks and identification of these within particular reservoirs or pools of FMD activity can greatly assist globally informed regional FMD control programmes. It also follows that if vaccination is to be a major tool for control, each pool could benefit from investigation into the use of tailored or more specific vaccines relevant to the topotypes present in that pool, rather than a continued reliance on the currently more widely available vaccines. Current trends show that globally the serotype most commonly identified is type O, with more than 80% of isolates characterized by the OIE/FAO FMD reference laboratory network in 2010-2011 being of this serotype (Hammond *et al*., 2012). In 2012 so far WRLFMD have observed that more than 25% of samples tested were found to be type Asia 1 and 14% to be SAT-2 (Hammond *et al*., 2012).

The SAT serotypes have never established outside of Africa, although in 2000, SAT 2 was found in Saudi Arabia and in 2012 in Palestine Autonomous Territories. A total of 902 outbreaks of FMD were reported to the African Union Interafrican Bureau for Animal Resources (AU-IBAR) from 28 countries in 2011 compared to 454 outbreaks from 24 countries in 2010 and 378 outbreaks from 26 countries in 2009. In 2011, a total of 86,185 cases leading to 2804 deaths, with an estimated case

fatality rate of 3.25% were reported from the infected countries. Ethiopia (721), followed by Eritrea (404), Benin (355), Burkina Faso (305) and Uganda (137) reported the highest number of fatalities within the year. With regards to the monthly distribution in Africa, FMD appears to occur throughout the year. However, the level of FMD occurrence showed a sharp increase between April and September, and a decreasing trend between October and April.

During 2011, Mauritius and Madagascar maintained their "FMD freedom without vaccination" status as per the OIE code, whereas Botswana and Namibia maintained "FMD free zones where vaccination is practiced". However, the FMD free zone status of South Africa without vaccination is no longer recognized by the OIE as it was suspended on the 25th February 2011 following outbreaks of FMD in the districts of Jozini and Umhlabuyalingana in KwaZulu-Natal province. It is probably the geographical location of Madagascar and Mauritius as islands that enabled them to establish and maintain their FMD country freedom status. Cattle production systems across Africa largely fall either in the pastoral or sedentary categories. The predominantly sedentary system in Southern Africa has enabled Botswana, Namibia and South Africa to establish OIE recognized free zones. Furthermore, these zones have largely been supported by the erection of fences which are not practical in the predominantly pastoral systems of production practised elsewhere in Africa (OIE, 2012).

North America (the United States, Canada, and Mexico), Central America, Western Europe, Australia, and New Zealand are all free of FMD. From 2013 to 2016, FMD outbreaks have occurred in countries including (but not limited to) Egypt, China, Vietnam, North and South Korea, Russia, South Africa, Iraq, Nepal, Cambodia, Venezuela (OIE. 2016). The last FMD outbreak in the United States was in 1929 (CFSPH 2014).

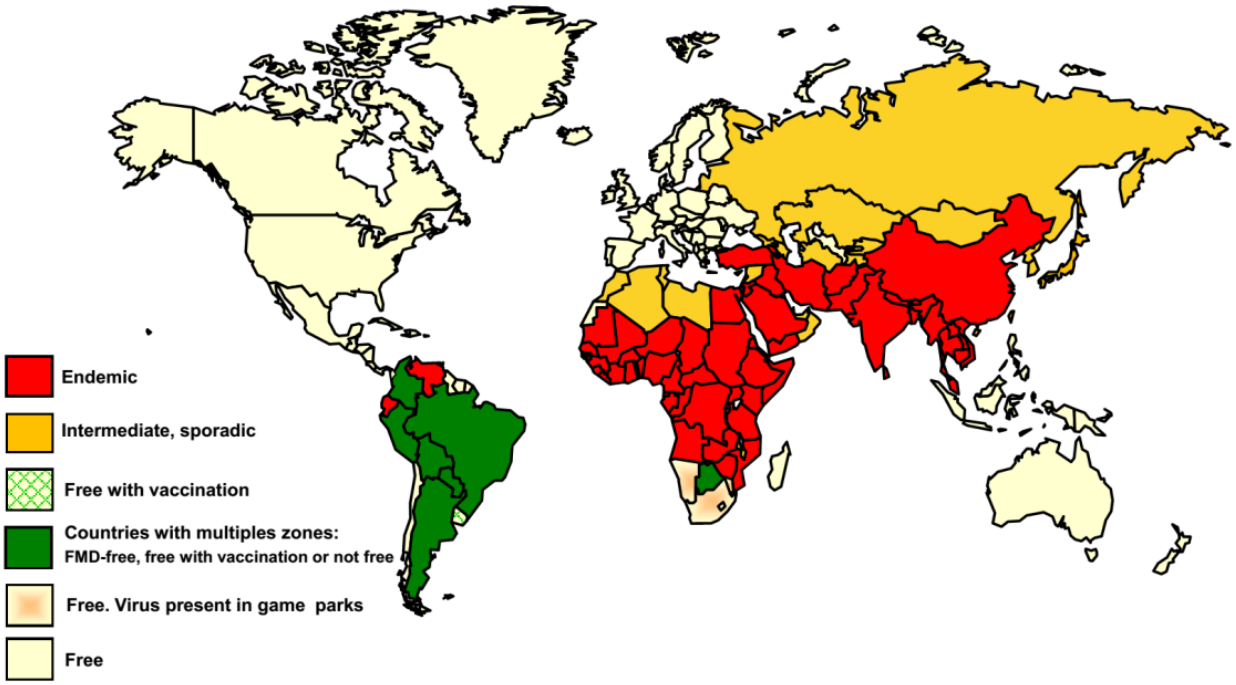


Figure 2: Conjectured status of FMD in 2012 (www.pirbright.ac.uk).

2.11. 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 comparisons.

2.12. Pathogenesis

2.12.1. Sites of primary infection

Several lines of evidence point to the pharyngeal area as the usual primary site of infection (Alexanderson *et al*., 2001). Many of the epithelia of the oral cavity are of the stratified squamous type and are, moreover, cornified (that is, have a layer of dead cells overlying the outer surface. The special epithelial cells in this region of the pharynx appear also to be responsible for supporting persistent infection in carrier animals. Virus may be demonstrated in the pharynx for 1–3 days before a viraemia can be detected (Alexandersen *et al*., 2002b). Viraemia usually lasts for 4–5 days (Alexandersen *et al*., 2002c) and is the means by which virus is distributed to secondary sites.

Under certain circumstances, for example after injection of virus into the skin or tongue or when infection takes place through damaged skin, replication will take place at the site of entry. After this initial replication and spread through regional lymph nodes and into the circulation, a number of secondary sites will be infected, especially the skin (both with and without hair) and the epithelia of the tongue and mouth, that is, cornified epithelia, where the main viral amplification occurs. As, Alexandersen *et al*. (2003) they had found no evidence to suggest that the lung is either a primary or secondary site of viral amplification. Nasal mucosa has also been suggested as a site of initial replication (Korn, 1957), but although nasal fluid contains significant amounts of virus during the prodromal and acute clinical phases, even though Alexandersen *et al*. (2003) had found no evidence to support this hypothesis.

2.12.2. Secondary sites of replication

The earliest sites of FMDV infection and replication in contact-exposed animals appear to be in the pharynx. Viral replication may reach a peak as early as 2-3 days after exposure (Alexandersen *et al*., 2001). After initial replication the virus enters through regional lymph nodes and into the bloodstream. The greater part of the viral amplification occurs subsequently within the cornified stratified squamous epithelia of the skin (including the feet and mammary gland) and mouth

(including the tongue), or in the myocardium of young animals. Secondary phase of replication occurs in lymph nodes. Some evidence from in-vitro studies has indicated that FMDV infectivity may persist in macrophages for 10–24 h (Rigden *et al*., 2002). However, experimental investigations in vivo and more studies suggest that lymph nodes as well as lymphocytes and macrophages (including alveolar macrophages) play little or no part in FMDV replication and that any virus present in lymphoid organs is produced elsewhere, that is, in the pharynx and the epithelia of the mouth and skin.

2.13. Diagnosis of FMD

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained (OIE, 2012).

In acutely infected animals, FMDV, its antigens or nucleic acids can be found in a variety of samples including vesicular fluid, epithelial tissue, nasal and oral secretions, esophageal-pharyngeal fluids, blood and milk, and in tissue samples such as myocardium collected at necropsy. The OIE-recommended samples at this stage are epithelium from unruptured or freshly ruptured vesicles, or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected usually from the tongue, buccal mucosa or feet. Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation (OIE, 2012).

In cases Where epithelial tissue is not available or with no vesicles cases from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, the OIE recommends blood (serum) and esophageal–pharyngeal fluid samples, collected by means of a probang (sputum) cup from ruminants, or as throat swabs from pigs) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Carrier animals can only be identified by collecting esophageal-pharyngeal fluids for virus isolation and/or the detection of nucleic acids. Viral antigens are usually identified with enzyme-linked immunosorbent assays (ELISAs), and nucleic acids by reverse transcription

polymerase chain reaction (RT-PCR). Virus isolation can be performed in primary bovine thyroid cells, primary pig, calf or lamb kidney cells, or BHK-21 or IB-RS-2 cell lines. Nucleotide sequence analysis can identify viral strains (Clavijo *et al*., 2004).

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], and mycostatin [100 IU]). If 0.04 M phosphate buffer is not available, tissue culture medium or phosphate-buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6 because FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection and preservation. Samples should be kept refrigerated or on ice until received by the laboratory (OIE, 2012).

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. Diagnosis is based on clinical sign but, the clinical signs can be confused with other diseases (example, Differential diagnosis for FMD should include vesicular stomatitis, rinderpest, malignant catharal fever, the bovine herpes 1 infections, swine vesicular disease, vesicular exanthema of swine and bluetongue) and therefore laboratory based diagnosis is necessary for confirmation (Blood *et al*., 1994).

Laboratory diagnosis of FMD is carried out using both classical and molecular virological techniques coupled serological investigations. The classical technique uses virus propagation and isolation. The grown viruses were then subjected to molecular and/or serological techniques for identification of serotypes. The serotype of a virus involved in an outbreak cannot be ascertained on the basis of clinical signs. Various techniques have been used to diagnose the disease and to ascertain the serotype of the virus (Blood *et al*., 1994; Quinn *et al*., 2005).

2.13.1. Tests for antigen/virus/genome

The current techniques used for FMD diagnosis are highly sensitive and specific. The type of sample recommended for testing is based on knowledge of the amount of virus present in various tissues, excretions and secretions. For many of the tests, especially the ELISA methods, vesicular epithelium or vesicular fluid is preferred, as these materials contain high titres of FMDV. The more sensitive methods, such as cell culture and RT-PCR, are appropriate for samples likely to contain smaller amounts of infectivity or viral RNA, example,, blood, swabs, milk, probang samples, tissues and faeces (Quinn *et al*., 2005)

Given a satisfactory sample, a positive result for FMD can be obtained in 3–4 h by an antigen ELISA, which also will identify the serotype of the virus present (Ferris *et al*., 1990). However, less satisfactory samples may yield weak, inconclusive or negative results, and small amounts of virus must be amplified in cell cultures. Depending upon the amount of virus present, two 48 h passages of each test inoculum may be required before a final result can be declared. The antigen ELISA detects 70–80% of positive samples (that is, samples subsequently being positive in cell culture), this is probably due to high quality of the samples and the short transport time between the field and the laboratory. However, for samples obtained from sheep, the percentage was lower, probably due to the difficulty of finding adequate amounts of suitable epithelium lesion. Consequently, the specificity of a sample directly yielding a positive ELISA result is high, but a negative sample requires further examination in highly susceptible cell cultures (OIE, 2004).

2.13.2. Antibody detection

The liquid phase blocking ELISA was routinely used for FMD antibody detection in World Reference Laboratory (WRL) in UK (Hamblin *et al*., 1987). The sensitivity of this assay is close to 100% and the specificity ~ 95%. Samples giving inconclusive results are tested by a virus neutralization test (VNT) (Golding *et al*., 1976). At present the VNT is recommended by the OIE as the definitive “gold standard” for the final assessment of such results. The relatively low specificity of the liquid phase ELISA makes the method less than optimal for large-scale screening purposes, as numerous confirmatory VNTs are likely to be required. Alternatively, a solid phase competitive

ELISA (SP-C ELISA) has been developed, validation tests showing high sensitivity and specificity (99.8%) at the chosen "cut-off" (Mackay *et al*., 2001). This method detects all experimentally infected animals (cattle, sheep and pigs) at 5–8 days after infection and for several months thereafter.

2.13.3. Virus isolation

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 rpm for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures or into un weaned mice. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IB-RS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The sensitivity of any cells used should be tested with standard preparations of FMDV. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease virus (SVDV) from FMDV (as SVDV will only grow in cells of porcine origin) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. Un weaned mice are an alternative to cell cultures and should be 2–7 days old and of selected inbred strains. Some field viruses may require several passages before they become adapted to mice (Skinner, 1960). In the case of OP fluids, pre-treatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes (OIE 2012).

2.13.4. Enzyme linked immunosorbent assay (ELISA)

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Roeder and Le, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with rabbit antisera to each of the seven serotypes of FMDV. These are the 'capture' sera. Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMDV are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results are usually read by spectrophotometrically at an appropriate wavelength (Alonso *et al*., 1993).

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVDV or vesicular stomatitis virus (VSV). Ideally a complete differential diagnosis should be undertaken in all vesicular conditions. Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMDV (plus SVDV or VSV if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6. Guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (OIE, 2012).

2.13.5. Nucleic acid recognition method

RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and OP samples. RT combined with real-time PCR or with convectional PCR has sensitivity comparable to that of virus isolation and automated procedures enhance sample throughput (Reid *et al*., 2003). Serotyping primers have also been developed (Vangrysperre and De, 1996). Simplified RT-PCR systems for potential field-use are under development (Callahan *et al*., 2002).

The reverse transcription-polymerase chain reaction (RT-PCR); has been shown to be a useful tool for the diagnosis of FMD as it offers the advantages of fast, sensitive and reliable diagnosis. A variety of RT-PCR methods have been reported in recent years for the early detection of FMDV RNA in epithelium, cell culture isolates and other tissues using universal primers for all seven serotypes (Meyer *et al*., 1991).

Typing of FMDV by RT-PCR was first demonstrated by Rodriguez *et al*. (Rodriguez *et al*., 1992) for the differentiation of the serotypes O, A and C. Serotype specific primers have since been designed for the detection of all seven FMDV serotypes by RT-PCR (Vangrysperre and De, 1996; Callens, 1997). Primers designed for these assays target various regions of the virus genome, including the 5′ UTR, the open reading frame and the 3′ UTR. In order to improve the diagnostic sensitivity of RT-PCR, multiplex assays, incorporating more than one set of primers have been developed (Giridharan, *et al*., 2005). Thus the conventional RT-PCR is not sufficiently sensitive and specific to replace methods using virus propagation in cell culture and ELISA. Recently, real time/quantitative RT-PCR (rRT-PCR) methods have been developed which do not require post-PCR processing (example, gel analysis). Other advantages of the rRT-PCR include high throughput capability and the ability to quantify the genetic material in the starting sample. A TaqMan assay is very robust and effective for primary detection of FMDV as virus isolation in conjunction with antigen ELISA (Reid *et al*., 2003). Currently, two different rRT-PCR TaqMan assays are in common use, one targeting the internal ribosomal entry site (IRES) within the 5′ UTR (Reid *et al*.,2002) and the second targeting the 3D (RNA polymerase) coding sequence (Callahan *et al*., 2002).

The speed and accuracy of detection of the rRT-PCR assay was further improved by coupling the assays with robotic methods for extraction of nucleic acid from the samples and for set up of the assays. This has made the assay highly suitable for the diagnosis of the primary index case and for use in an on-going outbreak. The rRTPCR assays are currently used as a routine test for FMD diagnosis and quantification of the virus in many developed countries (King *et al*., 2006). No single standalone assay is capable of detecting FMDV with 100% sensitivity. Recently, Tam and colleagues (Tam *et al*., 2009) reported fluorescence-based multiplex rRT-PCR assays for the detection of FMDV and virus typing. The assay was found to have greater sensitivity for detection

but some cross reactivity between some serotypes was also noted. Further work is in progress to develop rRT-PCR assays for serotyping of the virus.

2.13.6. Serological test

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination (Reid *et al*., 2002).

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs). The virus neutralisation test (VNT) is used to detect as the virus or antigen used in the test is closely matched or not, to the strain circulating in the field (Golding *et al*., 1976). The solid-phase competition ELISA and the liquid-phase blocking ELISA are serotype-specific and are highly sensitive (Mackay *et al*., 2001; Paiba *et al*., 2004). An approach combining screening by ELISA and confirming the positives by the VNT minimises the occurrence of false-positive results.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, and whether the animal has not been vaccinated. Therefore the tests can be used to confirm suspected cases of FMD and to detect viral activity or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti-NSP tests, causing false-negative results. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (Mackay *et al*., 1997). Antibody to 3ABC (non-structural protein) is found only in virus infected animals but not in vaccinated animals (Ding, 2013).

2.14. Differentiation between Infected and Vaccinated Animals (DIVA)

Detection of animals that have been infected with FMDV is important for the control of FMD especially in a previously FMD free country or in a country with sporadic outbreaks. Both previously infected and vaccinated animals can have neutralizing antibodies in their sera, but it is important for trade purposes to be able to distinguish previously infected animals from those that have just been vaccinated against the disease. This is because a high proportion (up to 50%) of animals infected with FMDV can become "carriers", these are defined as animals which continue to have infectious virus present within the oropharynx more than 28 days post-infection. The animals are clinically normal and can maintain this state for a long period (2–3 years in cattle). It is possible that such animals can act as a source of infection for other animals, viral replication during infection results in the production of both structural (SP) and nonstructural (NSP) proteins. Like the SPs, some NSPs are immunogenic (Tesar *et al*., 1989). Vaccines consisting of purified preparations of inactivated 146S virions induce antibodies almost exclusively against the SP of the virus. Thus, it can be possible to discriminate between infected and vaccinated animals based on the detection of antibodies to NSPs.

2.15. Characterization of FMDV below the Level of Serotype (strains/subtypes)

Within each serotype of FMDV, there is a spectrum of variants with their own antigenic and biological characteristics. In some cases there is poor cross-protection between variants within a serotype and thus characterization of sub-serotypes/strains becomes necessary to ensure selection of appropriate vaccines to control an outbreak. Subtype variants were distinguished by the fact that immunization against one subtype did not confer the same level of immunity to another variant of the same serotype as to the homologous strain. Animals vaccinated with one strain withstood homologous challenge but were only partially protected against challenge with a heterologous strain. Antigenic characterization was used to compare field viruses with vaccine strains by determination of the serological relationship (r^1 value) using hyper immune sera in liquid phase ELISA (Kitching *et al*., 1988) or in VNTs using cell culture (Rweyemamu, 1984).

Using VNTs, r_1 values of ≥ 0.3 have been shown to reflect a close antigenic relationship between the field isolates and vaccine strains, indicative of good protection by the vaccine, whereas values < 0.3 reflect a more distant antigenic relationship, indicating that the vaccine is unlikely to protect against the field isolates. Antigenic characterization using serological tests like VNT and ELISA, using defined sera/MAbs, are useful in showing antigenic diversity but they are unable to characterize strains individually and cannot be used to trace the origin of an outbreak (Rweyemamu, 1984).

Nucleotide sequence analysis has now become the definitive technique for characterization of FMDV strains. The first phylogenetic analysis of FMDV using nucleotide coding sequences for VP1 was reported by Dopazo *et al*. (1988). Since then a number of studies have been published on nucleotide sequence analysis for all the seven serotypes of FMDV (example, Ayelet *et al*., 2009; Jamal *et al*., 2011; Bari *et al*., 2014; Mishamo, 2016), some of these have used complete genome sequences for tracing of outbreaks (Valdazo *et al*., 2011).

The serotypes of FMDV have on average 86% nucleotide sequence identity to each other across the whole genome (Fry *et al*., 2005) but the VP1 coding region is substantially more variable and shows only about 50-70% identity (Knowles and Samuel, 2003). Serotypes O, A, C and Asia-1 have been further classified into genotypes based on up to 15% difference in VP1 coding sequences. Thus, FMD serotype O viruses from around the world have been classified into eight genetically and geographically distinct genotypes, called topotypes, on the basis of the VP1 coding sequence (Samuel and Knowles, 2001). FMDV serotype Asia-1 is considered to be genetically and antigenically the least diverse serotype (Knowles and Samuel, 2003).

2.16. Disease Treatment

There is no specific treatment once disease has become established, other than supportive therapy, and antibiotics to prevent secondary infections. Supportive care is recommended in endemic areas where there is no slaughter policy and may greatly reduce the development of secondary infections. In areas of the world where slaughter of animals with clinical signs of foot-and-mouth disease is not mandatory, and in the case of infection in very important animals where an exception to an

automatic slaughter policy might be given, nursing and supportive care are important for the welfare of the individual animal and to minimise the time for which it is clinically affected. However, with severe disease and particularly if there are severe secondary infections and complications, culling may be preferable on the grounds of the welfare of the animal and also the prevention of additional losses to the farmer due to animals which are "poor doers"(Murphy *et al*., 1999).

Various novel approaches for treatment and prevention of FMD and other viral infections have been proposed. Inhibition of FMD virus replication and reduced production of escape mutants can be demonstrated in vivo using, for example, small interfering RNA targeting the conserved regions of viral genome or lethal mutagenesis using pre-extinction viral RNA (Gonzalez-Lopez *et al*., 2004). Other experimental options include mutagenic antiviral drugs such as ribavirin, or targeting drugs at viral components such as the highly conserved 3C protease of FMDV, which is required to cleave the precursor virus into functional proteins (Murphy *et al*., 1999).

2.17. Management Techniques Available

2.17.1. Environmental control measures

Cleaning and disinfection of infected premises is an important part of foot-and-mouth disease control. Essential points to remember for effective disinfection are thorough cleaning is required as the presence of organic matter/dirt may stop the applied disinfectant being effective, Disinfectants effective against FMD virus generally rely for their action on being acids or alkalis. These must not be mixed, Prolonged times may be required for the action of disinfectants on some substances (example, slurry), Small amounts of detergent (example, 1 ml detergent per litre of disinfectant solution) may be added to disinfectants to increase effectiveness, and It is important to use effective (approved) disinfectants and to use these at their effective (approved) concentrations (OIE, 2013).

2.17.2. Population control measures

Population control measures aim to prevent the introduction of foot-and-mouth disease (FMD) into FMD-free areas and to limit the spread of the disease where it is endemic or following introduction. Culling operations should be designed to minimise the spread of disease while avoiding unnecessary slaughter. Because of the highly contagious nature of foot-and-mouth-disease virus (FMDV), and because animals are known to release large amounts of virus into the environment before starting to show clinical signs (up to five days before for cattle and sheep, as long as ten days for pigs), it is usual in FMD-free countries to cull not only those animals with clinical disease (lesions) but also all "dangerous contact animals" such as: All other animals on the same farm, All other animals which have been in contact at example, a market, and Other animals considered likely to have been infected by indirect contact (example, transported in same lorry immediately after it has been used for infected animals, or tended by stockman immediately after tending infected animals) .This is because there is a high chance that these animals will already be infected and may already be producing virus and releasing it into the environment. This policy, the slaughter and burial or burning of all affected and in-contact susceptible livestock (dangerous contact animals) on an affected premise is also known as "stamping out". Effective rapid disposal of carcasses following culling is recognised to be an essential part of effective FMD control (OIE, 2013

2.17.3. Isolation and quarantine

The aim of Isolation and Quarantine is to prevent susceptible animals from coming into contact with the virus. As FMD is a particularly virulent and infectious virus, strict physical separation of susceptible animals from animals, animal products, vehicles, clothes etc. must be enforced. The FMD virus can be transferred through all of these means. Great care must be taken not to introduce the FMD virus through the movements of people and vehicles. Isolation and quarantine cannot prevent infection if animals are exposed to airborne virus. Quarantine of an individual animal or group of animals would frequently involve testing for disease (screening) to reduce any possible risk of introducing the virus to a previously unexposed population (OIE, 2013).

2.18. Progressive Control Pathway for FMDV

Determination of the factors responsible for maintenance and spread of the disease and knowledge about circulating subtypes of FMDV are essential for effective control of the disease. In order to monitor FMDV circulation effectively, each participating country should, have sufficient FMD diagnostic and surveillance capacity. Countries where the disease is endemic with no reliable information on the disease status are classified as in stage 0. In order to move from stage 0 to 1, a comprehensive study on the epidemiology of FMD is required to be planned (FAO, 2011).

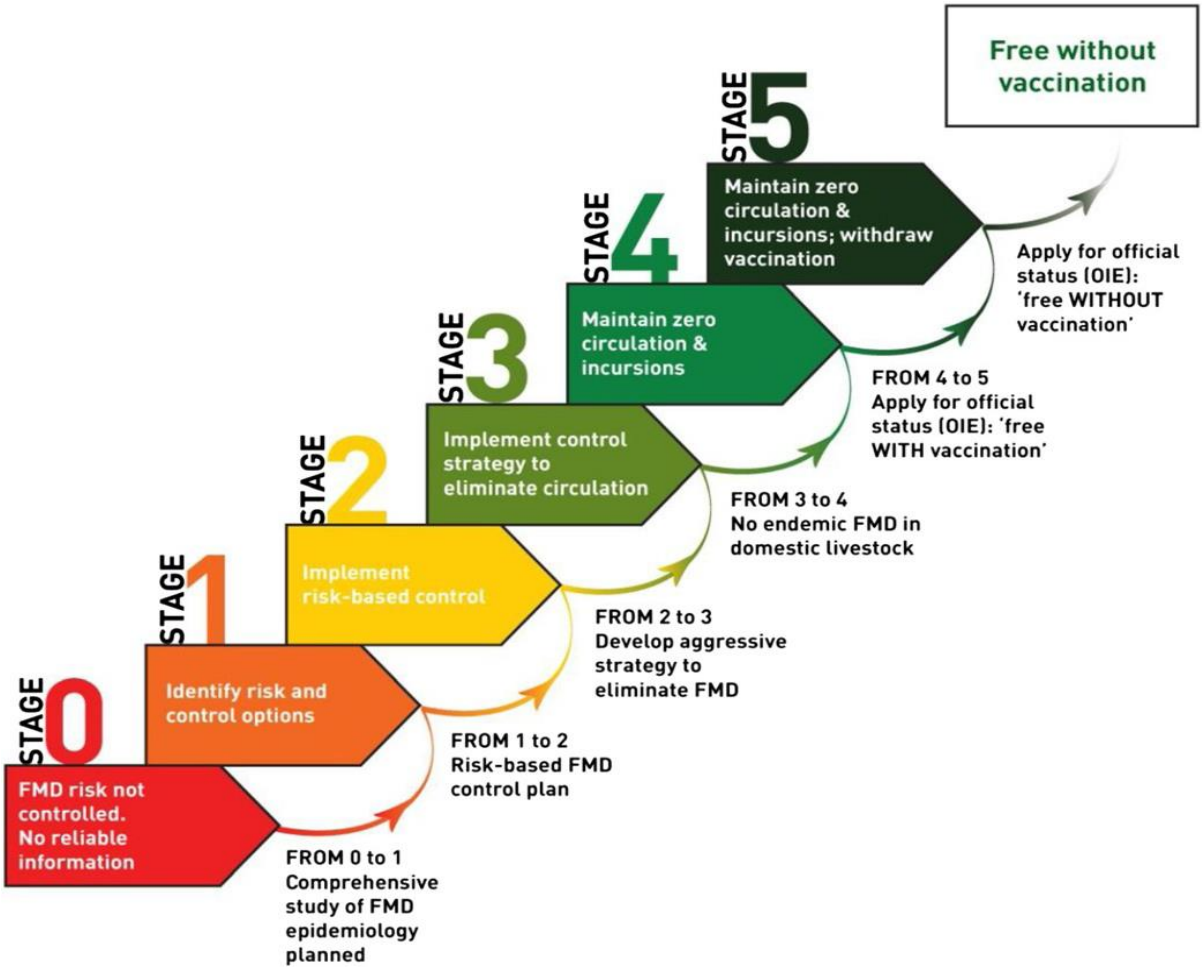


Figure 3: The FAO/EuFMD/OIE Progressive Control Pathway for FMD. The status of countries on the PCP-FMD is evaluated according to defined criteria. Countries with endemic disease are in stages 0 to 3 while countries with no endemic disease within livestock are at stage 4 or above.

Source: FAO, (2011)

Vaccination plays a vital role in controlling FMD for countries in stages 2–4. Normally vaccine quality control is determined by the producer and vaccine batches are only released when they pass the quality control parameters. However, lack of maintenance of the cold chain before, during or after transport/importation may reduce the vaccine efficacy, even if the vaccine initially contained sufficient payload of serotype(s), matching with the circulating strain(s). Furthermore, as the dose–response relationship in FMD vaccination is influenced by the serotype and type of adjuvant present in the vaccine and the quality of the adjuvant in formulated vaccine need to be established. An independent quality control of the formulated vaccine is therefore necessary for effective control of the disease (Jamal *et al*., 2008).

Vaccination alone may not be able to contain the disease unless it is coupled with restrictions on animal movement (biosecurity measure in animal movement). Control of animal movements is, however, complicated by many factors including social customs, religious festivals, and trade of animals in live animal markets and both formal and informal animal movement. Progression from stage 4 to 5, and from Stage 5 to Pathway completion, would be through the existing official OIE recognition processes of freedom from FMD with or without vaccination, respectively. In order to target resources available for surveillance and control of the disease, each stage of the PCP requires risk assessment and risk management activities (Jamal *et al*., 2012).

2.18.1. Vaccines of FMD

Nearly all fully licensed, commercially produced FMD vaccines are inactivated (killed) vaccines containing chemically inactivated virus. Similar vaccines have been manufactured since the 1950s, and have been used successfully in a number of control or eradication programs. Both monovalent and multivalent FMD vaccines are produced (Clavijo *et al*., 2004).

Conventional live attenuated vaccines are unacceptable for FMD. When attempts were made to produce such vaccines in nonsusceptible hosts, the attenuated viruses tended to revert and become virulent. In addition, infections of susceptible animals would not be recognized in vaccinated ones, and there would be a risk of shedding the vaccine virus and further spreading the disease (Margarita *et al*., 2002).

2.18.2. Types of FMD vaccine

Vaccines of FMDV are; Inactivated FMD vaccines and Live attenuated vaccines. Conventional live FMD vaccines are not acceptable due to the danger of reversion to virulence (OIE, 2013).

2.18.3. Vaccine matching

The serological relationship between a field isolate and a vaccine virus (r1 value) can be determined by VNT or ELISA (Kitching *et al.,* 1988; Mattion *et al.,* 2009; Pereira, 1977; Bari *et al*., 2014). 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. So far, VNT using chequer-board titration method gave more accurate results. *In-vitro* neutralisation may be more relevant to predict *in-vivo* protection by the vaccine than other measures of virus-antibody interaction. VNT is the method of choice compared with the ELISA, which can be used only as a screening method for vaccine matching, but according to recent comparative evaluation by Tesfaalem *et al* (2013/4), there is no significant difference between liquid phase blocking ELISA and VNT for vaccine matching. For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after inoculation. The titre of antibody to the vaccine strain is established for each serum. Sera are used either individually or pooled, after excluding low responders i.e sera with low titre (Mattion *et al.,* 2009).

Selection of field virus for vaccine matching; for vaccine matching, preferably, more than one representative isolate should be evaluated from any outbreak. Viruses should be selected based on epidemiological information, for instance isolation at different stages of an outbreak, from different geographical locations, or from different hosts. Field evidence for a suspected lack of vaccine efficacy, as shown by reduced apparent protection, is an important criterion for vaccine matching (Alonso *et al*., 1987).

Selection of vaccine strains to be matched; the serotype of the virus, the region of origin and any information on the characteristics of the field isolate and, as appropriate, the vaccine strain used in the region of origin, may give indications as to the vaccine strains to be selected for vaccine

matching tests. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. Antigenic characterisation has two purposes; first, to choose the most effective vaccine strain for use in a particular circumstance and, second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves (OIE 2012).

Relationship between field isolate and vaccine strains; the recommended standard test is the VNT. The ELISA can be used as a screening method (OIE, 2012).

Vaccine matching by virus neutralization test; this test uses antiserum raised against a vaccine strain. The titres of this serum against 100 TCID50 of the homologous vaccine strain and the same dose of a field isolate are compared to determine how antigenically "similar" the field virus is to the vaccine strain (OIE, 2012).

Biological reagents required are monovalent 21–30 day post-vaccination bovine sera (inactivated at 56°C for 45–60 minutes), the homologous vaccine strain, and the test virus which is a field isolate of the same serotype as the vaccine strain. First field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log10 TCID50/ml) by end-point titration. Then, for each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed (OIE, 2012). Lastly, antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman–Kärber method (Dougherty, 1964). The titre of the vaccine serum against 100 TCID50 of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an "r" value as:

$$r = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithemetic titer of reference sera against vaccine virus}}$$

Interpretation of the results: It is generally accepted that in the case of neutralisation, 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 (Rweyemamu, 1984).

Tests should always be repeated more than once. 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 (Rweyemamu and Hingley, 1984).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in areas where outbreak of FMD occurred in centeral parts of Oromia from September 2016 to March 2017 to isolate and characterize FMDV serotypes responsible for the outbreak. Outbreaks samples have been collected from Adama and its sorruondings, Bishoftu, Addis Ababa and Addis Ababa areas.

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 ºC, 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 (CSA, 2009). The city is fully surrounded by Oromia Regional Government in all directions (CSA, 2009).

The second study area was Bishoftu, which is located at 9°N and 40° East, 47 km Southeast of Addis Ababa. Bishoftu is the center of Ada'a Liban district (Woreda), and the district has a total land area of about 1610.56 km2 and is divided into three agro-ecological zones, namely, mid land (94%), high land (3%), and low land (3%) (Oromia Regional Government Bureau, 2006). The altitude is about 1850 m.a.s.l. and it is where many commercial dairy farms are located. Bishoftu experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and short rainy season from March to May with an average annual rain fall of 800 mm. The mean annual minimum and maximum temperatures are 12 and 27°C, respectively with an overall average of 18.7°C. Highest temperatures are reached in May and the mean relative humidity is 61.3% (NMSA, 2003).

The third study area was Adama and its surroundings which is located in East Shoa zone of Oromia regional state of Ethiopia. Absolute location of East Shewa 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 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 4307 meter 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).

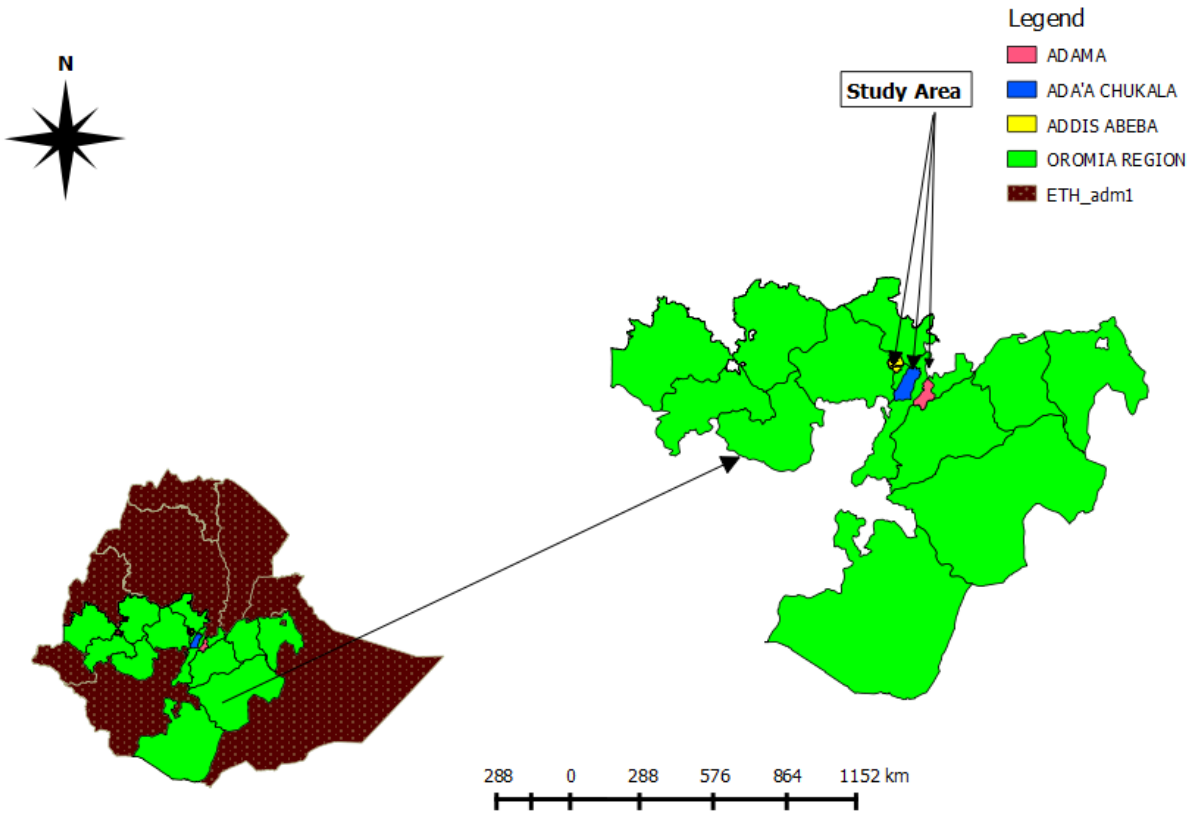


Figure 4: Map of Ethiopia showing the study areas

Source: GIS

3.2. Study Animal population

The study was conducted in cattle that have experienced outbreaks of FMD in the selected sites. Cattle of all age, sex and breed which were reared under intensive, semi-intensive and extensive

production and management system were included in the outbreak investigation in different parts of central Oromia.

3.3. Study design

At the beginning of an investigation proper information channel to Veterinary professionals, District Veterinary officers, and Regional laboratories were organized. All these were informed to report FMD outbreaks to the NVI and to me. When an outbreak was reported, a field investigation was conducted at specific site of an outbreak. FMDV clinical sign manifesting cattle were physically examined for presence of FMD lesions on the mouth and feet, teat and udder for specimen's collection for diagnostic purpose. Therefore, based on the occurrence of FMD outbreak report and active outbreak finding, a cross sectional study design was employed to collect tissue samples. Breed, age, sex, type of farm, and vaccinated or none vaccinated history of sampled animals were considered. Sampled animals were grouped as three age categories based on their dental eruption status (ANNEX 1).

3.4. Ethical Consideration

Ethical clearance for this study was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Minutes of Animal Research Ethics and Review committee. After the committee evaluated the significance of this research, approval was given (minutes number VM/ERC/27/06/09/2017) (ANNEX 17). Before sampling all animals included in this study, consent was asked from the owners and only animals from volunteer owners were sampled.

3.5. Sampling Technique

Using report base, purposive sampling strategy, outbreak investigation and active disease search were conducted. The target areas (Central Oromia) were purposively selected and identified based

on transport accessibility, geographical location and on the abundance of dairy farms that constituted the known milk sheds. Clinically diseased animals were sampled for isolation and characterization of FMD virus serotypes. During FMD outbreak, a field investigation were conducted purposively at the specific site of the outbreak, within the study districts and animals with the clear signs, symptoms and suspected to be diseased with FMDV were sampled.

3.6. Methodology

3.6.1. Field observation

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

3.6.2. Clinical examination

After arriving at the specific outbreak sites during disease outbreak investigation, Cattle were carefully examined for presence of characteristic clinical signs of FMD (ANNEX 2). In each outbreak, cattle manifesting the characteristic signs of FMD like salivation and lameness in each individual animal owner's farm (small holding) areas were examined from distance. The hooves of lame animals were thoroughly washed with water and then carefully examined for similar lesions particularly on the coronary bands and inter-digital spaces. Salivating and/or limping animals were restrained for thorough examination and sampling. The mouth cavities of salivating animals were opened and examined for evidence of intact and/or ruptured vesicles, erosions and ulcers on the tongue, dental pad and mucosa of the oral cavity. Other cattle in the herd with slight of these signs and history of animals from the owner were taken and examined.

3.6.3. Sample Collection

During September 2016 to March 2017, at least 1gram of 20 tissue samples were collected from the legs (11 samples), gum and tongue (9 samples) of cattle in outbreak case from different parts of study area. Tissue were collected from un-ruptured or freshly ruptured vesicles and placed in a bottle with transport medium composed of equal amount of glycerol and 0.04M phosphate buffer with antibiotics (OIE, 2014). After samples were taken, lesion parts were washed with salt solution. Samples of oropharyngeal fluid (OP fluid) (21 samples) were collected from affected and cured cattle that were found in close proximity to the affected and health herd.

The OP fluid were collected by a probang cup and poured into a 20 ml bottle. The fluid was then examined and about 2 ml of it containing cellular material was added to 20 ml tube containing about 2ml of transport medium (OIE, 2004). Then, both tissue in 0.04 M phosphate buffer (pH 7.2-7.6) with 50% glycerol and OP fluid samples were labeled and transported at 4°C from the collection site in cold chain to NVI, Bishoftu as per international recommendation (OIE, 2008) and stored at –20°C until tested (Kitching *et al*., 1988). 10 samples which were tested and being positive at the NVI were submitted to the International atomic energy laboratory in Berlin Germany for generation of sequence data.

Table 2: List of pathological samples collected in the present study from clinically diseased cattle suspected of FMD virus infection in the outbreak areas.

No	Country sample code	Type of sample	Area sample collection	Date of collection	Species animal
31	O/ETH-31/16-1	tongue epithelium	Bishoftu	08/11/16	Bovine
32	O /ETH-29/16	foot epithelium	Mulo	11/11/16	Bovine
33	ETH-33/16-05	foot epithelium	MuloTiro	12/11/16	Bovine
34	ETH-34/16-07	Probang	Alaltu	10/12/16	Bovine
35	ETH-35/16-010	Probang	Sheno	19/12/16	Bovine
36	O/ETH-36/16-012	foot epithelium	Asela	12/12/16	Bovine
37	O/ ETH-23/16	foot epithelium	Adama	28/10/16	Bovine
38	ETH-38/16-014	foot epithelium	Dun F	30/10/16	Bovine
39	O /ETH-19/16	foot epithelium	Bishoftu	20/12/16	Bovine

40	ETH-40/16-55	tongue epithelium	Gelan	17/12/16	Bovine
41	O /ETH-21/16	tongue epithelium	Sandafa	04/01/17	Bovine
42	ETH-42/16-65	Probang	Sendafa	19/12/16	Bovine
43	ETH-43/16-29	Probing	Mogoro	22/10/16	Bovine
44	ETH-44/16-66	Probing	Sululta	27/12/16	Bovine
45	ETH-45/1667	Probing	Mukaturi	13/12/16	Bovine
46	ETH-46/16-74	Tongue epithelium	Onoda	09/12/16	Bovine
47	O/ETH-47/16	tongue epithelium	Bishoftu	3/11/16	Bovine
48	O/ETH-48/16	tongue epithelium	Bishoftu	5/11/16	Bovine
49	O/ETH-48/16	tongue epithelium	Bishoftu	30/12/16	Bovine
50	O/ETH-39/16	tongue epithelium	Bishoftu	04/01/17	Bovine
51	ETH-49/16	Probang	sheno	18/12/16	Bovine
52	O/ETH-71/17	Foot epithelium	Adama	10/1/17	Bovine
53	O/ETH-70/17	foot epithelium	Adama	12/1/17	Bovine
54	ETH-50/16	Probang	Onoda	11/12/16	Bovine
55	ETH-51/16	Probang	Onoda	5/10/16	Bovine
56	ETH-52/16	Probang	Onoda	6/10/16	Bovine
57	ETH-53/16	Probang	sheno	23/12/16	Bovine
58	ETH-54/16	Probang	sheno	24/12/16	Bovine
56	ETH-55/16	Probang	sheno	1/12/16	Bovine
60	ETH-56/16	Probang	sheno	2/12/16	Bovine
61	ETH-57/16	Foot epithelium	Mogoro	25/10/16	Bovine
62	ETH-58/16	Probang	Mogoro	23/10/16	Bovine
63	ETH-59/16	Probang	Mogoro	24/10/16	Bovine
64	ETH-60/16	Probang	Sululta	28/12/16	Bovine
65	ETH-61/16	Probang	Mulo (Dunburi	28/10/16	Bovine
66	ETH-62/16	Foot epithelium	Mulo (Dunburi	29/10/16	Bovine
67	ETH-62/16	Probang	Mulo (Dunburi	27/10/16	Bovine
68	ETH-62/16	Probang	Mulo (Dunburi	1/10/16	Bovine
69	ETH-63/16	foot epithelium	Mulo (Tiiro)	14/11/16	Bovine
70	O/ETH-64/16	Probang	Asala		Bovine
71	ETH-65/16	Probang	Sheno	3/12/16	Bovine
72	ETH-66/16	Tongue epithelium	Mulo (Tiro)	13/11/16	Bovine

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

BHK-21 cell line were subcultured to required confluent monolayer and used to inoculate FMDV. The old medium overlaying the cell monolayer was poured off in a sterile beaker under sterile conditions under class II cabinet. The confluent monolayer cell was washed twice with PBS and cover the whole cell surface with trypsin/versin immediately decant 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 pipetted for complete media mixing and finally dispensed in to another new sterile tissue culture flasks.

3.6.5. Testing strategy

Virus isolation; A 10% epithelial suspension (w/v) was prepared by grinding the tissue sample (ANNEX 3) with sterile pestle and mortal with small volume tissue culture medium containing antibiotics (penicillin, streptomycin and Amphotericin B solution) (OIE, 2014). The suspension was centrifuged at 3500 rpm for 10 minutes and filtered by Millipore filter membrane of 0.22µm pore size. All procedures were conducted under the Biosafety cabinet level 2. About 0.5 ml of filtered tissue suspension was inoculated (ANNEX 5) on confluent cultured Baby hamster kidney (BHK-21) (ANNEX 4) monolayer cells grown on 25cm^2 tissue culture flasks and incubated at 37°C for 1 hour for adsorption of the virus then hanks virus media (10 ml) was added and incubated at 37°C with 5% CO_2 in a humidified incubator for 24-48 hour. The appearance of virus induced cytopathic effect (CPE) was observed daily under the inverted microscope. The inoculated cell line was harvested when 85 - 100% of CPE was observed. Whenever no CPE was detected, the cells were frozen and thawed, and used to inoculate fresh cultures and examined for CPE before the samples were declared to be negative (OIE, 2014). Samples not exhibiting CPE by 72 hours post infection on the third passage were considered virus negative.

3.6.6. Polymerase chain reaction (PCR) assay

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 (ANNEX 6). 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 spine centrifuge for three minutes. Next, to centrifuged, 350µl of 70% ethanol was added and mixed by vortexing as before. 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 centrifuged at 13400 rpm with 500µl RPE buffer subsequently. After the flow through was discarded the RNAase spine column was centrifuged at 13,400 rpm speed for 2 min to dry the membrane. Then, the RNAase mini spin column was transferred to new clean and labeled eppendorf tube. RNA free water (elution buffer, 50µl) was added and then centrifuged at 13400 rpm for 1 minute. Finally, the RNA was elute with RNA free water in to eppendorf tube and stored at -20^{o}c until used.

Complementary DNA Synthesis (cDNA); the complementary DNA was synthesised (ANNEX 7) 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°C for 3 minutes (PCR machine) and chilled on ice for 3 minutes. Then 10x RT buffer, 25mM MgCL2, 0.1M DTT and RNase out were added with the rate of 2µl, 4µl, 2µl and 1µl, respectively and incubated at 42°C for 2 minutes. Finally, 1.5µl Superscript III (Reverse transcriptase) was added and incubated at 42°C for 50 minutes, followed by heating at 85°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°C until needed.

3.6.7. Polymerase Chain Reaction (PCR) and Primers Used

Specific primers were synthesized by VBC Biotech (Vienna, Austria) and purified by reverse phase high-performance liquid chromatography. At NVI, the samples were screened by universal primer set FMDV7- forward (FMDV7F) and FMDV7- reverse (FMDV7R) (Table: 2) for the Reverse Transcription Polymerase Chain Reaction (RT-PCR) of FMDV and for typing of the suspected serotypes of FMDV. The primers and thermal profiles used for amplification of the 5′ UTR to screen the FMDV were shown in bellow table 3.

Table 2: Universal primers and thermal cycle used consecutively

	Primer-FMDV7 universal-Forward 5pm/ µl 5′- GCCTGGTCTTTCCAGGTCT-3′
	Primer-FMDV7universal-Reverse-5pm/ µl 5′-CCAGTCCCCTTCTCAGATC-3′

	Temperature	**Time**	**Cycle**
Initial Denaturation	95^{o}c	5 mints	1-Cycle
1st Denaturation	94^{o}c	1mint	30 cycles
Annealing	54^{o}c	1mint	
Elongation	72^{o}c	1mint	
Final Elongation	72^{o}c	10mints	1-Cycle

After screening; positive FMD samples were amplified using a specific primer for serotypes O, A, SAT2 and SAT1 viruses (Table; 3). The thermal cycling conditions for serotypes O, A, SAT2 and SAT1 were indicated in tables 4 respectively. The amplified PCR products were visualized on gel electrophoresis using 1.5% Agarose gel.

Table 3: Location and sequences of oligonucleotide primers used for reverse transcription–PCR and sequencing for the serotypes O, A, SAT2 and SAT1.

Oligonucleotide primers	Sequence (5′→3′)	Sense	Location	Serotype	Method used	bp
VMDVOF	CAGGCGCCACTATCTTCTGT	+	VP3	O	RT-PCR and sequencing	591
FMDVOR	TACCAAATTACACACGGGAA	-	2B	O	RT-PCR	
1C562F	TACCAAATTACACACGGGAA	+	VP3	A	RT-PCR	866
EUR2BS2R	GACATGTCCTCCTGCATCTG	-	2B	A	RT-PCR	
1C445	TGGGACAMGGIYTGAACTC	+	VP3	SAT2		1145
2B208R5	ACAGCGGCCATGCACGACAG	-	2B	SAT2	RT-PCR	
1C559Fow	CAAGACCGTGGACAACAAGA		VP3	SAT1	RT-PCR	506
2B208R	GCGTGGTCTTGTACCTGTCA		2B	SAT1	RT-PCR	

Table 4: Thermal cycling profiles for the serotypes O, A, SAT2 and SAT1 viruses were shown in bellow tables consecutively.

Thermal cycling profiles for O

	Temperature	Time	Cycle
Initial Denaturation	95^{o}C	5 mints	1-Cycle
Denaturation	95^{o}C	1 mint	30 Cycles
Annealing	58^{o}C	1 mint	
Extension	72^{o}C	1:30mints	
Final Elongation	72^{o}C	7 mints	1-Cycle

Thermal cycling profiles for A

	Temperature	**Time**	**Cycle**
Initial Denaturation	95^{o}C	5 mints	1-Cycle
Denaturation	95^{o}C	1 mint	35 Cycles
Annealing	55^{o}C	1 mint	
Extension	72^{o}C	1:30mints	
Final Elongation	72^{o}C	7 mints	1-Cycle

Thermal cycling profiles for SAT2

	Temperature	**Time**	**Cycle**
Initial Denaturation	95^{o}C	5 mints	1-Cycle
Denaturation	95^{o}C	1 mint	35 Cycles
Annealing	50^{o}C	1 mint	
Extension	72^{o}C	1:30 mints	
Final Elongation	72^{o}C	7 mints	1-Cycle

Thermal cycling profiles for SAT1

	Temperature	**Time**	**Cycle**
Initial Denaturation	95^{o}C	5 mints	1-Cycle
Denaturation	95^{o}C	1 mint	35 Cycles
Annealing	55^{o}C	1 mint	
Extension	72^{o}C	1:30mints	
Final Elongation	72^{o}C	7 mints	1-Cycle

3.6.8. Agarose Gel Electrophoresis of post polymerase chain reaction manipulations

Agarose Gel Electrophoresis determines the charges and molecular weights of all sorts of macromolecules. Negatively charged molecules will migrate in an electric field, over time, toward the positively charged cathode. Agarose gel is used to conduct heat evenly and provide an extra sieving effect. Electrophoresis is useful as a qualitative tool for estimation of molecular weights,

separation of complex mixtures of macromolecules into their components. 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 band was visualized by UV illumination, using desktop according to the base pair (bp), and then the size was determined and documented. (ANNEX 9)

3.6.9. Sequencing of the VP1 gene and Phylogenetic tree reconstruction

The positive PCR products of the amplified VP1 gene were purified using the Wizard SV Gel and PCR clean-up system kit (Promega, Germany). The concentration of the purified PCR product was quantified using the Nano Drop 2000c spectrometer (Thermo Scientific, USA). The concentration of each purified product was adjusted and prepared according to the instruction recommended by the sequencing providing company. The purified PCR products were mixed with the sequencing primers and submitted for sequencing to the commercially sequencing LGC Genomics (Berlin, Germany).

The raw sequence data were obtained from LGC Company and the fragments were edited and assembled using Vector NTI Advance™ 11.5 software (Invitrogen, Carlsbad, CA, USA). For each isolate, the fragments produced with both forward and reverse primers for VP1 gene fragment were edited and assembled together and the clean gene sequence was extracted. Multiple sequence alignments were performed using the ClustalW algorithm implemented in BioEdit software package to compare the VP1 gene of the outbreak isolates and the reference strain. For comparative studies, BLAST was used to collect additional FMDV serotype O sequences from GenBank NCBI (National Center for Biotechnology Information) for inclusion in the data set.

For construction of phylogenetic tree, the multiple sequence alignments were performed to align the sequences as codons using the Muscle algorithm in MEGA 6 (Koichiro *et al*., 2013). The Neighbour-Joining algorithm was used with the maximum composite likelihood nucleotide substitution model with the pairwise deletion option. For construction, 1000 bootstrap replicate was used. Lastly selected virus isolates were further characterized by sequencing of the VP1 gene

between serotype O FMD viruses in Ethiopia as well as with other serotype O FMDV isolates from other countries.

3.6.10. Vaccine preparation

Vaccine was prepared for the selected vaccine strain using standard protocol of vaccine production recommended by OIE (2009 and 2012) manual (ANNEX 10). The virus strain was inoculated into a monolayer cell culture of BHK-21 cells and the resulting preparation was clarified by centrifugation inactivated with formalin and adjuvanted with aluminium hydroxide and saponin. The virus inactivated with formalin at a final concentration of 0.06% for 48 h at 26°C. The inactivated virus was clarified using chloroform at a final volume of 0.5% at +4°C overnight. The safety of the inactivated and clarified virus was tested *in vitro* by three serial blind passages in BHK 21 cell cultures (Iyer *et al*., 2001). In addition, the safety of the monovalent vaccine was checked for being free from residual live virus in vivo by subcutaneous administration of 4ml of the prepared vaccine in two calves. The calves were previously screened by 3ABC ELISA for the presence of antibody against FMDV and only calves that were free from Antibody to FMD were included in the experiment. The vaccinated calves were followed for evidence of FMD clinical signs development up to 15 days. The prepared vaccines free from residual virus were kept at +4^{o}C until used.

3.6.11. Serum collection and preparation

A total of five young cattle (6-12 months old) were used after being screened for the absence of FMD specific antibodies using 3ABC-ELISA kit (ANNEX 11). FMD Virus specific antibody negative animals were selected, quarantined in a separate area (barn), ear-tagged and vaccinated with 4 ml subcutaneously in dewlap region with the vaccine prepared from 'O' vaccine strain on day 0. Booster dose was also given on day 7 and 21 post initial vaccination. For 'O' vaccine strain 5 animals were vaccinated. Sera were collected following booster dose separately for O vaccine strain on day 7, 14, 28 and 35 (ANNEX 12). The titer of antibody to the vaccine strain was established for each serum. Sera with strong titer was selected, excluding low responders and stored at -20ºC for the intended vaccine matching. The sera rise against the vaccine strain virus in cattle were also titrated

against known titer of the homologous strains to exclude those with lower titer and inactivated at +56ºC for 30 minutes before testing (OIE, 2009).

3.6.12. Virus and Serum titration

For a good vaccine strain, the virus should grow readily in baby hamster kidney (BHK) monolayer cells, high virus yield and the ability to elicit an antibody response which is broadly cross-reactive within a subtype are the most important (Rweyemamu and Hingley, 1984; Doel, 2003; OIE, 2009 and 2012). Accordingly, FMD virus field isolates were serotyped both at NVI and LGC Genomics (Berlin, Germany) for FMD. This strain was propagated in BHK-21 cell culture three times (Alonso *et al*., 1993). Both FMDV vaccine strains and field isolates were titrated after being adapted on monolayer of BHK-21 cell culture and showed 100% CPE in 24 hours, using tenfold serial dilution beginning with 10^{-1} by taking 0.5 ml of the virus suspension to 4.5 ml of the diluents minimum essential base medium (MEM) ANNEX 14. Using a sterile pipette tips, 0.5 ml from the first dilution was taken and transferred to the next 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 BHK-21 cells. 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 Spearman Karber formula (Dougherty, 1964).

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

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).

3.6.13. Vaccine matching by one way VNT and r value determination

Vaccine matching was conducted to determine the serological antigenic relationship between the field isolates of FMDV circulating in the country and vaccine strain. The 'r' value can be determined by liquid phase ELISA or VNT. 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 (OIE 2009 and 2012). In vitro neutralisation may be more relevant than in vivo protection because of virus-antibody interaction (McCullough *et al*., 1992).

It was performed by using the one-way virus neutralization test, according to the standard protocol of the OIE (2012) manual (ANNEX 14). The field isolates were assessed for their serological relationships to the reference vaccine strain of O/ETH/38/2005 against antisera that was raised for it in bovine. All tests were repeated three times and average 'r1' value was taken for each test virus on different days as per the recommendation of Rweyemamu and Hingley (1984) to increase the confidence with which 'r1' values could be taken to indicate differences between strains was related to the number of times that the examination was repeated.

3.6.14. Data management and analysis

The data obtained from virus isolation were taken forward for serotype identification by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and the isolates were used for vaccine matching study. The data obtained by one-step/way virus neutralization assay were stored in Microsoft Office Excel spread sheet and statistical analysis to calculate arithmetic mean of 'r1' value. The antigenic relationship between a field strain and a vaccine virus (r1-values) was determined by VNT test. The r1- values were calculated as followed:

$$r1 = \frac{serum\ titre\ against\ heterologous\ virus}{serum\ titre\ against\ homologous\ virus}$$

The molecular sequences generated in this study were done at LGC Genomics (Berlin, Germany). The sequences were edited and aligned using MEGA 6 software (Koichiro *et al*., 2013).

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. Phylogenetic tree were constructed using methods of analysis included in MEGA version 6.0 (Koichiro *et al*., 2013) 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).

4. RESULTS

4.1. Clinical Examination

Cattle 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 lameness, and rise in temperature were considered as clinically affected by FMD. Most cattle body temperature was between 39.5-41ºC. Out of 60 cattle examined physically, 41 (68.30%) animals showed clinical signs and lesions suggestive of FMD. The principal clinical signs were salivation and lameness.

Mouth lesions consisted of erosions and ulcers mainly on the tongue and dental pad. Foot lesions comprised of erosions on the interdigital spaces and the coronary bands; on the latter, the lesions were so severe that the hoof tended to separate from the coronary band. In one of study area so-called Mulo, one crossbred bull hoof separated from the coronary band. In this study area (Mulo) cattle affected were local breed and they mostly showed foot lesions with few mouth lesions. In Adama and its surrounding area cattle affected with interdigital spaces lesion and especially in Assala, all affected cattle were exotic breed. In Bishoftu cattle were affected only with oral lesions. In all study area animals affected were with good body condition (3). In all study areas from the total of 41 cattle, the most commonly affected age was between 1-<2 years and above 5years. During this outbreak investigation cattle owners were interviewed for history of vaccination and in all except one farm, in all extensive production system; animals weren't vaccinated against FMD.

4.2. Virus Isolation

From a total of 42 samples (Table 2) one of them was a vaccine seed subjected for virus isolation using BHK21 cell culture and CPE was developed on 17 representative samples; while virus did not inoculated on the rest of 25 samples because two or three samples were collected from same area of for the same outbreak (Table 6). Different types of samples taken from cattle at different date of

collection from outbreaks occurred at Addis Ababa area, Bishoftu, Adama and its surrounding area were isolated in the BHK-21 cell culture (Table 6). From a total of 41 field, tongue epithelial, foot epithelial and oropharyngeal fluid, 16 (100%) were isolated (show cytopathic effect) and one vaccine strain 'O' was also grown in the cell culture.

The entire isolated virus showed CPE which was characterized by a fast destruction of BHK-21 monolayer cell and infected cells were round and formed singly as indicated in arrow (Figure 5). The majority of the tested samples showed CPE within 24hours of inoculation. Of 16 samples that showed CPE, 10 of them were sent and amplified using classical PCR targeting the VP1 gene and sequenced by LGC Genomics (Berlin, Germany) for further serotyping, topotyping and phylogenetic analysis. On those samples that showed CPE, further examination was done to identify the type of the virus at NVI. Thus, only serotype O was recorded from all samples collected from outbreaks (Table 7).

Table 5: Representative list of FMD virus suspected samples collected from cattle isolated using BHK-21 cell culture.

Name of areas of outbreak (Districts)	No of sample collected	Represent ative (s)	Code of Representative	Suspected FMDV Sample	Name of virus show CPE	Date of collection
Adama	3	1	O ETH-23/16	Foot epithelium	ETH-37/16-013	28/10/16
Alaltu town	1	1	ETH-34/16-07	Probing	ETH-34/16-07	10/12/16
Galan	1	1	ETH-40/16-55	Foot epithelium	ETH-40/16-55	17/12/16
AsalaGara gutata	2	1	ETH-36/16-012	Foot epithelium	ETH-36/16-012	12/12/16
Onoda (Alaltu)	4	1	ETH-46/16-74	Probing	ETH-46/16-74	09/12/16
MukaTuri	1	1	ETH-45/1667	Probing	ETH-45/1667	13/12/16
Bishoftu	7	3	ETH-31/16-1	Tongue	ETH-31/16-1	08/11/16
			O/ ETH-19/16	epithelium	ETH-39/16-41	04/01/17
			ETH-42/16-65		O /ETH-19/16	20/12/16

Sheno	7	1	ETH-35/16-010	Probing	ETH-35/16-010	19/12/16
Mogoro (sheno)	4	1	ETH-43/16-29	Probing	ETH-43/16-29	22/10/16
Sandafa	1	1	O/ ETH-21/16	Probing	ETH-42/16-65	7/11/2016
Sululta	2	1	ETH-44/16-66	Probing	ETH-44/16-66	28/12/16
Mulo (DunburiFurda)	6	2	O ETH-29/16 and ETH-38/16-014	1 foot epithelium & 1 tongue epithelium	O ETH-29/16 and ETH-38/16-014	11/11/16 30/10/16
Mulo (Tiiro)	2	1	ETH-33/16-05	Foot tissue	ETH-33/16-05	
Total	**41**	**16**				

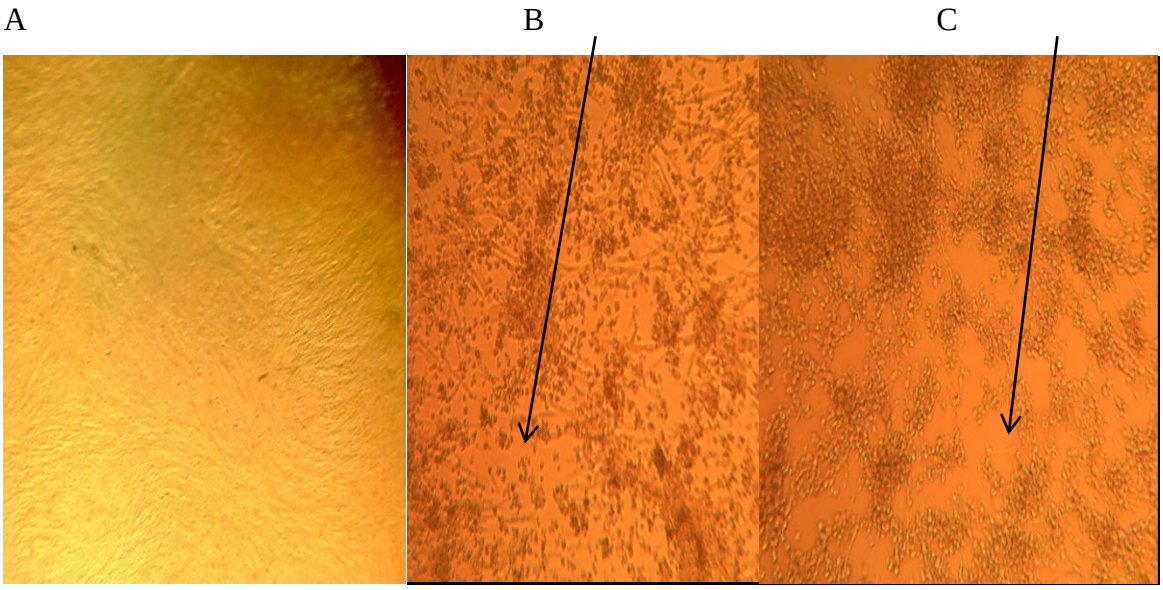


Figure 5: Picture taken during infectious FMDV virus isolation. A: BHK21 cell control (cell without FMDV infection), B and C: showed virus induced cytophatic effect (indicated by arrow) inoculated with FMDV outbreak isolates.

4.3. Molecular Characterization

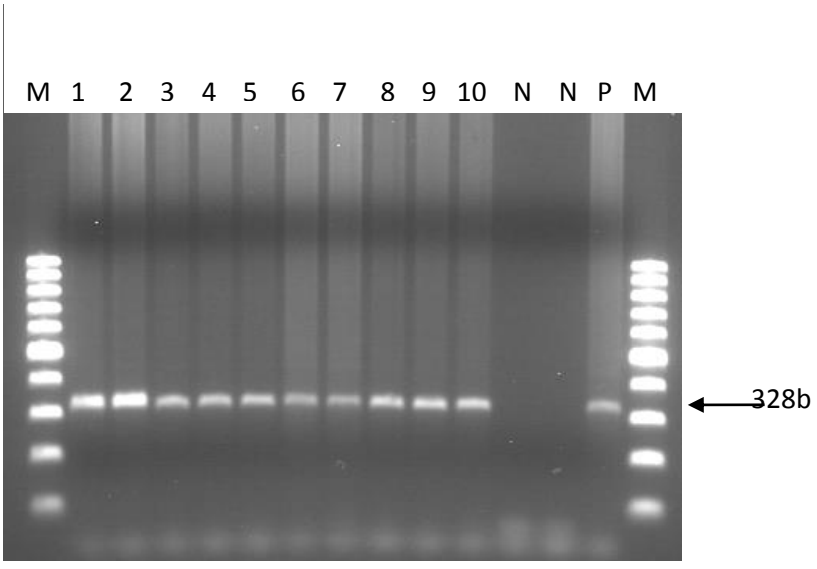
For molecular characterisation, the harvested cell supernatants were detected by RT-PCR to amplify FMDV genome from field isolates and RT PCR positive results (Figure 6) were used for serotype identification. All isolates collected at different times from outbreaks occurred in Addis Ababa and its surroundings, Bishoftu, and Adama and its surroundings were found to be serotype O (Table 7).

Table 6: RT- PCR and sequence analysis results of FMDV isolates.

ID No	Type of sample	Area	P	RT-PCR result	Serotyping result by RT-PCR from P3 cell culture suspension	Topotype identified
1. **O/ETH-29/16**	Tissue suspension	Mulo	P3	+ve	O	EA-4
2. ETH-33/16- 05	Tissue suspension	Tiro	P3	+ve	O	-------
3. ETH-34/16-07	Probang	Alaltu	P3	+ve	O	-------
4. ETH-35/16-010	Probang	Sheno	P3	+ve	O	-------
5. ETH-36/16-012	Tissue suspension	Asala	P3	+ve	O	-------
6. **O /ETH-23/16**	Tissue suspension	Adama	P3	+ve	O	EA-4
7. ETH-38/16-014	Tissue suspension	Dunbur i F	P3	+ve	O	-------
8. **O/ ETH-19/16**	Tissue suspension	Bishoft u	P3	+ve	O	EA-4
9. ETH-40/16-55	Tissue suspension	Gelan	P3	+ve	O	-------
10. **O /ETH-21/16**	Tissue suspension	Sandafa	P2	+ve	O	EA-4

Positive- +ve, P- passage number, -----, not serotyped

Foot-and-Mouth Disease virus genome was amplified from 10 samples of P2 and P3 cell culture suspension sample; no samples were negative from BHK-21 cell culture positive suspension. The samples were amplified using FMDV universal primers (FMDV7F/FMDV7R). Samples O/ETH-29/16,ETH-33/16-05, ETH-34/16-07,ETH-35/16-010,ETH-36/16-012, O/ETH-23/16, ETH-38/16-014, O/ETH-19/16, ETH-40/16-55 and O /ETH-21/16 were given a positive result on RT- PCR (DNA bands on gel electrophoresis around 328 bp) (Figure 6 and ANNEX 9). The samples positive for screening with universal primer were tested for serotype specific primer for each serotype O, A, SAT2 and SAT1 (Figure 7 and Annex 9). Figure 7; revealed that the different FMDV samples were typed as Serotype O positive of samples number O/ETH-29/16, ETH-33/16-05, ETH-34/16-07,ETH-35/16-010,ETH-36/16-012, O/ETH-23/16, ETH-38/16-014, O/ETH-19/16, ETH-40/16-55 and O /ETH-21/16. No Serotype A, SAT1 and SAT2 positive sample was found. Serotypes O of the above samples were given a DNA band at around 600 bp. 10 serotype O were recovered in the field samples, in other word; from the total of 10 samples molecularly diagnosed, 10(100%) serotype O; serotype A, SAT1 and SAT2 serotype also were not detected according to LGC Genomics (Berlin, Germany) and all of the 10 samples were detected as O serotype.



M= Marker started with 100bp,

N. Negative control- No amplification

P. Positive control

1. O/ETH-29/16 2. ETH-33/16- 05

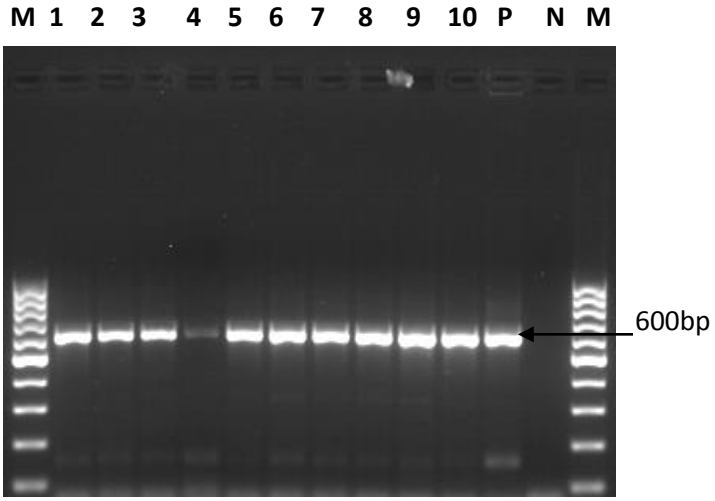
3. ETH-34/16-07 4. ETH-35/16-010

5. ETH-36/16-012 6. O /ETH-23/16

7. ETH-38/16-014 8. O/ ETH-19/16

9. ETH-40/16-55 10. O /ETH-21/16

Figure 6: Detection of FMDV genome by RT–PCR using primer mixture of FMDV7F/ FMDV7R was used targeting the 5′ UTR coding region of the virus.



M= Marker started with 100bp,

N. Negative control- No amplification

P. Positive control

1. O/ETH-29/16 2. O/ETH-33/16- 05
3. O/ETH-34/16-07 4. O/ETH-35/16-010
5. O/ETH-36/16-012 6. O /ETH-23/16
7. O/ETH-38/16-014 8. O/ ETH-19/16
9. O/ETH-40/16-55 10. O /ETH-21/16

Figure 7: Detection of FMDV genome by RT– PCR. Primer mixture FMDVOF/FMDVOR was used for targeting the VP1 coding region of the virus.

4.4. Phylogenetic Analysis

The VP1 gene sequence was used to construct the phylogenetic tree and study the genetic relationships between the current outbreak isolates with the isolates retrieved from the GenBank. The three serotype O isolates of central Oromia, Ethiopia falls to a single topotype i.e. East Africa-4 (EA-4) as shown in Figure 8.

Comparing with other topotypes serotype O isolated during the study periods were also compared with each other and with other countries type O isolates. Isolates from Bishoftu, Adama and Sandafa showed 99-100% sequence similarity.

4.5. Vaccine matching by one step virus neutralization test

The extent of *in vitro* cross-neutralization of 4 field's isolates by antiserum against serotype O/ETH/38/2005 vaccine strains was evaluated (Table 8). The match against O/ETH/38/2005 vaccine strains were above the cut off r1 value of 0.3. The vaccine matching result revealed that

O/ETH/38/2005 was antigenetically close to O/ETH-21/16, O/ETH-23/16, O/ETH-19/16 and O/ETH-29/16 field isolates with r1-values of 0.77, 0.74, 0.67 and 0.74, respectively, using one step VNT (Table 9). So the results presented in Table 9 revealed that there was close antigenic relationship between vaccine strains and the 4 FMDV field isolates. The mean 'r1' value of the serotype O field isolate was 0.73.

Table 7: FMDV serotype selected for vaccine matching study

No	Name of the virus	Site of isolation	Specious	**Serotype**	**Topotype**
1	O/ETH-21/16	Sandafa	Bovine	O	EA 4
2	O/ETH-23/16	Adama	Bovine	O	EA 4
3	O/ETH-19/16	Bishoftu	Bovine	O	EA 4
4	O/ETH-29/16	Mulo	Bovine	O	-------

Table 8: 'r1' value obtained between serotype O field isolate and vaccine strain

No	Field virus (Name of the virus)	Antigenic relationship ('r1'value) between Vaccine strain (O-ETH/38/2005) and field isolates
1	O/ETH-21/16	r1=0.77
2	O/ETH-23/16	r1=0.74
3	O/ETH-19/16	r1=0.67
4	O/ETH-29/16	r1=0.74
	Mean 'r1' value	0.73

4.6. Phylogenetic tree

The 1D or VP1 gene characterization was used to study phylogenetic relationships between 3 serotype O FMD viruses in central Oromiya, as well as with other serotype O isolates from Ethiopia. This serotype O isolated in different districts of central Oromiya, during the study periods were compared with each other and also with other countries type O isolates of the world. Serotype O

isolated in this study clustered within East Africa topotype 4 strains. Serotype O recovered from O/ETH-21/16, O/ETH-23/16, and O/ETH-19/16 Sandafa, Adama and Bishoftu respectively were compared based on the complete 1D sequence (639 nucleotides) data of VP1 gene.

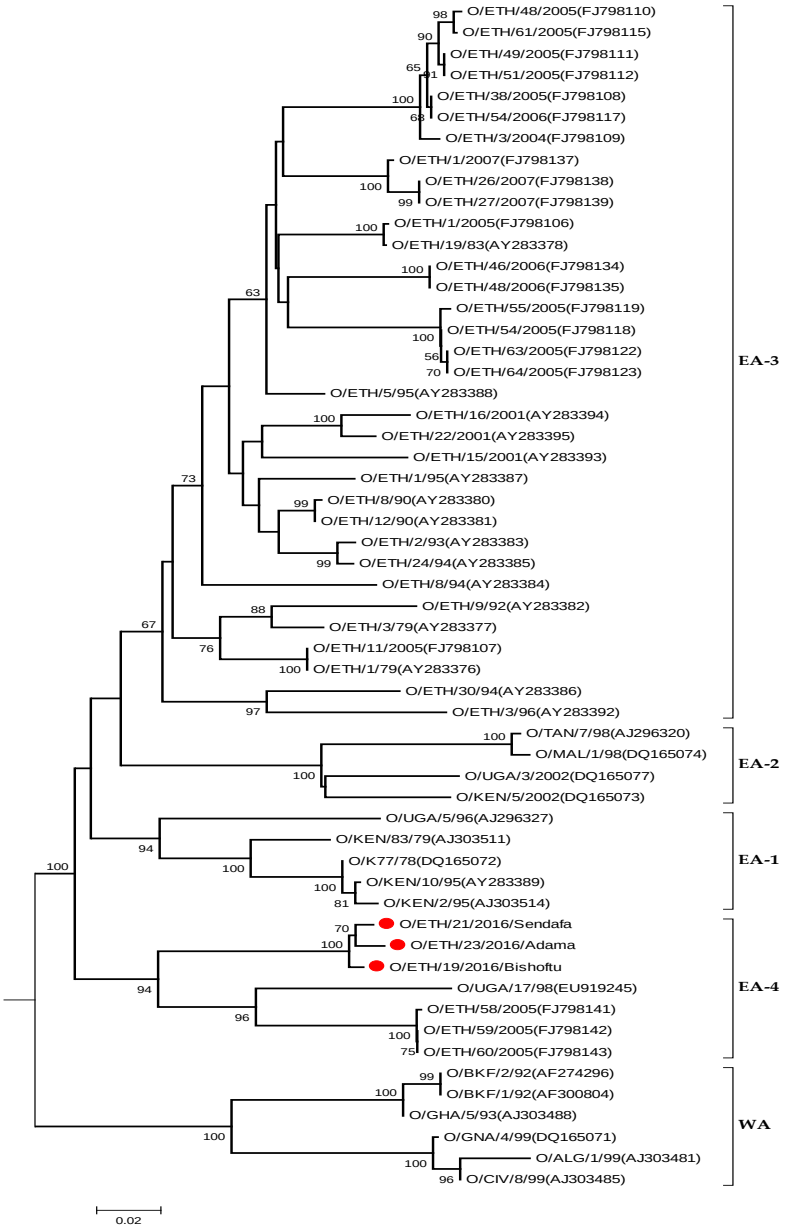


Figure 8: Midpoint-rooted neighbor-joining tree based on the complete virus protein (VP)-1 coding sequence (639 nucleotides) showing the genetic relationship between the FMD virus serotype O isolates of the current Ethiopian virus isolates with the previously characterized Ethiopian isolates and other reference virus African isolates downloaded from the GenBank.

The current 3 isolates collected in 2016 clustered together with the previously characterized Ethiopian FMDV isolates in East African (EA)-4 topotype. The analysis involved 56 FMDV VP1 coding nucleotide sequences. The Neighbor-Joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option was computed using Molecular Evolutionary Genetic Analysis software (MEGA6). The percentage bootstrap scores above 50% (out of 1000 replicates) are shown next to the branches. The 3 isolates sequenced in the present study are indicated in color circle.

5. DISCUSSION

Complete destruction of the BHK-21 cell monolayer was mostly seen with in 24 hrs but in some cases the cells presented obvious CPE within 4 hrs. A longer infection time indicated more evidence of CPE. At 8 hrs, more cells floated in the culture solution which was appreciated in passages 2 and 3. In this study from a total of 41 clinical samples collected and subjected to BHK-21 cell line adaptation, all 16 (100%) field samples showed of the cytopathic effect (CPE) which appeared as rounding in cells culture, swelling, clumping of the cells, breakdown of intercellular bridge, finally cell death indicated the presence of FMDV in the sample (Figure 5) and the result obtained was in agreement to previous work by Huang *et al*. (2011), Haileleul *et al*. (2013), Yenene (2014) and Mishamo (2016) in that the CPE was characterized by a fast destruction of the cell monolayer. Some cell culture supernatants corresponding to 10 samples were further analyzed for FMDV serotyping using RT-PCR (Table: 7. It was reported that RT-PCR is a reliable, rapid, highly sensitive and specific tool for the molecular detection of infectious agents including FMDV (Reid *et al*., 2000; Mehran *et al*., 2006; Hossen *et al*., 2014) that detected presence of the virus in 10 cell culture supernatants showing CPE.

The obtained results revealed that the recovered serotype O FMD virus was identified in Finfinnee and its surrounding zones of Oromia, Bishoftu, and Adama and its surroundings. This was subsequently confirmed by generation of sequence data and serotyping of FMDV as serotype O from samples of clinically infected animals either from tongue epithelium, interdigital epithelium or oropharyngeal fluid of infected cattle. Only serotype O (100%) was the dominant serotype prevailed through study areas were in agreement with pervious work who reported the predominant circulation of serotype O in Ethiopia (Haileleul *et al*., 2009, 2013; Sentayhu *et al*., 2014; Yenene, 2014). Gelaye *et al*. (2005) and Ayelet *et al*. (2009) also reported that serotype O was the most prevalent and dominant FMD virus serotype circulating in Ethiopia, causing most of the outbreaks in Ethiopia. Klein (2009) indicated that it is the most prevalent serotype worldwide. The current study indicated that serotype O is highly prevalent and a dominant serotype causing an outbreak in central Oromia, Ethiopia. The failure to identify serotype ‘A’ and ‘SAT2’ in this study might be due to small sample size. This observing similar serotype in one outbreak is in agreement with Mersie *et*

al. (1992) who reported that the high incidence of the disease in Ethiopia may be associated with extensive movement of livestock and the high rate of contact between animals at marketing and common grazing places as well as at watering points. Those statements again supported by Samuel *et al*. (1999) who demonstrated that closely related viruses could either form the same outbreak or originate from viruses temporally closely related.

Serotype O isolated from Adama O/ETH/23/2016, Sandafa O/ETH 21/16 and Bishoftu O/ETH/19/2016, had 99.808% sequence similarity with vaccine strain isolate virus we used for vaccine matching also the three isolate from Adama, Sandafa and Bishoftu were closely related and < 1% nt. sequence difference and this indicated that they are antigentically related. Also this indicated the outbreaks due to these isolates were from the same origin. These might be due to free movement of livestock and livestock products among various markets in different regions and states and this plays an important role in the dissemination of the virus. Furthermore, serotype O isolated f rom Finfinnee special zones of Oromia O/ETH/21/2016 Sendafa, O/ETH/23/2016 Adama and O/ET H/19/2016 Bishoftu were closely related with each other (> 99% nt. sequence similarity), which indicated that these viruses isolated from the same outbreaks.

These isolates were antigenetically related (< 1% nt. difference) with viruses isolated from Ethiopia in 2005, this indicates they share a common ancestor. Further these isolated viruses were antigenically more closely related (<1% nt sequence difference and >99% bootstrap value) with viruses isolated in Uganda in 1998 and Kenya in 1995 indicating that they share a common ancestor or they belong to the same epizootics (common origin). This suggested that the outbreaks due to these isolates were most probably spread by uncontrolled transboundary movement of animals, and these have a big risk on the transmission of the virus across the border in both directions because there is no strong animal movement regulation across the border and the ability of the virus to transmit with the wind. This statement is supported by Samuel *et al*. (1999) who demonstrated that closely related viruses could either be from the same outbreak or from viruses temporally closely related. Sangare (2005) also reported on the presence of transboundary and transcontinental transmission of viruses in west and southern Africa.

The serotype O isolated from Bishoftu, Adama and Sandafa belonged to East Africa – topotype 4. These indicated that EA-4 topotype has wider distribution and highly prevalent in central Oromiyaa, Ethiopia. This is inconsistent with Haileluel *et al*. (2009) who found serotype O and only EA-3 topotype which had wider distribution and was highly prevalent in Ethiopia. But the current finding is in agreement with study on serotype O by Samuel and Knowles (2001) who verified the existence of EA-3 and EA-4 topotypes in Ethiopia based on the comparison of sequence data of the VP1 gene; additionally this finding is in agreement with Ayelet *et al*. (2009) who demonstrated the existence of EA-4 in Ethiopia with the highest rate of EA-3 topotype.

Serotype O FMDV isolated strains within this study were matched with reference vaccine strains of serotype O FMDV isolated from four serotypes O FMDV; O/ETH/23/2016/ isolate from Adama, O/ETH/19/2016/ isolate from Bishoftu, as well as O/ETH/21/2016 isolate from Sendafa and O/ETH-29/16 isolate from Mulo were used for vaccine matching study.

The findings of high degree of serological matching between the four serotype O FMDV field isolates and vaccine strain O-ETH/38/2005 depicted dependable protection level conferred for circulating O serotypes in the country. The serological match of O-ETH/38/2005 topotype EA-4 to the field isolates had mean 'r1' value of 0.73. Serotype O of Addis Ababa area, Bishoftu, Adama and its surroundings field isolate were antigenic similarity to the reference vaccine strain of Ethiopia where r1 value greater than 0.5. Serotype O/ETH/21/2016/Sendafa was highly significant antigenic similarity to reference vaccine strain of Ethiopia r1 were 0.77. Field isolates strain O/ETH/21/2016/Sendafa was antigenetically related or matched to O/ETH/23/2016/Adama and O/ETH/19/2016/Bishoftu where r1 values were 0.739 and 0.666 respectively using one step VNT. These suggested that there was a close relationship between field isolate and vaccine strain and a potent vaccine containing the vaccine strains were likely to confer protection. This supported by Ayelet *et al*. (2013) finding that the new topotype O/ETH/58/2005 EA-4 and O-ETH/38/2005 topotype EA-3 of Ethiopian vaccine strain assessed for their serological relationship with antisera raised against both revealed a good serological match of r1= 0.81 and r1= 0.76, respectively. Vaccine strain was genetically different from most field virus isolate as Gelagay *et al*. (2009) reported but antigenic study (r1 value $\geq$ 0.3) showed that the field virus types O were highly related to vaccine strain as Ayelet *et al*. (2013) confirmed. Generally, despite the genetic variation observed

for serotype O virus worldwide, the antigenic variation is not extensive and the current vaccine strains can protect against most outbreak which supported by the Esterhuysen (1994) statement that differences in the genetic composition of the viruses from the same serotype don't necessarily reflect differences in antigenicity.

6. CONCLUSION AND RECOMMENDATION

FMD is endemic in Ethiopia as in most parts of Africa. Regular investigation of FMD outbreaks to have more detailed information about the serotypes and topotypes circulating in Ethiopia is important for effective vaccine development. Serotype O was the only identified serotype in this finding and they were isolated from central Oromiya lies on East Africa-4 topotype. Despite the genetic variation observed for serotype O virus worldwide, the antigenic variation is not extensive also the finding revealed the vaccine candidate depicted high antigenic similarity above the mean "r1" value, to its own serotypes in the studied serotype population. These suggested that there was a close relationship between field isolate with vaccine strain and a potent vaccine containing the vaccine strain was likely to confer protection; therefore the current vaccine strains found at NVI FMD laboratory can protect against this strain in the study areas. Although control of FMDV in Africa is challenging, control by vaccination seems the best option in that animal movement control will be difficult. Not only a good quality and vaccine matching is needed, but also a good delivery system and effective vaccination coverage and frequency of vaccination. This should go along with active participatory support from the farmers, governmental decision makers and manufacturers of vaccine to make FMD control a success. An all-inclusive region-based FMDV control strategy along the OIE/FAO progressive control pathway for FMDV control in Africa, when implemented in a well-coordinated manner, would effectively reduce the occurrence and transmission of FMDV. But the prevailing situations in different countries is different and cost need to control FMD would definitely be very high. This risks the control efforts of many countries. But if successfully implemented, these efforts would improve national and regional economies and food security and protect livelihoods.

Based on the above conclusion the following recommendations were forwarded:

- Awareness creation for livestock farmers, small holder and commercial dairy farms regarding the effect of FMD on animals performance and national economy is very important.
- Restriction of animal movement must be enforced to minimize further spread and transmission of the disease.
- Outbreak investigation, serotyping of isolates, molecular characterisation and vaccine matching studies of field isolates should cover the larger areas of the country so that representative information of the circulating strains and vaccine protection potential could be generated and understood. Availability of such data are critical for control of the disease.

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8. ANNEXIS

ANNEX 1: Study animals affected (sick) age estimation on the basis of their dental eruption

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

Source: (Merck veterinary manual, 1998)

ANNEX 2: FMD clinical signs observed in cattle during outbreak investigation;

Lesion in mouth and interdigital space Figures

ANNEX 3: Tissue processed

- Tissue sample is taken from -20^{O}cdeep freeze and thawed at room temperature
- BSL is cleaned and disinfected with 70 % ethanol and dry using sterile gauze and on UV light for 30 minute
- PBS is taken from refrigerator and disinfected with 70 % ethanol and dry using sterile gauze
- sample bottle is disinfected outer surface with 70% ethanol alcohol

- the sample , scissors , forceps, scalpel blade , scalpel handle , Petri dish, centrifuge tubes , beaker pipette , are putted in laminar flow hood class II
- The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed
- 1g of sample is taken from sample bottle and put sample on Petri dish and add PBSA with antibiotic
- Tissue sample is washed three time with PBS having antibiotic
- Tissue suspension homogenized is transferred to centrifuge tube
- Sample is centrifuged at 3500RPM for 10 minute and It is filtered supernatant with 0.22µl filter paper into universal bottle
- The filtered sample is labelled date processed , coded, and typed
- The sample is used immediately or preserved at -20^{O}c until to inoculate to cell.

ANNEX 4: Preparation BHK -21

- Pre-warm both Trypsin 0.05% /EDTA 0.02% solution
- Sub culturing working (trypsine/ versine) and complete media(800ml + 100 calf serum + 100ml tryptose phosphate broth (TPB) + 2ml antibiotic solution) for cell growth at +37^{o}c
- Take cell culture flask with confluent monolayer from the incubator
- Decant old media
- Wash the cells briefly with PBS (Which must be free of Ca, Mg if version is to be used as dispersant) to remove residual serum (which would inactivate the trypsin) & residual bivalent Ions (which would inactivate the versene). Sometimes, this is followed by a brief wash with trypsine/versine (trypsine 0.05%, EDTA 0.02% solution).
- Add slowly enough amount of trypsine/ versine on the opposite wall of attached cell monolayer culture flask and observe the action of trypsine/ versine for two minutes
- Decant the trypsine/ versine,wait by placing the flask inverted until the cell detached from it inside the laminar flow hood
- Add growth medium (sterile new borne calf serum) and pipette vigorously with Pipette ball bulbs, trypsinization mechanically until monolayer become dispersed to a single cell.
- Add growth medium(10% MEM) after trypsinization enough of the amount
- The BHK cell suspension decant on trough to dispense in to other flask

- Incubated at $37^{O}C$

Daily observe the cell line by using inverted microscope for any contamination or growth

ANNEX 5: Virus inoculated on BHK cell

- The tissue suspension virus sample is thawed
- 2% GMEM media is kept in water bath at 37 ^{O}C
- the sample bottles is disinfected outer surface with 70% Ethanol and dry
- BSL is cleaned and disinfected with 70 % ethanol and dry using sterile gauze and on UV light for 30 minute
- Put the cultured flask, media and other solution in laminar flow hood, After Disinfection cells were forms monolayer one day .and debris as possible and then decant and discard growth medium or old medium
- BKH -21 cell is Washed monolayer gently with 2-3 ml of pre warmed PBS and discard.
- Add or inoculate 0.5-1ml tissue suspension virus sample to BKH -21 cell Washed monolayer flask. Rock each flask gently to distribute inoculum evenly over the cell monolayer.
- Incubate inoculated cultures in 37 ^{O}C incubators for one hour to allow virus to adsorb.
- Rock tray once or twice during incubation if possible.
- Add 10 ml maintenance medium to $25cm^2$ inoculated tissue flask.
- Incubate at 37 ^{O}C. Check inoculated tissue flask daily for cytopathogenic effect (CPE) and condition of cells.
- Microscope readings may be feasible after 48 hours and read any CPE
- Inoculated samples are harvested that have 80% CPE and freeze-thaw 2-3 times and collected.

ANNEX 6: Extraction of total RNA from FMD suspected epithelial tissue suspension

RNeasy Mini Kit

The RNeasy Mini Kit (Catalogue No. 74104 and 74106) can be stored at room temperature (15-$25^{0}C$) for at least 9 months.

Protocol: Extraction of total RNA from the FMD suspected epithelial tissue culture suspension samples was conducted using the RNeasy Mini Kit (RNease mini Spin-column) following the manufacturer's instruction (Catalogue No. 74104 and 74106, Qiagen, USA) as follow:

1. Sample taken from 4^0C and thawed by shaking b/n the palm and vortex (2-3) times.
2. Take to the safety cabinet, label the epindurf tube
3. Extraction control (RNA free water) 350 microliter taken to epindurf tube.
4. 350 microliter sample pipetted well and added into epindurf tube.
5. Equal RLT Buffer 350 microliter with sample was taken and added on each sample, mix well. (RLT buffer has enzyme and salt).
6. After adding RLT Buffer,vortex well and centrifuge for 3 min at 13400 rpm
7. 70% ethanol added on each sample, pipet it well and transfer it to RNeasy Mini Spin Column tube.
8. Centrifuge for 90 s at 12500 rpm (this is used as DNA, cell, protein and others go down and RNA is bind to RNeasy Mini Spin Column.
9. Take out of the centrifuge and discard the water, add again the left over sample.
10. Add washing buffer RW1 700 microliter centrifuge at 12500 rpm for 90 s.
11. Add 500microliter washing buffer RW2 (type 2), wash by this two times.
12. Again add 500microliter RW2 and centrifuge at 3 min for 13400 rpm. (To wash clearly and remove ethanol and during this centrifugation lid was not used to cover, we put it out.
13. Discard the previous collection tube and change it to the labelled one, 50microliter RNase free water (elusion Buffer) was added and centrifuge for 3min at 13400 rpm. (Water adding should be at the center=this is to disperse the RNA).
14. Lastly discard the RNease Mini Spin Column membrane and store the RNA in the collection tube and the extracted RNA was stored at -80ºC until analysis

ANNEX 7:*Two-step RT-PCR* (cDNA Synthesis)

Scriptum First cDNA Synthesis kit-Bio gell

Lot no- BS169581

I, Two-Steps RT-PCR (cDNA Synthesis) Procedure

1. 100 µl Scriptum primer(oligodT) = 1 µl/reaction (sample)
2. Add Rnase free water 8 µl/reaction

3. Add 5 µl each sample (RNA)
4. Incubate at 70^{o}c/10mint
5. Incubate 25^{o}c / 15mints

II, Prepared –cDNA sections

1. Scriptum buffer 5x=4ml/reactions(sample)
2. *40* µl dNTPS mix =1 µl/reactions
3. *DTT 100m MScriptum=1*µl/reactions
4. *RNase inhibitor 40units/* µl=*1*µl/reactions
5. *Reverse Transcriptase 200units/* µl =*1*µl/reactions
6. Rnase free water = 4 µl/ reactions

III, From step II above take 12 µl mixture and add to reaction above sample i.e *step I (cDNA Synthesis.)*

1. Incubate at 55^{o}c/ 90mints
2. Incubate at 70^{o}c/10 mints –to inactivate remaining reverse transcriptase enzyme
3. Lastly cDNA is synthesised and store this cDNA at -20^{o}c until PCR run.

ANNEX 8.1: Conventional PCR for FMDV Isolation and Identity test of universal primer, O, A, SAT-2 and SAT-1 strain by two step RT-PCR Master Mix preparation

Two step RT-PCR Master mix preparation for universal primer

Ser.no	**Type of reagent**	**For one reaction**	**Total reaction**
1	RNase free water	3µl	
2	Primer-FMDV7 universal-Fow-5pm/µl 5′ GCCTGGTCTTTCCAGGTCT 3′	2 µl	
3	Primer-FMDV7universal-Reverse-5pm/ µl 5′-CCAGTCCCCTTCTCAGATC-3′	2 µl	
4	IQ super mix	10	
5	Add Template (cDNA)	3 µl	
	Total volume	20 µl	

ANNEX 8.2. Two step RT-PCR Master mix preparation for serotype O

No	Type of reagent	For one reaction	Total reaction 13
1.	RNase free water	3µl	42µl
2.	Primer-FMDV Sero type"O"l-Fow-5pm/ µl 5′-CAGGCGCCACTATCTTCTGT 3′	2 µl	26 µl
3.	Primer-FMDV Sero type"O -Reverse-5pm/µl 5′ TACCAAATTACACACGGGAA-3′	2 µl	26 µl
4.	IQ super mix	10 µl	130 µl
5.	Add Template (DNA)	3 µl	
6.	Total volume	20 µl	

ANNEX 8.3. Two step RT-PCR Master mix preparation for serotype A

No	Type of reagent	For one reaction	Total reaction 12
1	RNase free water	3µl	36µl
2	Primer-FMDV Sero type "A" -Fow-5pm/ µl 5-'TACCAAATTACACACGGGAA-3′	2 µl	24 µl
3	Primer-FMDV Sero type" "A"-Reverse-5pm/µl 5-GACATGTCCTCCTGCATCTG-3′	2 µl	24 µl
4	IQ super mix	10 µl	120 µl
5	Add Template (DNA)	3 µl	
	Total volume	**25 µl**	

ANNEX 8.4. Two step RT-PCR Master mix preparation for serotype "SAT_2"

No	Type of reagent	For one reaction
1	RNase free water	3µl
2	Primer-FMDV Sero type"SAT2"l-Fow 5pm/µl 1C-445 5'-TGGGACAMGGIYTGAACTC-3′	2 µl
3	Primer-FMDV Sero type"SAT2"-Reverse-5pm/ µl 5′- 2B 208 5′-ACAGCGGCCATGCACGACAG -3′	2 µl
4	IQ super mix	10
5	Add Template (cDNA)	3 µl
	Total volume	**20 µl**

ANNEX 8.5. Two step RT-PCR Master mix preparation for serotype SAT_1

No	Type of reagent	For one reaction	Total reaction 12
	RNase free water	3µl	36µl
	Primer-FMDV Sero type"SAT1"-Fow-5pm/ µl-5-CAAGACCGTGGACAACAAGA-3′	2 µl	24 µl
	Primer-FMDV Sero type"SAT1"-Reverse-5pm/ µl -5-GCGTGGTCTTGTACCTGTCA-3′	2 µl	24 µl
	IQ super mix	10 µl	120 µl
	Add Template (cDNA)	3 µl	
	Total volume	**20 µl**	

ANNEX 9: ***Agarose Gel Electrophoresis Protocol***

Equipment

To run a gel you will need the following:

1. Two 1L orange cap bottles.
2. 250 mL flask
3. Volumetric cylinders
4. Spatula
5. Gel casting tray
6. Gel combs
7. Tape
8. Electrophoresis tank
9. Power supply and cables

The first six items are used to pour the gel, and the last three are required for running the gel.

2. Pouring the gel

Agarose gels will always be made in 0.04M Tris-Acetate-EDTA, pH 8.3 (1X TAE) buffer. The percentage gel (w/v) will very depending on the size of molecules we are trying to resolve.

1. Weigh out the appropriate amount of agarose (ex. a 1.5% gel would be 1.5g agarose in 100 mL). Usually we will make 40-50 mL of gel solution. Add the appropriate amount of 1X TAE. Make the mixture in a 250 mL flask, cover it with Saran Wrap, and microwave for 1 minute and 20 seconds on high power.
2. Visually check to see that all the agarose has melted. Unmelted agarose looks like tiny refractive lenses floating around. If not completely melted, nuke it a little longer. Try 20 second intervals.
3. Tape both sides of the casting tray so that it will hold the liquid gel.
4. If the solution is clear and fully liquefied, bring it to your bench and let it cool to about 60ºC – it should feel quite warm to your hand, but not hot.
5. Add Ethidium Bromide (EtBr) to a final concentration of 0.05 μg/mL. So for a 100 mL gel add 5 μL of 10 mg/mL stock (you can also stain later). Note that EtBr is a carcinogen (it intercalates DNA) and so must be handled with care and gloves at all times!

6. Pour the warm liquid agarose. Place the comb into the casting tray by placing the sides into the notches.
7. Wait until the gel polymerizes. It usually takes about an hour. The gel should look opaque and uniform. Use this time to do other things, finish preparing samples, or get the power supply if you haven't done so already.
8. Carefully remove the comb to expose the sample wells.

3. *Running the gel*

1. Place the gel into the tank.
2. Add EtBr to the ~900 mL 1x TAE to make it 0.05 μg/mL (45 μL of a 10 mg/mL stock solution). Pour the buffer into the tank high enough to cover the gel. Be careful since the buffer now has EtBr in it.
3. Add 1 μL loading dye per 5 μL sample (because the dye is 6x).
4. Add samples:
   a) Loading 100-500 ng of DNA per lane is usually sufficient.
   b) Total sample volume should be from 10-35 μL (depends on the gel thickness of the gel and well size used).
   c) One of the samples should be a marker that contains DNA fragments of known lengths that are in the range of your samples.
5. Connect the tank to the power supply and Set the voltage at ~150 V.
6. Run for about an hour or until the faster dye (BB) migrates most of the way through the gel. You can monitor the progress of the DNA directly (if the EtBr was added) by shining UV light on the gel as it is running. Just be careful with the UV lamp.

4. Staining a gel

If you did not add the ethidium bromide earlier, you will need to do so before you can visualize it. The advantage of staining it after running is reduced probability of DNA damage and perturbed migration. The disadvantage, however is that you can't visualize the DNA directly during the run. If you didn't add EtBr, put the unstained gel in a container and pour some TAE buffer (you can reuse the one from the tank you just used to run the gel) just enough to cover the gel, and add ~50μL EtBr (from 10 mg/mL stock solution). Incubate for about an hour with mild shaking.

5. Imaging the gel

To visualize the DNA bands, you must look at them under UV light. Use the UV transilluminator next to the STORM in the Schepartz hot room. **WEAR GLOVES**. You can carry your gel in the casting tray or transfer it onto saran wrap first. Be very careful with gels, as they can be very fragile.

1. Open the plastic cover.
2. Transfer gel from the casting tray by sliding onto the transilluminator (if you're using saran wrap, you can leave it on the saran wrap).

Close the plastic cover. This should protect you from UV, but you can use a face mask, a plastic shield, or other protection as well.

3. Turn on UV to 100%. You should see bands at this point.
4. TURN OFF UV. You want to minimize the UV exposure time as it may damage DNA. Turn off UV before you open the plastic cover.

ANNEX 10: Vaccine preparation procedure

1. Virus for antigen production in BHK-21 cell monolayer or suspension cultures under sterile conditions at +37Oc until the 100% CPE observed within 24 hours. When the virus had reached its maximum titer, which was determined by infectivity, in BHK-21 cell the culture was clarified, by chloroform treatment.
2. Centrifuge at 1500 – 2000 revolution per minute for 15 minutes, and inactivated at 30OC by addition of formaldehyde for 36 hours.
3. The preparation was blended by adsorbing the inactivated virus on to aluminum hydroxide gel and saponin, finished vaccine was checked to be free from residual live virus by vaccinating calves after being checked for the presence of FMD specific antibody, and then followed for clinical sign development.

ANNEX 11: Procedure for CHEKIT- FMD-3ABC BO-OV ELISA

Procedure for CHEKIT- FMD-3ABC BO-OV ELISA

1. Whole blood collected without anticoagulant; half day put at room tempreture; and then +4^{o}covernight (preparation of sample)
2. Preparation of reagent (all reagent taken to room temperature 16-26^{o}c)
3. Serum is diluted by 1:100 in pre plate (1 µl sample with in99 µl diluent)
4. 100 µl sample as well control diluted was transferred to coated plate (coated by known FMDV Ag) and incubated for 1 hour at 37^{o}c
5. 3 times washed by washing buffer 300 µl
6. conjugate 100 µl that was antispecious IgG had been added
7. 100 µl conjugate (mix of IgG and enzyme) had been added and incubated for 1hour at 37^{o}c, lastly washed 3 times
8. 100 µl substrate added (chromogenic) this bind with enzyme in the conjugate and incubated for 15 minutes at dark place at room tempreture.
9. Then add 100 µl stop solution and read the result using a photometer at a wavelength of at 450 nanometer (nm) filters.

10. H. Interpretation of results

If tested in duplicates, the OD of the respective sample or control must be averaged. The OD of the positive control (ODpos) as well as the OD of the samples (ODsample) is corrected by subtracting the OD of the negative control (ODneg):

$$\text{Formula} = \frac{\text{Posetive control:ODpos} - \text{ODneg}}{\text{Sample:ODsample} - \text{ODneg}}$$

Analyze the samples in relation to the negative and positive controls with the formula:

Interpretation: Value	**< 20%**	**20% - 30%**	**> 30%**
Interpretation	negative	ambiguous	Positive

If a sample remains ambiguous after a second run, a new sample of the same animal should be collected and analyzed again. If the new sample is again ambiguous, the epidemiological situation should be considered. Re-analyzed the sample with a different method if available.

ANNEX 12: Serum sample collection procedure for both serum titration and 3ABC ELISA

Whole blood was collected from a jugular vein of selected cattle into 10ml sterile vacutiner tube (without anticoagulant) and store overnight at room temperature for serum separation. The serum was then transferred into a single sterile cryovails, bearing the identification number of the animal using sterile pipette. In the laboratory, the serum was stored at -20oc until laboratory investigation.

ANNEX 13: Protocol of Hanks Virus Media Preparation (Sterilization by filtration)

Hanks virus media is media for inoculation of FMD virus

	10 liters	**5 liters**
NaC1	80.0gm	40.0 gm
KC1	4.0 gm.	2.0 gm
$MgSO_4(7H_2O)$	2.0 gm.	1.0 gm
$CaC1_2$ ($2H_2O$)	1 4 gm. (1.85 gm.)	0.7 gm (0.93 gm.)
$Na_2 HPO_4$ 12 H_2O	1.5 gm.	0.75 gm.
$KH_2 PO_4$	0.6 gm.	0.3 gm.
Glucose	10.0 gm.	5.0 gm.
Yeast extract	10.0 gm.	5.0 gm.
Lactalbumin hydrolysate	50gm	25 .0 gm.
$NaHCO_3$	30.0 gm.	15.0 gm.
Red phenol 10% Solution	1.5 ml	0.75 ml
Distilled water	10,000 ml.	5,000 ml.

Adjust the pH 7.4 –7.5 and sterilize by filtration using 0.22µm pore size Pad.

ANNEX 14: The *Virus neutralisation test procedure for vaccine matching*.

1. Virus dilution series

The pre-determined virus titer (example, $10^{5.5}$) 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(mls)
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.

Above all is the same for both field isolate and vaccine strain serial dilution in universal bottle

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.

VT plate layout

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

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

2.1. Add 100µl/well of media to well column 12 (cell control) and 50µl/well to columns 1-11.

2.2. Add 50µl/well of reference sera to the top well rows A1-A11, 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 from our working dilution (starting from H wells to A wells) to each well columns.

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

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

3. VNT plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	***Homologues virus***	Homologues virus			***Heterologous virus***	***Heterologous virus***				***Cell control***		***Virus control***
B												
C												
D												
E												
F												
G												
H												

4. After incubation, add 50µl/well of BHK21 cells at a seeded rate of between $0.7^{-1}x10^6$ 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.

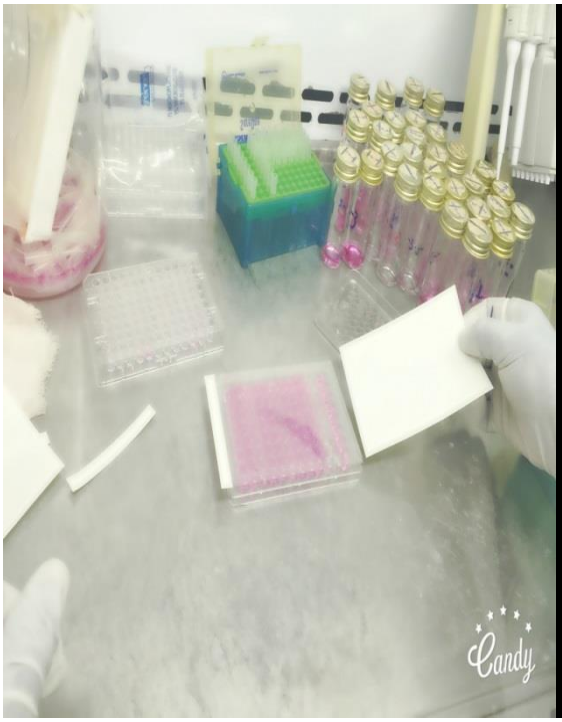
9. After each series of procedures when the cabinet is no longer required, thoroughly disinfect the cabinet with an approved disinfectant, and leave to circulate for at least one hour before re-use. Log all actions in the cabinet log book.

ANNEX 15: Sample collection format of FMD cases in cattle

No	Owner name	address	History of cattle						Sample collected			Clinical sign				parameters			other
31			ID	Breed	Sex	Age	Bod	Reprd. sta	Tissue	probing	Epithelia	lameness	salivation	Lesion(o/I	severe	Temp.	H/r	R/r	

ANNEX 16: Miscellaneous pictures Work at Cell culture, Virology and Molecular rooms

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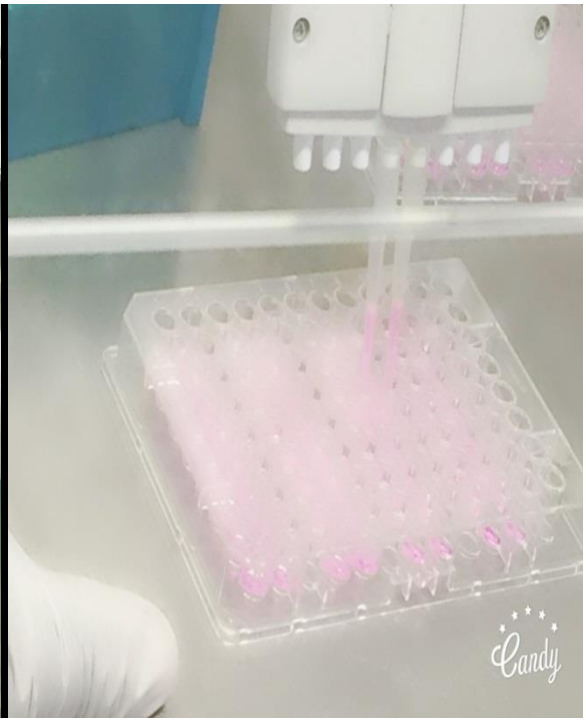
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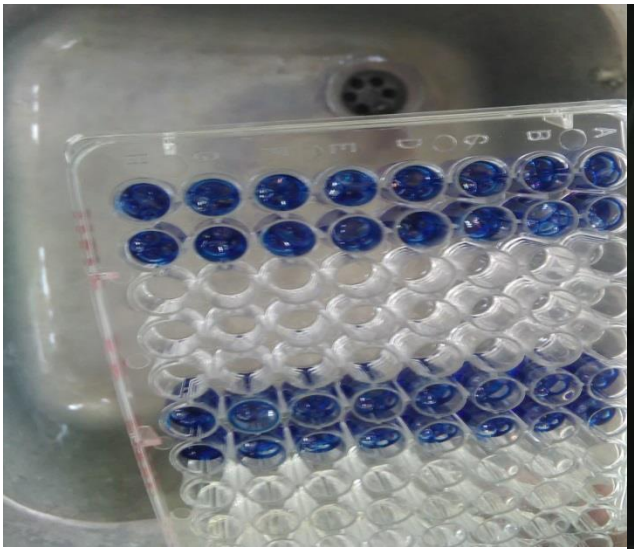

Signature and Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

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Title: ***Molecular characterization and Vaccine matching test of foot and mouth disease viruses isolated from outbreak cases in cattle in selected sites of central part of Oromiya, Ethiopia*.**

Submitted by: Motuma Debelo _____ _____

Name of Student Signature Date

Approved for submittal to MSc research thesis assessment committee

1. Fufa Dawo _____ _____

Major Advisor Signature Date

2. Esayas Gelaye _____ _____

Co- Advisor Signature Date

3. Dr. Gezahegne Mamo _____ _____

Department chairperson Signature Date