Thesis Ref No _____

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

MSc Thesis

By

YENENEH TESFAYE ALEMU

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

JUNE, 2014

BISHOFTU, ETHIOPIA

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

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

By

Yeneneh Tesfaye Alemu

JUNE, 2014

BISHOFTU, ETHIOPIA

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health.

As member of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Yeneneh Tesfaye Alemu titled Isolation, Molecular characterization and Vaccine Matching of Foot-and-Mouth Disease Virus circulating in Ethiopia and recommend that it be accepted as fulfilling the thesis requirement for the degree of master of Science in Veterinary Microbiology.

Dr. Biruke Tesfaye	_____	_____
Chairman	Signature	Date
Dr. Bekele Megarsa	_____	_____
External Examiner	Signature	Date
Dr. Bedaso Mamo	_____	_____
Internal Examiner	Signature	Date
Dr. Gezahegne Mamo	_____	_____
Major advisor	Signature	Date
1. Dr. Gelagay Ayelet	_____	_____
2. Dr. Esayas Gelaye	_____	_____
3. Dr. Shiferaw Jenberie	_____	_____
Co- advisor	Signature	Date
Dr. Bedaso Mamo	_____	_____
Department chairperson	Signature	Date

TABLE OF CONTENTS

CONTENTS	PAGES
TITLE PAGE	i
APPROVAL PAGE	ii
TABLE OF CONTENTS	iii
SIGNED DECLARATION SHEET	vi
ACKNOWLEDGEMENTS	vii
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF TABLES IN THE ANNEX	x
LIST OF FIGURES IN THE ANNEX	xi
LIST OF ANNEXES	xii
LIST OF ABBREVIATIONS	xiii
ABSTRACT	xiv
1. INTRODUCTION	1
2. LITERATURE REVIEW	3
2.1. Definition	3
2.2. Etiology	3
2.2.1. Taxonomy	3
2.2.2. Physicochemical Properties	3

2.2.3. Virus Morphology 3

2.2.4. Genomic Organization 5

2.2.5. Antigenic Variation 5

2.2.6. Serotypes and Sub type 5

2.3. Epidemiology 5

2.3.1. Distribution 5

2.3.2. Host Range 8

2.3.3. Carriers 8

2.3.4. Source of Infection and Transmission 8

2.4. Clinical sings 9

2.5. Diagnosis 9

2.6. Economic Importance 10

2.7. Prevention and Control 10

2. 8. Status of Foot-and-Mouth Disease in Ethiopia 10

2.8.1. FMD Sero-prevalence, Outbreak and Viral Serotypes 11

2.8.2. Risk factor 14

2.9. Vaccine Strains in East Africa 15

2.9.1. Type of Vaccine strain 16

2.9.2. Efficacy of FMD Vaccines 17

3. MATERIALS AND METHODS 19

3.1. Study Areas 19

3.2. Study Animal Population 20

3.3. Study Design 20

3.3.1. Method of sampling and Samples collection 20

3.3.2. Cell culture and Virus isolation 21

3.3.3. Polymerase Chain Reaction (PCR) assay 21

3.4. Culturing FMD vaccine strain and field isolates 22

3.5. Vaccine preparation 23

3.6. Antiserum preparation in cattle 23

3.7. Titration of foot- and-mouth disease virus and antisera collection 25

3.8. Vaccine matching by two-dimensional virus neutralization test 25

3.9. 'r1' value determination and interpretation 26

3.10. Data management and analysis 27

4. RESULT 28

4.1. Virus Isolation 28

4.2. Molecular Characterization 30

4.3. Vaccine matching by two-dimensional virus neutralization test 34

5. DISCUSSION 36

6. CONCLUSION AND RECOMMENDATION 38

7. REFERENCES 39

8. ANNEXES 47

SIGNED DECLARATION SHEET

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

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the dean of the college when in his or her judgment the proposed used of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Yeneneh Tesfaye Alemu Signature: _____

College of Veterinary medicine and Agriculture, Bishoftu

Date of submission: 09/6/2014

ACKNOWLEDGEMENTS

This research would not have been possible without the support and contribution of many people. I would like to express gratitude to my advisors; Dr. Gezahegn Mamo for devotion of his time in advisement and correction of this manuscript; Dr. Esayas Gelaye and Dr. Shiferaw Jenberie also for their encouragement and helpful comments on correction of this manuscript and Dr. Gelagaye Ayelete, for fund support from his FMD project.

My special thanks go to Dr. Marta Yami, Head of National Veterinary Institute, for permission to attend the Graduate program.

Finally, and most importantly, I would like to express my deepest appreciation to Mr. Alebachew Belay and FMD vaccine production laboratory staff, my families for their patience, support and love throughout the entire study period.

LIST OF TABLES

PAGES

Table 1: Serotypes of FMD commonly isolated from certain geographical regions 6

Table 2: Sero-prevalence of FMD at herd and individual levels in Ethiopia 11

Table 3: Samples tested for FMD for export certification 12

Table 4: Topotype of FMD serotype A, O, C, SAT1, SAT2 reported in Ethiopia 13

Table 5: Result of virus typing (Serotyping and Molecular typing) 14

Table 6: Eastern Africa Vaccine strain 16

Table 7: FMDV Candidate Vaccine Strain Selected for vaccine 17

Table 8: FMD virus cases from different part of Ethiopia 29

Table 9: Result of Reverse Transcription-Polymerase Chain Reaction (RT- PCR) 31

Table 10: FMDV serotype selected for vaccine matching 34

Table 11: 'r1' value obtained between serotype O field isolate and vaccine strain 35

LIST OF FIGURES

PAGES

Figure 1. Figure 1: Foot-and-mouth disease virus genome 4

Figure 2. Location of cases of various FMDV serotypes in the outbreaks of FMD, Ethiopia, 1981–2007 7

Figure 3. Map of Ethiopia where the outbreak occur showed in red and deposited samples used for Vaccine match showed in green 19

Figure 4. Cytopathic effect (CPE) for FMDV during the Virus Isolation 30

Figure 5. Detection of FMDV genome by RT – PCR. Primer mixture FMDV7F/ FMDV7R was used for targeting around 328 bp 5' UTR coding of the virus 32

Figure 5.1. Detection of FMDV genome by RT – PCR. Primer mixture FMDVOF/ FMDVOR was used for targeting around 600 bp VP1 coding of the virus 33

Figure 5.2. Detection of FMDV genome by RT – PCR. Primer mixture FMDVAF/ FMDVAR was used for targeting around 866 bp VP3 coding of the virus 33

Figure 5.3. Detection of FMDV genome by RT – PCR. Primer mixture FMDVSAT2F/ FMDVSAT2Rwas used for targeting around 730 bp VP1 coding of the virus 34

LIST OF TABLES IN THE ANNEXES

PAGES

Table 1: FMD samples identified, sequenced and stored at National veterinary Institute were involved in the vaccine match 47

Table 2: Technical Appendix Table. Location and sequences of oligonucleotide primers used for reverse transcription–PCR and sequencing 61

Table 3: Sample collection format 63

LIST OF FIGURES IN THE ANNEXES

PAGE

Figures: Results of gel electrophoresis 64

LIST OF ANNEXES **PAGES**

Annex I: FMD sample identified, sequenced and stored at NVI 47

Annex II: Procedure for CHEKIT-FMD-3ABC BO-OV ELISA 47

Annex III: Procedure for Two Dimensional Virus Neutralization Test (2dm VNT) 50

Annex IV: Test procedure for RT-PCR 56

Annex V: Oligonucleotide primers used for reverse transcription–PCR 61

Annex VI: BHK-21 cell growth and maintenance medium preparation protocol 62

Annex VII: Serum sample collection procedure 63

Annex VIII: Vaccine preparation procedure 63

Annex IX: Sample collection format 63

Annex X: Gel electrophoresis results 64

LIST OF ABBREVIATIONS

ELISA	Enzyme Linked Immuno Sorbant Assay
ETH	Ethiopia
FAO	Food and Agriculture Organization of the United Nation
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
ICTV	International Committee on Taxonomy of Viruses
KEVEVAPI	Kenya Veterinary Vaccine Production Institute
MENA	Middle East and North Africa
MoARD	Ministry of Agriculture and Rural Development
NCR	Non-coding Region
NVI	National Veterinary Institute
OIE	Office International des Epizooties (The International Office for Epizootics)
PCR	Polymerase Chain Reaction
SAT	South Africa Territories
ssRNA	Single Stranded Ribonucleic Acid
SPS-LMM	Sanitary and Phytosanitary Standards - Livestock and Meat Marketing Program
Vpg	Viral Genomic Protein
SNNP	Southern Nations, Nationalities and Peoples
WRLFMD	World reference laboratory for foot-and-mouth disease

ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral and economically important disease that infects wide variety of domestic and wild life hosts and occurs as multiple non cross-protective virus serotypes. FMD suspected samples were collected from Addis Ababa, Debre Berhan and Debre Zeit, with the aim of Isolation, identification of FMDV serotype and conduct vaccine matching. FMD virus was isolated on Baby Hamster Kidney cell culture at National Veterinary Institute (NVI). From the total of 32 samples (22 swine foot epithelium, 8 bovine tongue epithelium and 2 vesicular fluid samples) which were obtained from eight field FMD outbreaks, 21 (65.63%) samples showed cytopathic effect (CPE). Further analysis using Reverse Transcription Polymerase Chain Reaction (RT- PCR) at National Veterinary Institute (NVI) revealed that the FMD field outbreaks reported in this study were caused by FMDV serotype O and one of each serotype O, A and SAT2 were vaccine strains but not field samples included in the study. Two isolates from each outbreak were matched by two dimensional virus neutralization test (2dm VNT) in order to check the antigenic similarity of the field isolate to the vaccine strain. The result indicated that vaccine strains found at National Veterinary Institute (NVI) can protect against field serotype O of Ethiopia isolate except ETH03/12 and ETH04/12 had antigenic variation from the vaccine strain. The finding discovered most of the field isolates have antigenic similarity with the average "r1" value = 0.60 with the vaccine strain. Therefore, FMDV serotype O caused the outbreak in the sample collecting area which were antigenically similar with the vaccine strain and vaccine produce at NVI was still protecting the existing outbreak but further study of antigenic match conducted at certain interval covering a wide area and targeting different animal species since the virus serotype circulating in the country might be changed and all the dominant circulating viruses are incorporated into the vaccine formulation.

Key words: Ethiopia, Foot-and-Mouth Disease virus, FMDV isolation, FMD Serotypes, RT-PCR, Vaccine Match, vaccine strain.

1. INTRODUCTION

Foot-and-Mouth disease (FMD) was the first animal viral disease to be discovered by Loeffler and Frosch in 1898 (David and Howley, 2001). FMD virus was defined in 1963 by the International Committee on Taxonomy of Viruses (ICTV) as belonging to the genus *Aphthovirus*, family *Picornaviridae* (OIE, 2004). It has seven serotypes, namely: O, A, C, South-African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1; and by one time or another, one type or the other was widely reported in most parts of the world (Quinn *et al.*, 2005).

Essentially all cloven-hoofed animals and *Camelidae* (i.e., members of the order *Artiodactyla*) are susceptible to infection with FMD viruses (Thomson, 1994) and amongst the C*amelidae*, bactrian camels and new world camelids have been shown to be susceptible (Larska *et al*., 2009). Foot-and-Mouth disease is an endemic disease in Ethiopia affecting mainly cattle in the majority of cases, but also causing problems in small ruminants at infrequent intervals. Historically the disease was first reported in 1957 (Gulima, 2011), although the disease had, undoubtedly, been in the country for longer time prior to this report, as most of livestock keepers were familiar with the disease and some were using traditional methods of immunization against it, i.e. 'mouthing' (Gulima, 2011). In Ethiopia reports indicated that FMD serotypes O, A, and C were responsible for FMD outbreaks during the period of 1957 to 1979 (Martel, 1974) and in another report of genetics characterization of FMDV from 1981-2007 disclosed additional serotypes of the virus like SAT 1, and SAT 2 circulate in which four host species (cattle, sheep, goats, and pig) are involved, and high numbers of wildlife (especially African buffalo) which has uncontrolled cross the borders of neighboring countries (Gelagay *et al.*, 2009).

Today, many countries have either eliminated FMD by compulsory slaughter of infected animals and movement control or have reduced its incidence greatly by extensive vaccination programs (Radostits *et al.*, 1997). Infection or vaccination against one serotype fails to cross-protect against other types. Furthermore, antigenic differences within a serotype may be so great that there is little or no cross-protection between strains of the same serotype (Araujo *et al*., 2002; Mumford, 2007). Ethiopia was used serotype O281 and A110 locally to produce bivalent vaccine since 2009/2010 (OIE/FAO, 2011) and changed vaccine production to trivalent vaccine of serotype O-ETH/38/2005, A-ETH/7/2008, and SAT2-ETH/76/2009 (Ayelet *et al*., 2013). Recently there are some complains coming to the National Veterinary Institute (NVI) from

different areas of the country concerning the appearance of the disease in those vaccinated animals and this may be due to the existence of other serotypes or new subtypes responsible for that particular outbreak as a result of mismatch with produced vaccine strain (unpublished report)

Polymerase chain reaction was the most widely used nucleic-acid-based diagnostic techniques since its invention (Mullis and Faloona, 1987). RT-PCR developed to amplify RNA targets; many workers have assessed the usefulness of it as a reliable tool for FMD diagnosis (Rodriguez *et al*., 1994) and in parallel with conventional assays such as virus isolation, serology and virus neutralization test (Marquardt *et al*., 1995). However PCR offers potential advantages over other conventional tests. There are different causes of false negative result such as poor sample handling and virus inactivated during RNA extraction. Further, cell culture loses the sensitivity due to presence of inhibitors like interferon and presence of some enzymatic inhibitors. Several studies have compared Reverse Transcription (RT-PCR) methods with FMDV isolation (Callens *et al*., 1998). The sensitivity of the methods reach similar to or greater than virus isolation techniques and these can supplement or go in parallel, but not replace, the routine procedures for diagnosis of FMDV. Viruses for vaccine matching can be selected based on epidemiological information, including stages of an epidemic, geographical locations or range of host species (Alonso *et al*., 1993; Paton *et al*., 2005) by incorporating minimum of two isolates per outbreak (OIE, 2008). The protective capacity of FMD vaccine could be evaluated through vaccine matching using indirect serological methods (Rweyemamu, 1984; Rweyemamu and Hingley, 1984; Paton *et al*., 2005) or alternatively on the calculation of the relatedness between the field isolate and available vaccine strains using *in vivo* challenge tests (Brehm *et al*., 2008; Goris *et al*., 2008).

Therefore, the main Objectives of this study were:

- To isolate and identify the serotypes of Foot-and-Mouth Disease virus from the outbreak samples
- Molecular characterization of Foot-and-Mouth Disease Viruses of the isolate identified from new outbreaks
- To determine Vaccine matching of Foot-and-Mouth Disease vaccine strains to field isolate circulating in the country and to the virus stored at National Veterinary Institute (NVI)

2. LITERATURE REVIEW

2.1. Definition

Foot-and-mouth disease is the most contagious viral disease of mammals caused by virus and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. It is characterized by fever, loss of appetite, salivation and vesicular eruptions on the feet, mouth and teats (Thomson, 1994).

2.2. Etiology

2.2.1. Taxonomy

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

2.2.2. Physicochemical Properties

The FMD virus is susceptible to two percent solutions of NaOH or KOH and 4% Na_2CO_3 are effective disinfectants for FMD contaminated objects, but the virus is resistant to alcohol, phenolic and quaternary ammonium disinfectants (Sahle *et al*., 2004).

2.2.3. Virus Morphology

The diameter of 22 - 25 nm capsids is composed of 60 capsomers each consisting of four proteins (VP1-4). VP1 - VP3 is exposed on the surface, whilst VP4 is located internally at the pentametric apex of the icosahedrons and contains a myristic acid molecule attached to the amino terminal glycine (Robert and Bruce, 1981).

2.2.4. Genomic Organization

FMDV has single stranded, positive sense RNA that is approximately 8500 bases long and consists of a 5' non-coding region (NCR), a single open reading frame, and a short 3' NCR. It is polyadenylated, on the 3' end and has small virus encoded protein, Vpg, covalently attached to

the 5' terminus. Four distinct regions are distinguished for the polyprotein namely the L, P1, P2, and P3. Another characteristic, unique to FMDV, is that there are three species of Vpg encoded by protein 3B, termed 3B1, 3B2, and 3B3. All encoded Vpg variants have been shown to be attached to the 5' terminus of viral RNA (King *et al*., 1982).

The L protein represents the leader protein, where 2 initiation sites (AUG codons) have been identified in FMD virus, namely Lab and Lb (Sangar *et al*., 1988). The P1 gene product is the precursor of the capsid proteins 1D, 1B, 1C, and 1A. Firstly, the intermediate P1 precursor is processed with the help of viral protease 3Cpro to produce VP0, VP1, and VP3 where the products combine to form empty capsid particles. The mature virion is produced after the encapsidation of the virion RNA that is accompanied by the cleavage of VP0 to VP2 and VP4. The P2 (2A, 2B, 2C) and P3 (3A, 3B, 3C, 3D) regions encode for non-structural proteins that are involved in viral RNA replication and protein processing (Belsham, 1993).

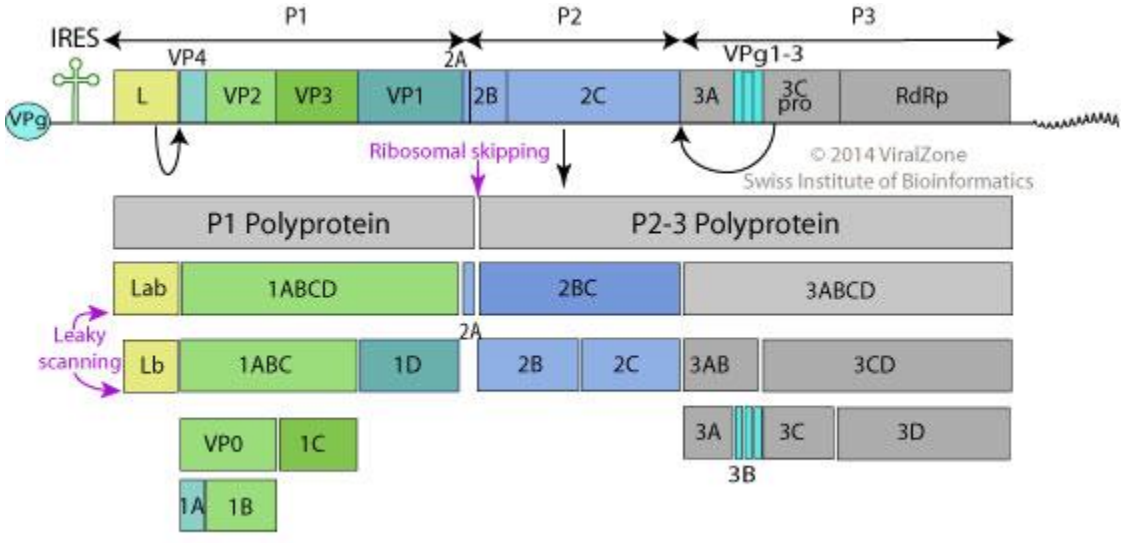


Figure 1: Foot-and-mouth disease virus genome. Source: Adapted from Viral Zone (2014).

2.2.5. Antigenic Variation

Changes to the genes encoding capsid proteins through mutation can result in antigenic variation and evolvement of new subtypes (Haydon *et al*., 2001). This may give rise to immunological distinct variants that can re-infect individuals that have been previously infected by related viruses.

2.2.6. Serotypes and Sub types

Currently there are seven serotypes of Foot and Mouth disease virus (FMDV), namely O, A, C, Southern African Territories (SAT) 1, 2 and 3, and Asia 1, which infect cloven-hoofed animals (Quinn *et al*., 2005). Within these serotypes, over 60 subtypes have also been described using biochemical and immunological tests; and new subtypes occasionally arise spontaneously. However, at a specific time, there are only a few subtypes causing disease throughout FMD endemic areas. The importance of subtypes is that a vaccine may have to be tailored to the subtype present in the area in which the vaccine is being used (OIE, 2004). Foot-and-Mouth disease in Ethiopia has evidenced by laboratory diagnosis for multiple serotypes of the virus O, A, C, SAT 1, and SAT 2 (Gelagay *et al*., 2009).

Infection with any one serotype does not confer immunity against another (OIE, 2004). Antigenic variation within a type occurs as continuous process of antigenic drift without clear-cut demarcation between sub types (Quinn *et al*., 2005) and there are also biotypical strains (strains which become adapted to particular animal species) and topotypes (antigenic entity specific to a given topography (Samuel and Knowles, 2001).

2.3. Epidemiology

3.3.1. Distribution

Foot-and-mouth disease has a global distribution, with the exception of North America, Western Europe and Australia (Kitching, 1999). It is endemic in Africa, Asia, and South America, the Middle and Far East and parts of Europe (Radostits *et al.*, 1997) (Table 1).

Table 1: Serotypes of FMD commonly isolated from certain geographical regions

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

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

In Ethiopia, 1981–2007, a total of 5 serotypes (O, A, C, SAT 1, and SAT 2) were identified in bovine, swine, ovine, and caprine samples collected from the outbreak areas of Amhara, Oromia, Beneshangul-gumuz, South Nation Nationalities People, Addis Ababa and Gambella (Gelagay *et al.,* 2009). SAT 2 was recorded in 2007, after an apparent gap of 16 years, from a bovine sample collected from Bambas, Beneshangul-Gumuz, western Ethiopia bordering Sudan (Figure 1, panel B). The first recorded occurrence of FMDV type SAT 1 in Ethiopia was identified from cattle sample collected in November 2007 from the Mizan Teferi area bordering Kenya (Figure 1, panel B) (Gelagay *et al.,* 2009). Analysis of the samples collected from the same region one month later, in December 2007, showed involvement of 3 species: cattle, sheep, and goats (Gelagay *et al.,* 2009).

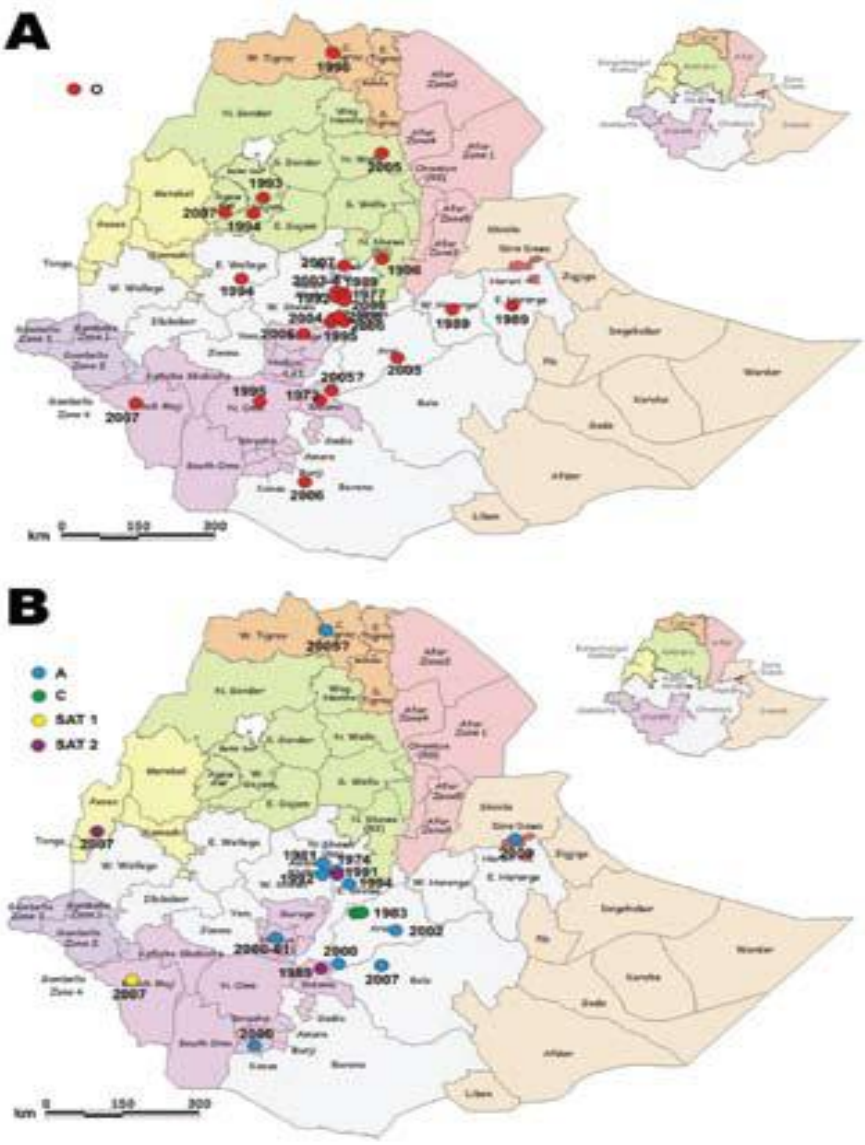


Figure 2: Location of cases of various foot-and-mouth disease (FMD) virus serotypes in the outbreaks of FMD, Ethiopia, 1981–2007, as evidenced by laboratory diagnosis. A) Serotype O, B) serotypes A, C, Southern African Territories (SAT) 1, and SAT 2. Source: adapted from (Gelagaye *et al.*, 2009).

Recent seroprevalence studies of FMD in different districts and localities in the central, south west, northwest and in south pastoral areas of Ethiopia revealed seroprevalence of the FMD in the range of 2.5 to 21% (Rufael *et al.*, 2008; Hailu *et al.*, 2010). In another report from Borana and Guji zones of Oromia (Habtamu *et al.*, 2011) overall individual FMD prevalence was at

24.6% (113/460*100%). Significantly higher prevalence was recorded in Borana 53.6 % (82/153*100%) compared to Guji 10.1 % (31/307*100%) (Habtamu *et al.,* 2011).

2.3.2. Host Range

Foot-and-mouth disease (FMD) is a highly contagious disease affecting *Artiodactylae*, mostly cattle, swine, sheep, goats, and many species of wild ungulates (Brooksby, 1982). FMD in Ethiopia has occurred in four host species (cattle, sheep, goats, and pig), and high numbers of wildlife (especially African buffalo) have uncontrolled cross the borders of neighboring countries (Gelagay *et al.,* 2009).

2.3.3. Carriers

Carriers are defined as animals from which virus can be isolated from the oropharyngeal area more than 28 days after infection (Salt, 1993). Impala (*Aepyceros melampus*) seem to be the most susceptible species in South Africa and are considered an indicator host for the presence of SAT viruses because infection in impala in the past often presaged the occurrence of FMD in livestock (Meeser, 1962). The post infection carrier state is significant for FMD evolution, which is up 5 years for buffaloes, 3 years for cattle, 9 months for sheep and 3-6 months for goats (Condy *et al*., 1985; Quinn *et al.,* 2005).

2.3.4. Source of Infection and Transmission

In the tropics, the most important method of FMD spread is believed to be by direct contact between animals, but under more intensive management systems, it is introduced often, via pigs feeding on contaminated material (Radostits *et al.,* 1997). Infection can be windborne and possibly across expanses of sea (Gloster *et al.,* 1982). Herd-to-herd transmission occurs either by direct movement of infected animals or possibly humans act as transportation means through contamination or indirectly by the transportation of virus on inanimate objects, particularly uncooked and unprocessed products; meat, milk, butter, etc (Blood *et al.,* 1994). Therefore, transmission of FMDV by cattle movement or from wild animals to domestic animals is likely and may play a role in FMD outbreaks and in the appearance of new topotypes in Ethiopia (Gelagay *et al.,* 2009).

2.4. Clinical sings

The incubation period of FMD is 2-8 days (OIE, 2004). In cattle, the initial signs are fever of 103-105ºF (39.4-40.6ºC), dullness, anorexia, and fall in milk production. There is abundant salivation, the saliva hanging in long, ropy strings, a characteristic smacking of the lips and drop in milk yield (Quinn *et al.,* 2005). The predilection sites for vesicles are areas where there is friction such as on the tongue, dental pad, gums, soft palate, nostrils, muzzle, interdigital space, coronary band, and teats (Woodbury, 1995; Sahle *et al*., 2004).

2.5. Diagnosis

In cattle, FMD should be considered whenever salivation and lameness occur simultaneously and when a vesicular lesion is seen or suspected. Due to highly contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a virus secure laboratory (OIE, 2004). Diagnostic samples include vesicular fluid epithelium, blood in anticoagulant, serum and esophageal/pharyngeal fluids collected with a Pro-probing (Quinn *et al.,* 2005). When epithelium tissue is not available from ruminant animals e.g. in advance or convalescent cases and infection is suspected in the absence of clinical sign, samples of oesophageal-pharyngeal fluids (OP) is collected by means of a probang and used for virus isolation (OIE, 2004).

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

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

2.6. Economic Importance

Foot-and-Mouth disease, therefore, threatens the livelihoods of simple 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 (James and Rushton, 2002; Paton *et al*., 2005; Bayissa *et al*., 2011) and therefore, FMD causes substantial economic loss to farmers and to the nation from embargoes of livestock and livestock product trade (Megersa *et al.,* 2009). Recently it has become the major constrain hampering the export of livestock and livestock products to the Middle East and Africa country (FAO, 2005). It is worth to mention that the Egyptian ban of 2003 on Ethiopia livestock alone cause market loss of 14.36 million USD (MoARD, 2007) and the disease threat to Ethiopia's live animal export trade to middle East and North Africa (MENA) which accounts for about 140 thousand heads, worth of 23.9 million USD (SPS-LMM, 2008) annually.

2.7. Prevention and control

Routine vaccination is used where the disease is endemic; in contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and slaughter of infected and in contact animals when outbreaks occur (OIE, 2004).

Immunity to one serotype provides protection only against the homologous viruses. In some cases, inactivated bi-, tri-, or polyvalent vaccine, which contains the representative strains of the serotypes that are in circulation in the region, must be used; therefore, active disease surveillance must be effective which needs a strong field service as well as proper laboratory facilities with efficient methods of detection and characterization of the virus (OIE, 2004).

2.8. Status of Foot and Mouth Disease in Ethiopia

Foot-and-Mouth disease is a notifiable disease in Ethiopia and the Federal Veterinary service of Ministry of Agriculture send monthly and annually office reports to OIE (Leforban, 2005).

2.8.1. Foot-and-Mouth disease Sero-prevalence, Outbreak and Viral Serotypes

Sero-prevalence of FMD at herd and individual levels assessed in different Regions of Ethiopia was a herd prevalence of 57.6% and individual prevalence of 11.9%. Addis Ababa had highest herd and individual prevalence compared to the other region (Table 2).

Table 2: Sero-prevalence of FMD at herd and individual levels in different Regions of Ethiopia.

Region	**Total sample**	**No of herd examined**	**Individual positive**	**Individual % positive**	**Herd positive**	**Herd % positive**
Oromia	3909	269	578	14.8	174	64.7
Benshangul	655	43	41	6.3	20	46.5
SNNP	2070	140	146	7.1	59	42.1
Amhara	1767	116	169	9.6	62	53.4
Tigray	390	26	51	13.1	18	69.2
Somali	272	18	29	10.7	12	66.7
Dire-Dawa	45	3	0	0	0	0
Addis Ababa	412	27	119	28.9	25	92.6
Total	9520	642	1133	11.9	370	57.6

Source: adapted from **(**Gulima, 2011).

Samples tested for FMD certification on 32284 animals for export showed an overall positivity of 14.82% as depicted in Table 3. Serotyping of 120 seropositive samples took from Borana pastoral herds indicated that serotypes O (99.2%), A (95.8%), SAT 2 (80%) and C (67.5%) in which the endemic nature of FMD is discussed in terms of the direct household-level impact of the disease and the increasing export of cattle and chilled beef from Ethiopia (Rufael *et al.*, 2008).

Table 3: Samples tested for FMD for export certification

Region	Zone	District	Species	No. Tested	No. positive	% Positivity
Amahara	North wollo	Desse	Ovine	2764	53	1.92
Dire Dawa	-	-	Caprine	1625	32	1.97
	-	-	Caprine	289	7	2.42
	-	-	Ovine	139	2	1.44
Oromia		Fentalle	Ovine	1000	22	2.20
		Fentalle	Ovine	9304	103	1.11
		Fentalle	Ovine	226	1	0.44
		Fentalle	Ovine	8468	1187	14.02
		Fentalle	Ovine	21	2	9.52
East Shoa		Fentalle	Caprine	399	2	0.50
		Fentalle	Bovine	1103	267	24.21
		Fentalle	Bovine	386	107	27.72
		Adama	Bovine	2943	1148	39.00
		Mekey	Bovine	3617	1850	51.14
Total				32284	4783	14.82

Source: adapted from (Bewket *et al.*, 2012).

It is important to note that FMDV types prevailing in the country changed the genetic material through time (Leforban, 2005) as summarized below:

Period, No Samples, Virus Types: 1957-1973, 98, O, A, C; 1973-1988, not available, O, A; 1988-1994, 16, O, SAT2; 1994-2000, 67, O, A, SAT2; 2000-2005, 7, O, A, SAT2.

In another report of genetic characterization of FMD viruses of Ethiopia from 1981-2007, it was disclosed that 5 of 7 FMDV serotypes circulated in Ethiopia, comprising O, A, C, SAT1, and SAT2. FMDV O was the dominant serotype (73.3%), followed by types A (19.5%), SAT 2 (4.1%), SAT 1 (1.8%), and C (1.3%). In this same report, it is stated that serotype C is not detected in the country after 1983. This report revealed that the phylogenetic analysis of virus protein 1 (VP1) sequences on samples from Mizan Teferi indicated the emergence of a new topotype within serotype O, East Africa 4 (EA-4), apart from the dominant topotype within

serotype O of EA-3 encountered in Eritrea, Ethiopia and Sudan. Serotype SAT1 is noted to have been detected in Ethiopia in 2007 and formed a new distinct topotype Table 4 (Gelagay *et al.,* 2009).

Table 4: Topotype of FMD serotype O, A, C, SAT1, and SAT2 reported in Ethiopia

Serotype	**Topotype**
O	East Africa 4 (EA-4) isolated from Benchi maji, apart from the dominant topotype within serotype O of EA-3 encountered in Eritrea, Ethiopia and Sudan.
A	Africa
C	Africa
SAT1	Serotype SAT1 is noted to have been detected in Ethiopia in 2007 and formed a new distinct IX topotype.
SAT2	VII, XIII and XIV

Source: adapted from (Gelagay *et al.,* 2009).

In very recent report (Bewket *et al.,* 2012) on 58 FMD outbreaks of 2011 in Addis Ababa, Amhara, Gambella, Oromia, SNNP and Tigray Regional States it is disclosed that there were 17577 cases comprising 13249 cattle, 4063 goats, and 265 sheep with mortality of 746 heads of animal (373 cattle, 309 goats and 64 sheep). This same report also revealed the involvement of swine in Adaa district (woreda), Eastern Shoa Zone of Oromia, in which 11 of 19 samples examined turned to be positive for FMD virus. All the virus serotypes and topotypes were similar, i.e. O serotypes, EA-3 except the Makelle outbreak topotype, which was identical with Sudan topotype (EA-3 Sudan) (Table 5).

Table 5: Outcome of virus typing (Serotyping and Molecular typing)

No.	Site of Outbreaks	Species of animal	No. samples	Showing CPE	Serotype identified	Topotype identified
1	Addis Ababa	Cattle	2	2	O	EA-3
2	Debre Zeit	Cattle, swine	14	7	O	EA-3
3	Makelle	Cattle, sheep and goats	11	1	O	EA-3 Sudan
4	Sidama	Cattle	7	3	O	EA-3
5	Adama	Cattle	2	1	O	EA-3
Total			36	14		

Source: adapted from (Bewket *et al.*, 2012).

2.8.2. Risk factor for FMD in Ethiopia

Risk factors for FMD may include factors that may change the level of risk (e.g. new serotypes or biotypes, or changing epidemiological or live stock husbandry patterns) and factors that may interrupt on the national veterinary service to respond effectively to the disease threats (Wondwossen and Tariku, 2000). According to Wondwossen and Tariku (2000) outbreak of FMD used to occur, frequently, in the pastoral herds of the marginal, lowland areas of the country. Currently this trend has changed and the disease is frequently being noted, with increasing incidence in the different part of the country.

The occurrence of new topotype and uncontrolled animal movement are some of the risk factor in Ethiopia. Hence from 58 FMD outbreak occurred in different regional state of country showed all the virus serotypes and topotypes were similar, i.e. O serotypes, EA-3 except the Makelle outbreak topotype, which was identical with Sudan topotype (Table 5) and its phylogenetic analysis indicated that the isolate was much related to the Sudan 1999, 2004 and 2008 isolates (Bewket *et al.,* 2012) and SAT2 serotype, VII, XIII and XIV topotype reappeared after an apparent gap of sixteen years (Gelagay *et al.,* 2009).

Foot-and-mouth disease is found in many parts of Ethiopia; including: Arisi Zone, Dire Dawa region, North wollo, round Debre zeit and Addis Ababa (Leforban, 2005). Extensive movement of live stock and the high rate of contact among animals at commercial markets, in communal

grazing areas and watering points, have been forwarded as cause for the increasing incidence in recent years (Wondwossen and Tariku, 2000).

Ethiopia has 43 million head of cattle, 47 millions sheep and goats, and considerable population of wild animals (FAO, 2005). Small ruminants are maintenance host of FMD (Uppal, 2004). Ovine and caprine FMD cases have been reported to OIE by the year 2000, from Ethiopia (Leforban, 2005). Four of the seven serotype (O, A, SAT1 and SAT2) has circulated in four host species such as cattle, sheep, goats, and pig (Gelagay *et al.,* 2009) and this complicates the control of FMD by vaccination (Sahle *et al*., 2004). The antibodies of SAT2 also were detected in 1971, in sera collected from cattle in the region now known as North Omo, Southwestern Ethiopia (Roeder *et al.,* 1994). This is the first record of the presence of SAT2 FMDV antibodies in Ethiopia from serum sample collected from Borana Southern Ethiopia whereas the first isolation of SAT2 was in 1989 from a sample collected from cattle raised on Leben Ranch, Borena zone, in southern Ethiopia (Roeder *et al.,* 1994) and recently antibody to SAT1 and SAT2 has also been isolated from serum samples from buffalos in the Omo National park, indicating the importance of various hosts in the Ethiopian epidemiology (Sahle *et al*., 2004).

2.9. Vaccine Strains in East Africa

.

Currently FMD vaccines are produced by infecting BHK21 cells with virulent FMDV, followed by chemical inactivation with binary ethyleneimine and purification by ultrafilteration. The effective formulation of FMD inactivated vaccines requires adjuvants, and Al(OH)3/saponin (for ruminants) and incomplete oil-based formulations (for pig and cattle) have been widely employed. Vaccine formulations frequently include viruses of different serotypes, and vaccine preparation implies the growth of high quantities of virus in tissue culture; therefore, high-containment facilities are required. The use of high quality vaccines, with an antigenic composition adapted to the circulating viruses, has decisively contributed to the eradication of FMD in several areas of the world. Inactivated vaccines elicit a consistent humoral response, though generally weaker than that induced in infected animals (Francisco *et al*., 2001). The vaccine was inactivated by formalin which used as inactivant for their production; however, its association with the emergence of viral escapes led to the use of binary ethyleneimine as an inactivant (Bahnemann, 1990).

2.9.1. Type of Vaccine strain

There are different types of FMD vaccine such as: Conventional vaccine, Emergency vaccine, Protein vaccine, protein fragments and viral subunits vaccine, peptide vaccine, Genetically-engineered attenuated strain vaccine, Expression of viral proteins in replicating vectors vaccine and DNA vaccine (Francisco *et al*., 2001) were used to control FMD. Inactivated FMD vaccines having different serotype were used in the East Africa (Table 6). In Ethiopia conventional type of Bivalent (Serotype O and A) vaccine produced at National Veterinary Institute (NVI) since 2009/10 (Table 6). FMD vaccination is applied only at a limited scale in 6 countries in the region: Kenya (KEVEVAPI vaccine, Kenya), Ethiopia (NVI Ethiopia and Indian Immunologicals), Uganda (KEVEVAPI vaccine, Kenya), Somalia (pre-export at Berbera port, KEVEVAPI vaccine Kenya), Burundi (KEVEVAPI vaccine, Kenya), and Sudan (KEVEVAPI vaccine, Kenya) Quadri-valent Vaccine (OIE/FAO, 2011).

Table 6: Eastern Africa vaccine strain

Vaccine strains that may be suitable for use in the region include:

Serotype	**Internationally available**	**Locally produced in 2009/2010**
O	O1 Manisa	Kenya 77/78, Egypt 2/72, Ethiopia O281
A	Eritrea 98	Kenya 5/80, Egypt 06, Ethiopia A110
SAT 1	(Rhodesia 12/78, Botswana 1/68)	Kenya T155/71
SAT 2	Saudi 2000, Eritrea 98, (Zimbabwe 7/830).	Kenya 52/84

Source: adapted from (OIE/FAO, 2011).

Previous studies showed that the new topotype O/ETH/58/2005 EA-4 and other field isolates of serotype O topotype EA-3 of Ethiopian isolates assessed for their serological relationship with

antisera raised against vaccine strains of O1 Manisa and O1 Lausanne in WRLFMD, UK, by virus neutralization test revealed a good serologic match (Negusssie *et al.*, 2010).

Table 7: FMDV Candidate Vaccine Strain Selected for vaccine

Name of candidate vaccine strains	**Site of isolation**	**Year of isolation**	**Serotype**	**Topotype**
O-ETH/38/2005	Addis Ababa	2005	O	EA-3
O-ETH/58/2008	Benchi maji	2005	O	EA-4
A-ETH/7/2008	Sinana	2008	A	A Africa
A-ETH/6/2000	Konso	2000	A	A Africa
SAT2-ETH/76/2009	Sululta	2009	SAT2	XIII
SAT2-ETH/64/2009	Debre Berhan	2009	SAT2	XIII

Source: adapted from (Ayelet *et al.*, 2013).

A total of 21 serotype O, 7 serotype A, and 8 serotype SAT 2 FMD viruses, which were isolated from cattle and swine. A couple of isolates from each serotype were identified as vaccine candidates in the trial (O-ETH/38/2005, O-ETH/58/2008, A-ETH/7/2008, A-ETH/6/2000, SAT2-ETH/76/2009 and SAT2-ETH/64/2009). All the tested candidate vaccine strains O (O-ETH/58/2005 and O-ETH/38/2005), A (A-ETH/6/2000 and A-ETH/7/2008), and SAT2 (SAT2-ETH/64/2009 and SAT2- ETH/76/2009) with their respective serotype field isolates realizes strong antigenic match except one serotype A field isolate which showed significant antigenic variation against the vaccine strains. Furthermore O-ETH/38/2005, A-ETH/7/2008, and SAT2-ETH/76/2009 provided better antigenic neutralization as compared with their alternative vaccine strain candidates (Ayelet *et al.*, 2013).

2.9.2. Efficacy of FMD Vaccines

The antigenic variation of FMDV creates a major problem for the control of FMD, as infection or vaccination with one serotype of FMDV does not protect against other serotypes and may fail to protect fully against other subtypes within the same serotype for this reason matching outbreak strains to the most appropriate vaccine held in an emergency vaccine bank and in vitro antigenic matching studies continue, to ensure the most appropriate antigen stocks are held (Paton *et al*.,

2005). The closeness of match between the outbreak strain and the vaccine applied will affect vaccine performance in an emergency situation, and more recent experimental work has attempted to also address the issue that outbreaks will involve isolates not homologous to the vaccine strain by using heterogonous challenges (Cox *et al*., 2005).

Previous studies at International Animal Health Institute, Pirbright (UK) in cattle, to assess the efficacy of a high potency O1 Manisa oil formulated vaccine applied 21 days prior to a direct contact challenge from infected cattle against both clinical disease and sub-clinical infection using larger group size, have shown more conclusively that vaccination greatly reduces the amount of virus recovered from vaccinated cattle as compared to unvaccinated cattle, both in terms of numbers of animals from which virus was recovered and the quantity of virus recovered from infected animals (Cox *et al*., 2005)**.**

3. MATERIALS AND METHODS

3.1. Study Areas

The study was conducted in sites where outbreak of FMD occurred in Ethiopia from September 2013 to May 2014. Outbreaks have been reported at Addis Ababa, Debre Zeit and Debre Berhan. Addis Ababa has an altitude of 2300 meter above sea level with a subtropical high land climate. The average annual rainfall and average maximum and minimum temperature for the area are 1180 mm, and 22.8ºC and 10.6^{o}C, respectively (CSA, 2009). Debre Zeit is located 45 km south east of Addis Ababa. The area is located at 9ºN latitude and 40ºE longitude at an altitude of 1850 maximum above sea level with annual rainfall of 866 mm of which 84% is in the long rainy season (June to September) (NMSA, 2010). Debre Berhan is a city and woreda in central Ethiopia. Located in the semien shewa zone of Amhara region, about 120 km North east of Addis Ababa, the town has a latitude and longitude of wikimini Atlav 9º41'N 39º32'E/9.683ºN 39.533ºE/9.683; 39.533 and an elevation of 2800 meters (Ethiopian Herald, 2009).

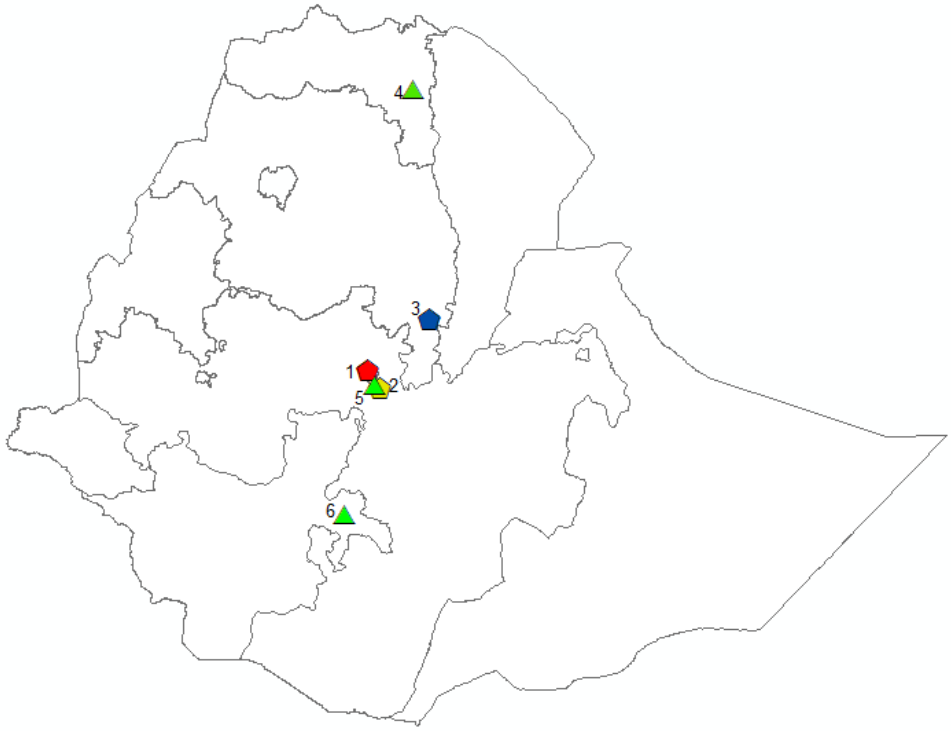


Figure 3: Map of Ethiopia showing the study areas where FMD outbreak samples were collected from cattle and pig. Where 1: Addis Ababa; 2: Debre Zeit; 3: Debre Berhan. FMDV isolates used for vaccine matching found in the NVI pathogen repository were originated from Mekele (4), Debre zeit (5), and Sidama (6).

3.2. Study Animal Population

The study was conducted in twenty two Pigs and ten Cattles that had experienced outbreaks of Foot- and- Mouth Disease and showed clinical signs of the disease. Pigs and Cattles of all age and breed rear under Semi-intensive and extensive production and management system were included in the study.

3.3. Study design

Based on the occurrence of FMD outbreak and active cases, a cross sectional study design were employed to collect samples for virus isolation and molecular characterization of the isolates from cattle and pig FMD cases. For evaluation of the vaccine matching *in vitro* experimental study conducted to evaluate antigenic or serological relationship between foot-and-mouth disease virus vaccine strains and representative FMDV field isolates. For this study, Foot-and-mouth disease virus (FMDV) vaccine serotype O, A, and SAT2 were selected to prepare vaccine. Field isolates FMDV serotype was collected and identified from outbreak in pigs and cattle. Dominant strains from different geographical locations of Ethiopia were selected to evaluate their serological match or antigenic relationship with vaccine strain. Both vaccine strains and representative field isolates were activated by adapting on BHK-21 cell monolayer until 100% cytopathic effect (CPE) observed within 24 hours to obtain a sufficient quantity of the virus. Vaccine was prepared from the vaccine strains using a standard protocol (OIE, 2009). FMDV specific antibody negative calves (n=15) were vaccinated with vaccine prepared from each vaccine strains to raise antisera. Vaccine matching test was conducted using two-dimensional virus neutralization test (Annex III). Finally, the serological relationships 'r' value between the vaccine strains and field isolates were determined.

3.3.1. Method of sampling and samples collection

Using Report base purposive sampling strategy, outbreak investigation and active disease search were conducted together with the professionals working in the dairy farm and swine farm. Those FMDV isolates which have been identified and sequenced previously and stored in the pathogen repository of National Veterinary Institute were involved in the vaccine match (Annex I). A total

of 32 tongue epithelial tissues, feet epithelial tissue and vesicular fluids were collected from FMD-suspected animals from Addis Ababa, Debre zeit and Debere Berhan and submitted to National Veterinary Institute (NVI) at Debre zeit. At least one gram of epithelial tissue was collected from an un-ruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet to isolate the virus. The samples were transport from the collection site to the diagnostic laboratory in 0.04 M phosphate buffer (pH 7.2–7.6) with 50% glycerol at +4°C (OIE, 2008) and stored at –20°C until tested (Kitching *et al*., 1988). The same samples collected at the same time were also submitted to World Reference Laboratory for foot-and-mouth disease (WRLFMD) in Pirbright (UK) for further molecular characterization (genomic sequencing).

3.3.2. Cell culture and Virus isolation

A confluent cultured monolayer of BHK-21 cells was used for virus isolation and propagation. The cell layer of BHK-21 (baby hamster kidney) was inoculated with the 10% (w/v) suspension of processed suspected material. If no CPE was detected within 48 hours of inoculation then the cells would be freeze and thaw and, inoculated into fresh cultures and examined for CPE for another 48 hours. Sample was declared negative if no CPE observed after second blind passages (Buxton and Fraser, 1977; Goel and Rai, 1985; OIE, 2004).

3.3.3. Polymerase Chain reaction (PCR) assay

The polymerase chain reaction (PCR) was applied to amplify the genome fragments of FMD virus. Virus specific primers had been designed to differentiate each of the seven serotypes and investigating the presence of FMD virus RNA in tissue samples (Woodbury *et al.*, 1995). The primer were synthesized by VBC Biotech (Vienna, Austria) and purified by reverse phase high-performance liquid chromatography. At National Veterinary Institute, the samples were screened by universal primer set FMDV7- forward (FMDV7F) and FMDV7- reverese (FMDV7R) for the Reverse Transcription Polymerase Chain Reaction (RT-PCR) of FMDV and for typing of the serotypes O viruses: a primer (Oligoname) set FMDVO – forward and FMDVO – reverse, Serotype A viruses a primer set FMDVA – forward and FMDVA – reverse, and Serotype SAT-2 viruses a primer set FMDV SAT-2 – forward and FMDV SAT-2 – reverse were used. Ribonucleic acid (RNA) extraction and Reverse transcription Polymerase Chain reaction (RT-

PCR) was conducted according to previously described protocol (Knowles *et al*, 2005). The thermal cycling profiles used for amplification of the 5' UTR coding region to screen the sample as positive for FMDV was: Initial denaturation at 94ºC for 4 min, 30 cycles of denature at 94ºC for 1 min, annealing at 54ºC for 1 min and extension at 72ºC for 1 min, followed by final extension 72ºC for 10 min to get the DNA band of 328bp. The thermal cycling profiles used for amplification of the VP1 of 591bp for the serotype O was as follow: Initial denaturation at 95ºC for 5 min, 35 cycles of denature at 95ºC for 1min, annealing at 58ºC for 1 min and extension at 72ºC for 1hour and 30 min, followed by final extension 72ºC for 7 min. The thermal cycling profiles used for amplification of the VP1 or 2B of 715 bp – 730 bp for the serotype SAT-2 was: Initial denaturation at 95ºC for 5 min, first round 15 cycles of denature at 95ºC for 1min, annealing at 60ºC for 1 min and extension at 72ºC for 1 min, followed second round 20 cycles of denature at 95ºC for 1min, annealing at 57ºC for 1 min and extension at 72ºC for 1 hour and 30 min by final extension 72ºC for 7 min. The thermal cycling profiles used for amplification of the VP3 or 2B of 863bp – 866bp for the serotype A was: Initial denaturation at 95ºC for 5 min, 35 cycles of denature at 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1hour and 30 min, followed by final extension 72ºC for 7 min.

3.4. Culturing FMDV vaccine strain and field isolates for vaccine matching

Field isolates serotypes and topotypes were selected based on their strong titer, and high prevalence and wide distribution throughout the country. The strains had already been shown to fulfill the criteria stated by (Rweyemamu and Hingley, 1984; Doel, 2003; OIE, 2009). 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. Accordingly, foot-and-mouth disease virus field isolates identified, serotyped both at NVI and World Reference Laboratory for foot-and-mouth disease (WRLFMD), Pirbright, UK, from 32 samples that were collected from cattle, and pigs, from Addis Ababa, Debre zeit and Debre Birhan including the 3 vaccine strain and samples collected at previous times from Mekelle, Sidama and Debre-zeit were also included in vaccine matching for the present study. All these strains were propagated in BHK-21 cell culture until three passages as previously described (Alonso *et al*., 1993). The selected known serotypes of FMD viruses of both vaccine strain and field strains were subjected to three times freeze-thaw

cycles to release the viral particles from the cells. In a biological safety cabinet, 0.5ml of suspension for each virus was inoculated into the establish monolayer of baby hamster kidney (BHK-21) cell lines and incubate at +37ºC for 30 minutes for adsorption of the virus to the cells. After 30 minutes 10 ml of Hanks virus medium was added into each cell of 25cm^2 plastic tissue culture flasks. After that all cell cultures were incubate at +37ºC for 48 hours and examined for cytopathic effects (CPE) using inverted microscope. After freeze and thaw the viruses were passed into 75cm^2 tissue culture flasks until 100% CPE (Cytopathic effect) observed within 24 hours of the inoculation to obtain a sufficient quantity of the virus for the study.

3.5. Vaccine preparation

Vaccine was prepared for the selected vaccine strain using standard protocol of vaccine production recommended by OIE (2009) manual. Briefly the virus strain was inoculated to a monolayer cell culture of BHK-21 cells and the resulting preparation was clarified, inactivate with formalin and aluminium hydroxide and saponin was added as an adjuvant. The virus was inactivated with formalin at a final concentration of 0.06% for 48 hrs at 26°C. It was then clarified using chloroform at a final volume of 0.5% at +4ºC overnight. The safety of the inactivated, vaccine was tested by three serial blind passages in suckling mice litters and BHK 21 cell cultures (Iyer *et al*., 2001) then the vaccine was checked again to be free from residual live virus by subcutaneous administration of 4 ml of each prepared vaccine in calves separately, after being screened for the absence of antibody against FMDV and following for clinical signs up to ten days. The prepared vaccines were kept at +4ºC until use.

3.6. Antiserum preparation in cattle and antisera collection

A total of fifteen young cattle (6-12 months old) were used after being screened for the absence of FMD specific antibodies using 3ABC-ELISA kit (Annex II). The CHEKIT 3ABC ELISA was used according to the manufacturer's instructions. Briefly, the serum collected from each test animals was diluted 1/100, added in duplicate to the wells of a 96-well microtiter plate pre-coated with the vector-expressed viral 3ABC antigen, and incubated for 1 hour at +37ºC in a humid chamber. Unbound antibody was washed away, and a horse radish peroxidase-labeled

guinea pig anti-bovine immunoglobulin G conjugate was added, and incubated for 1 hour at +37ºC in humid chamber. Unbound conjugate was removed by washing and the chromogen substrate was added and incubated for 15 minutes at room temperature or optimally at +25ºC for 15 minutes. Finally, 100 µl stopping solution was added to stop the reaction, and the result was read using a spectrophotometer at 450 nm wavelength within 2 hours of adding the stopping solution. The reader, connected to the computer loaded with excel packages, was used to automate the reading of optical density (OD) value. The percentage positivity (PP) for test samples in relation to the negative and the positive controls was calculated as per the formula given by the kit manufacturer.

$$\text{Value (\%)} = \frac{\text{OD Sample} - \text{OD Negative}}{\text{OD Positive} - \text{OD Negative}} \text{ X } 100\%$$

Where: OD sample was optical density of the test sera, OD negative was optical density of negative control, and OD positive was optical density of positive control. The Cut off value provided by the Manufacturer was used to determine the percentage positivity. OD values < 20% were considered as negative, OD values between 20% to 30% as ambiguous, and OD values > 30% were considered as positive.

Foot-and-Mouth Disease 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 each vaccine strain on day 0. Booster dose was also given on day 7 and 21 post initial vaccination. For each vaccine strain 5 animals were vaccinated. Sera were collected following booster dose separately for each vaccine strains on day 7, 14, 28 and 35. 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 viruses 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).

3.7. Titration of foot and mouth disease virus

Both FMDV vaccine strains and field isolates were titrated by tenfold serial dilution starting with 10^{-1} by mixing 1ml of virus in 9 ml of diluents (GMEM base medium or MEM base medium) and subsequent transfer of 1ml of previous virus dilution to the next using sterile micro pipette. Fifty micro liters (50 µl/well) of each virus dilution (10^{-1} to 10^{-8}) was distributed into the wells of their respective rows of micro titer plates containing establish cell layers of baby hamster kidney (BHK-21). Then all wells were over flown by dispensing hundred micro liter/well (100 µl/well) of minimum essential base medium (MEM) and incubated at 37^{o}C for 24 hours. The titer for each virus was determined by use of Spearmann Karber formula:

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

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

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

ni = Number of test monolayers use at each individual dilution

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

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

3.8. Vaccine matching by two-dimensional virus neutralization test

Vaccine matching was conducted to determine the serological antigenic relationship between the field isolates of FMDV circulating in the country and vaccine strain. It was performed by using the two-dimensional virus neutralization test, according to the standard protocol of the OIE (2009) manual. The field isolates were assessed for their serological relationships to the reference vaccine strain of O/ETH/38/2005 against antisera that would be raised for it in bovine. Briefly, both field isolates and vaccine strains were passage on monolayer of BHK-21cell cultures until adapt to give 100% CPE in 24 hours. The infected BHK-21 monolayer cells were subjected to three times freeze-thaw cycles to release the viral particles from the cells. Fifty micro liter (50 µl) of serum raise against the reference vaccine strain was added into top wells (A1 to A10) of row A in micro titer plate containing 50 µl/well of minimum essential base media

(MEM) and serially diluted in two fold down the plate (A-H) starting with ½ (1/2, ¼, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256) (OIE, 2009). Then constant amount (50 µl) of pretitrated field isolates of 100 $TCID_{50}$ dose was added in each well using two columns for each antigen, seal and incubates at 37°C. After 1 hour incubation, fifty micro liters (50 µl) of virus/serum mixture was transferred into their respective micro titer plate wells containing established monolayer BHK-21 cells, 50 µl of MEM added in all wells, seal with a semi permeable sealer and incubate in a 5% CO_2 incubator for 48 hours. Columns 11 was used as cell control and column 12 was used as virus control. Columns 1 and 2 of each microtiter plate were used for homologous virus of the vaccine strain. After 48 hours incubation, plates were observed for cytopathic effect using inverted microscope for protection. Finally, titers of the reference antiserum against the heterologous or field isolates and titer of reference antiserum against homologous virus was calculated for each test viruses and vaccine strains. The serological or antigenic relationship between the vaccine strains and field isolates was determined as 'r1' value.

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) and OIE (2009) 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.

3.9. 'r1'value determination and interpretation

The relationships of the field virus isolates to the vaccine strains were expressed as an 'r_1' value. The 'r_1' value was calculated by employing the following formula (OIE, 2009).

$$r1 = \frac{\text{Titer of the vaccine antiserum against the field isolate or heterologous virus}}{\text{Titer of the vaccine antiserum against the vaccine virus or homologous}}$$

For two-dimensional virus neutralization test, 'r_1' value was interpreted according to the criteria indicated by Rweyemamu (1984) and Paton *et al*. (2005) as:

- 'r_1' value $\geq$ 0.3 was considered as the field virus was sufficiently similar to the candidate vaccine strain and vaccine that contain vaccine strain virus was likely to confer protection against challenge with the field isolates.
- 'r_1' value < 0.3 was considered as field isolate was different from vaccine strain that the vaccine containing vaccine strain was unlikely to confer protection against field isolates. In these cases, either the field isolate should be examined against alternative

vaccine strains, tested in a heterologous cross-protection challenge test, or rarely eventually it will be necessary to adapt a suitable field isolate to become a new vaccine strain. 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). In practice, a minimum of at least three repetitions is advised.

3.10. Data management and analysis

The data obtained from virus isolation were analyzed for the type of serotype by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and the result was used for vaccine match study. The data obtained by two-dimension virus neutralization assay were stored in Microsoft Office Excel spread sheet and statistical analysis to calculate arithmetic mean of ‘r1’ value.

4. RESULT

4.1. Virus Isolation

From a total of 35 samples, three of them were a vaccine seed subjected for virus isolation using BHK21 cell culture and CPE was developed on 24 samples while virus did not grow on the rest of 11 samples (Table 8). Different types of samples taken from cattle and pigs at different date of collection from outbreaks occurred at Addis Ababa, Debre Berhan and Debre Zeit were isolated in the BHK-21 cell culture (Table 8). From A total of 32 field tongue epithelial, foot epithelial and vascular fluid samples, 21 (65.63%) were isolated (show cytopathic effect) and the three vaccine strain (O, A and SAT2) was also grown in the cell culture. Most of the isolated virus showed CPE which was characterized by a fast destruction of BHK-21 mono-layer cell and infected cells were round and formed singly as indicated in arrow (figure 4). Complete destruction of the cell sheet was mostly seen with 48 hours of inoculation.

Table 8: FMD virus isolated from outbreak cases from different parts of Ethiopia

No.	Site of Outbreak	Species	No. of samples	Suspected FMDV Sample	Date of sample Collection	Name of the virus show cytopathic effect (CPE)	
						Positive	Negative
1.	Addis	Bovine	1	TE	3/1/2014		ETH/01/14
	Ababa		1	TE	2003/04	ETH/11/13	
		Swine	10	HE	14/11/2013	ETH/21/13- ETH/30/13	
2.	Debre zeit	Bovine	4	2 TE, 2 VF	11/10/2013	ETH/03/13	ETH/01/13,ETH/02/13,ETH/04/13
			3	TE	17/10/2103	ETH/06/13, ETH/07/13	ETH/05/13
		Swine	5	HE	13/11/2013	ETH16/13, ETH/17/13	ETH/13/13- ETH/15/13
			1	HE	29/10/2013	ETH/18/13	
			2	HE	30/10/2013		ETH/19/13 and ETH/20/13
			4	HE	11/10/2013	ETH/02/12,ETH/03/12 and ETH/04/12	ETH/01/12
3.	Debre-Berhan	Bovine	2	TE	12/11/2013	ETH/08/13, ETH/09/13	
		Swine	1	HE	2009/10	ETH/12/13	
4.	Konso	Bovine	1	TE	2000/01	ETH/10/13	
Total			35			24	11

Note: HE (Hoof epithelium), TE (Tongue epithelium), VF (Vesicular fluid).

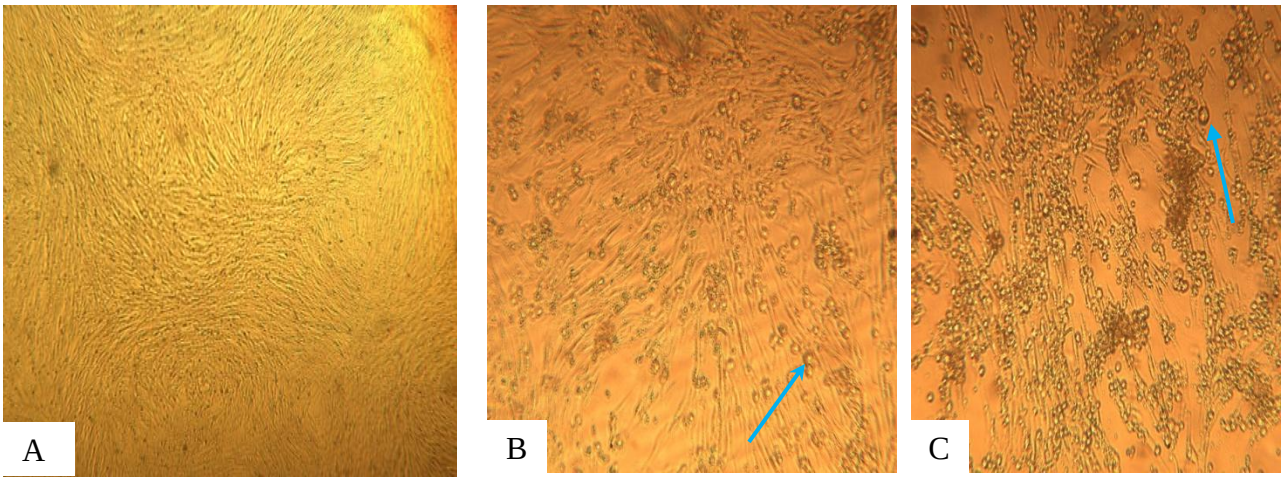


Figure 4: Picture taken during FMDV virus isolation. A: BHK21 cell control (cell with out FMDV infection), B and C showed cytopatic effect (indicated by blue arrow) inoculated with FMDV isolates of ETH08/13 and ETH06/13, respectively.

4.2. Molecular Characterization

Polymerase chain reaction was the most widely used nucleic-acid-based diagnostic techniques. The type of sample was taken from Bovine and Swine were tongue epithelium, foot epithelium and vesicular fluid. This samples passaged three time in BHK21 cell and the cell culture supernatant was checked by RT-PCR for serotyping. RT-PCR was used to amplify FMDV genome from field isolate and all isolates collected at different time from Addis Ababa, Debre-zeit and Debre Berhan were serotype O (Table 9) and the vaccine strain also checked for its identity.

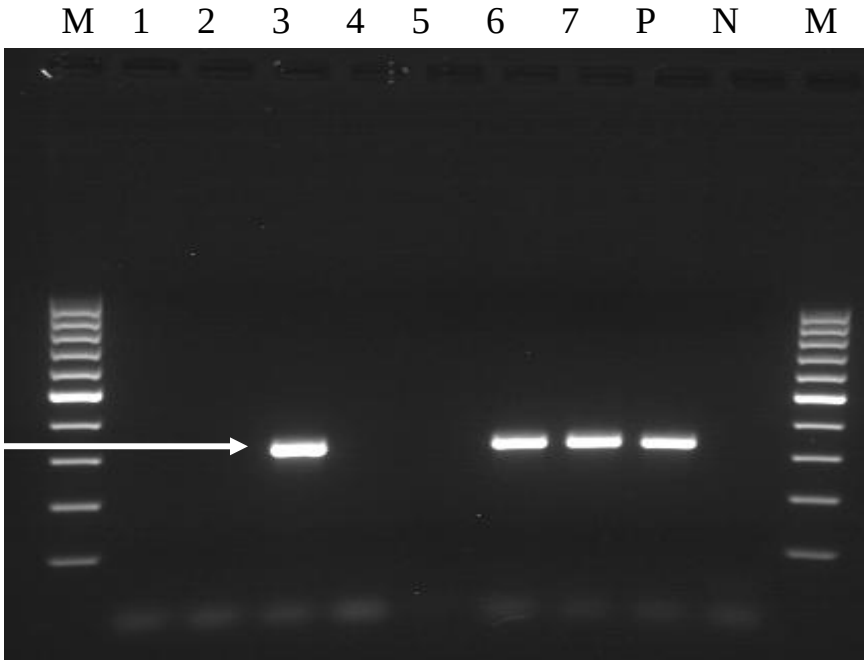
Table 9: Result of Reverse Transcription-Polymerase Chain Reaction (RT- PCR)

No.	Site of Outbreak	Species of animal	No. of samples	Date of sample Collection	RT-PCR result	Serotyping result by RT-PCR from P_3 cell culture suspension		Topotype identified
					Positive	Serotype	No FMDV GD	
1	Addis Ababa	Bovine	1	3/1/2014			1	-
			1	2003/04	1	O		EA3
		Swine	10	14/11/2013	10	O		-
2	Debre zeit	Bovine	4	11/10/2013	1	O	3	-
			3	17/10/2103	2	O	1	-
		Swine	5	13/11/2013	2	O	3	-
			1	29/10/2013	1	O		-
			2	30/10/2013			2	-
			4	11/10/2013	3	O	1	-
3	Debre-Berhan	Bovine	2	12/11/2013	2	O		-
		Swine	1	2009/10	1	SAT-2		XIII
4	Konso	Bovine	1	2000/01	1	A		Africa
	Total		35		24		11	

(-) Not done, (FMDV GD) Foot-and-Mouth disease Virus Genome detected

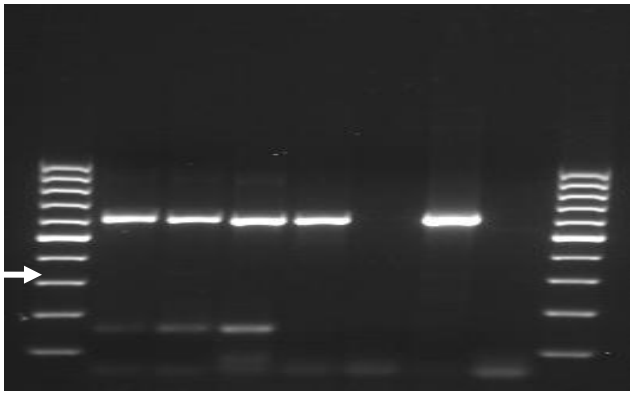
Foot-and-Mouth Disease virus genome was detected in 24 P_3 cell suspension sample and no virus genome were detected in the remaining 11 P_3 cell suspensions from a total of 35 samples by the universal primer (FMDV7F/FMDV7R). Samples number ETH03/13, ETH06/13, ETH07/13, ETH08/13, ETH09/13, ETH10/13, ETH11/13, ETH12/13, ETH16/13, ETH17/13, ETH18/13, ETH21/13 up to ETH30/13, ETH02/12, ETH03/12 and ETH04/13 were give a positive result on RT- PCR (DNA bands on gel electrophoresis around 328 bp) (Figure 5 and Annex X). The samples positive for screening with universal primer were tested for serotype specific primer for each serotype O, A and SAT2 (Figure 5.1 to 5.3 and Annex X). Figure 5.1 to 5.3 (Annex X) revealed that the different FMDV serotype were typed as Serotype O positive of samples number ETH03/13, ETH06/13, ETH07/13, ETH08/13, ETH09/13, ETH11/13, ETH16/13, ETH17/13, ETH18/13, ETH21/13 up to ETH30/13, ETH02/12, ETH03/12 and

ETH04/12. Serotype A positive of sample number ETH10/13 and Serotype SAT2 positive of sample number ETH12/13 was identified. Serotype O, A and SAT2 of the above samples were given a DNA band at around 600 bp, 866 bp and 730 bp respectively. 21 serotype O were recovered in the field samples, in other word from the total of 35 samples, 22 (62.86%) serotype O, 1 (2.86%) serotype A and 1 (2.86%) serotype SAT2 were detected.



M = Marker started with 100bp, P=Positive control, N. Negative control
1. ETH01/13=No amplification
2. ETH 02/13= No amplification
3. ETH03/13=Positive around 328bp
4. ETH04/13= No amplification
5. ETH05/13= No amplification
6. ETH06/13=Positive around 328bp
7. ETH07/13=Positive around 328bp.

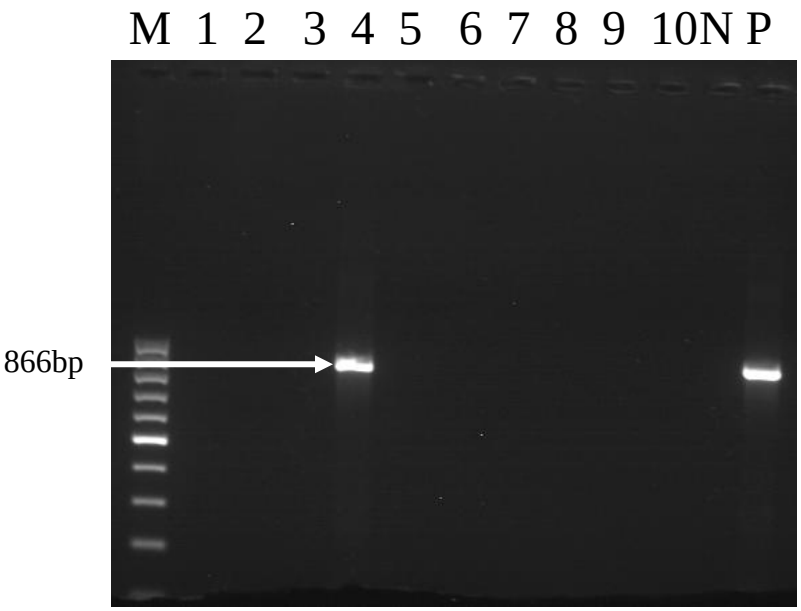
Figure 5: Detection of FMDV genome by RT– PCR. Primer mixture FMDV7F/ FMDV7R was used for targeting around 328 bp 5' UTR coding region of the virus.



M= Marker started with 100bp, P=Positive control, N=Negative control = No amplification

1. ETH03/13 Positive around 600 bp
2. ETH06/13 Positive around 600 bp
3. ETH07/13 Positive around 600 bp
4. ETH17/13 Positive around 600 bp
5. ETH10/13 No amplification

Figure 5.1: Detection of FMDV genome by RT– PCR. Primer mixture FMDVOF/FMDVOR was used for targeting around 600 bp VP1 coding region of the virus.



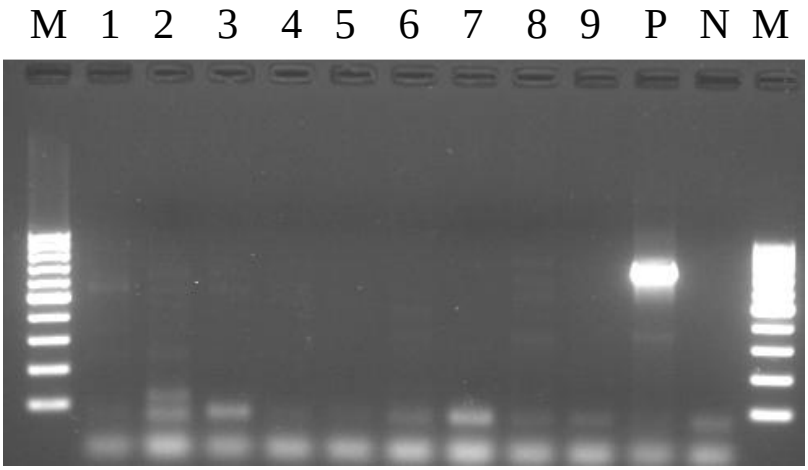
M= Marker started with 100bp, N. Negative control- No amplification and P. Positive control= positive around 866bp

1. ETH06/13 No amplification
2. ETH08/13 No amplification
3. ETH11/13 No amplification
4. ETH10/13 Positive
5. ETH18/13 No amplification
6. ETH24/13 No amplification

7. ETH03/13 No amplification 8. ETH12/13 No amplification

9. ETH03/12Noamplification 10. ETH04/12 No amplification.

Figure 5.2: Detection of FMDV genome by RT– PCR. Primer mixture FMDVAF/ FMDVAR was used for targeting around 866 bp VP3 coding region of the virus



M= Marker started with 100bp, P=Positive control (730bp) (ETH12/13) and N- negative control.

1. ETH03/13, 2. ETH06/13, 3. ETH09/13, 4. ETH18/13, 5. ETH16/13, 6. ETH22/13, 7. ETH04/12, 8. ETH10/13 and 9. ETH 11/13 were negative.

Figure 5.3: Detection of FMDV genome by RT – PCR. Primer mixture FMDVSAT2F/ FMDVSAT2R was used for targeting around 730 bp VP1 coding region of the virus.

4.3. Vaccine matching by two-dimensional virus neutralization test (2dm VNT)

The field isolate were selected from different area based on their titer strength, high prevalence and wide distribution throughout the country (Table 10). This isolates were taken from sample deposited at NVI and field outbreak from Bovine and Swine. The extent of *in vitro* cross-neutralization of 16 field's isolates by antiserum against serotype O/ETH/38/2005 vaccine strains was evaluated. The match against O/ETH/38/2005 vaccine strains were above the cut off r1 value of 0.3 except ETH03/12 and ETH04/12 with equal "r1" value of 0.13. So the results presented in Table 11 revealed that there is a closed antigenic relationship between vaccine strain and 14 field's isolates. The mean r1 value of the type O field isolate was 0.60.

Table 10: FMDV serotype selected for vaccine matching

No.	Name of the virus	Site of isolation	Species	Date of isolation	Serotype	Topotype
1	ETH/2/2011	Debre zeit	Swine	15/07/2011	O	EA -3
2	ETH/6/2011	Debre zeit	Bovine	29/07/2011	O	EA - 3
3	ETH/27/2011	Sidama	Bovine	8/10/2011	O	EA - 3
4	ETH/31/2011	Sidama	Bovine	8/10/2011	O	EA - 3
5	ETH/8/2011	Debre zeit	Bovine	14/10/2011	O	EA - 3
6	ETH/11/2011	Debre zeit	Bovine	14/10/2011	O	EA - 3
7	ETH/17/2011	Mekelle	Bovine	29/11/2011	O	EA-3
8	ETH/03/13	Debre zeit	Bovine	11/10/2013	O	-
9	ETH/06/13	Debre zeit	Bovine	17/10/2013	O	-
10	ETH/07/13	Debre zeit	Bovine	17/10/2013	O	-
11	ETH/08/13	Debre Berhan	Bovine	12/11/2013	O	-
12	ETH/11/13	Addis Ababa	Bovine	2003/04	O	EA-3
13	ETH/17/13	Debre zeit	Swine	13/11/2013	O	-
14	ETH/23/13	Addis Ababa	Swine	14/11/2013	O	-
15	ETH/25/13	Addis Ababa	Swine	14/11/2013	O	-

16	ETH03/12	Debre zeit	Swine	11/10/2013	O	-
17	ETH/04/12	Debre zeit	Swine	11/10/2013	O	-

(-) Not done, EA-3 (East Africa topotype 3)

Table 11: '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	ETH/11/13	1.00
2	ETH/2/2011	0.75
3	ETH/6/2011	0.81
4	ETH/27/2011	0.86
5	ETH/31/2011	0.75
6	ETH/8/2011	0.94
7	ETH/11/2011	0.88
8	ETH/17/2011	0.94
9	ETH/03/2013	0.69
10	ETH/06/2013	0.56
11	ETH/07/2013	0.31
12	ETH/08/13	0.44
13	ETH/17/13	0.38
14	ETH/23/13	0.50
15	ETH/25/13	0.56
16	ETH/03/12	0.13
17	ETH/04/12	0.13
	Mean 'r1' value	0.60

5. DISCUSSION

In this study from a total 32 field samples 65.63% of the samples showed of the cytopathic effect (CPE) which appeared as rounding in cells culture (Figure 3) and the result obtained was in agreement to previous work by Huang *et al*. (2011) and Haileleul *et al*. (2013) that the CPE was characterized by a fast destruction of the cell monolayer and infected cells were round and formed singly.

The obtained results revealed that the recovered virus was identified in Addis Ababa, Debre Zeit and Debre Berhan as serotype O but one serotype O, A and SAT2 of each was vaccine seed. This was subsequently confirmed by the World Reference Laboratory for Foot-and-Mouth Disease (FMD-WRL) in Pirbright, UK (United Kingdom). Virus isolation and serotyping of FMDV as O, A and Southern Africa Territories (SAT) 2 from samples of clinically infected animals either from tongue epithelium, foot epithelium or vesicular fluid of infected cattle and pig, located in swine farm, dairy farms and house hold cattle, showed that only serotype O (65.63%) was the dominant serotype prevailed through Addis Ababa, Debre Berhan and Debre Zeit were in agreement with pervious work who reported the predominant circulation of serotype O in Ethiopia (Bewket *et al.*, 2012) and the two other serotype A (2.86%) and SAT2 (2.86%) were vaccine seed. This indicated that serotype O was highly prevalent and a dominant serotype causing an outbreak in Ethiopia which agreed with the survey of SPS-LMM (2008) that there is a tendency for type O strain to occur most frequently in the outbreak. This similarity of the serotype in one outbreak might be due to the presence of uncontrolled transboundary animal movement and Ekboir (1999) also suggested that movements of infected animals are by far the most important dissemination and transmission means for Foot-and-Mouth Disease. Those statements 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.

Sample collected from Swine revealed only type O and this explains swine can act as a source of infection for other cloven-hoofed animal species. So in terms of species affected, from 32 field sample collected, 16 (50%) Swine had only serotype O which agreed with Gelagay *et al*. (2009) stated that, Cattle were found to be infected with all circulating serotype of FMDV, where as swine had only type O.

The findings of high degree of serological matching between 14 O serotype field isolates and vaccine serotypes O-ETH/38/2005 in the 2dmVNT test depicted dependable protection level conferred for circulating O serotype in the country. The serological match of (O-ETH/38/2005) topotype EA-3 to the field isolates had mean 'r1' value of 0.60. Serotype O of Addis Ababa, Debre Berhan and Debre Zeit field isolate were antigenic similarity to the reference vaccine strain of Ethiopia where r1 value greater than 0.3 except ETH03/12 and ETH04/12 of "r1" value was 0.13. These suggested that there was a close relationship between field isolate with vaccine strain except the two isolates and a potent vaccine containing the vaccine strain were likely to confer protection. Therefore, the vaccine strain was used if the animals were immunized more than once which agreed with Haileleul *et al*. (2013) of the idea that he stated as the vaccine strain might be suitable for use if no closer match could be found provided that a potent vaccine was used and animals were preferably immunized more than once.

Previously isolated sample (ETH/17/2011) which its topotype (EA-3) related to Sudan 1999, 2004 and 2008 (Bewket *et al.,* 2012) had been antigenic similarity with the vaccine strain ETH/11/13(O/ETH/38/2005) of r1 value more than the cutoff (≥0.3). 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, by 2dmVNT 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 ≥ 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

During the study period, 35 samples (field and vaccines strains) were tested for molecular characterization and identified as: 22 serotype O, 1 serotype A and 1 serotype SAT2. Serotype O was identified with highest prevalence. The vaccine O serotype isolated in Ethiopia lies on East Africa-3 topotype, while serotype A was laid in Africa topotype and SAT2 was laid in XIII topotype. The topotype of those vaccine strains were identified before this research. Adapting a new field strain on the *in vitro* method (cell line culturing) was challenging. Thus out of 32 field samples, 21 were adapted where as 11 not adapted on BHK-21 cell culture. Antigenic similarity among FMD viruses circulating in the field can increase the protective capacity of vaccine. The antigenic variation was not diverse for serotype O of vaccine strain ETH11/13 (O/ETH-38/2005) found at NVI can protect against serotype O occurred in Addis Ababa, Debre Zeit and Debre Berhan except ETH03/12 and ETH04/12. Previously isolated samples (ETH/17/2011) which its topotype (EA-3) related to Sudan 1999, 2004 and 2008 had been antigenic similarity with the vaccine strain ETH/11/13 (O/ETH/38/2005) of r1= 0.94 which was above the cutoff (≥0.3). On the other hand, the genetic variation in the immune dominant region (virus protein one) can alter the antigenic specificity of FMDV isolates and cause vaccine failure and severe disease outbreak.

Generally, despite the genetic variation observed for Serotype O virus worldwide, the antigenic variation is not widespread and the current vaccine strains can protect against most outbreaks if a potent vaccine is used.

Therefore, based on the present findings, the following recommendations are forwarded:

- The isolated sample ETH03/12 or ETH04/12 should be included as a vaccine strain because their "r1" value less than 0.3 and might be a new subtype on FMDV Serotype O.
- Vaccine matching should be conducted at a certain interval covering wide areas and targeting different animal species since the virus serotype of subtype circulating in the country might be changed.
- Continuous assessment and serotyping of the outbreak isolates should be conducted to check the introduction and circulation of new serotype in the country and to ensure that all the dominant circulating viruses are incorporated into the vaccine formulation.

7. REFERENCES

Alonso A., Gomes M. D., Ramalho A. K., Allende R., Barahona H., Sondhal M. and Osorio F. (1993): Characterization of foot-and-mouth disease virus by monoclonal antibodies. *Viral Immunology*, **6**: 219–228.

Araujo J. P., Montassier H. J. and Pinto A. A. (2002): Extensive antigenic and genetic variation among foot-and-Mouth disease type A viruses isolated from the 1994 and 1995 foci in Sao Paulo, Brazil. *Veterinary Microbiology*, **84**: 15–27.

Ayelet G., Soressa M., Sisay T., Belay A., Gelaye E., Jembere S., Skjerve E. and Asmare K. (2013): FMD virus isolates, the candidate strains for polyvalent vaccine development in Ethiopia. *Acta Tropica*, **126**: 244 – 248.

Bahnemann H. G. (1990): Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethyleneimine. *Vaccine*, **8**: 299-303.

Bayissa B., Ayelet G., Kyule M., Jibril Y. and Gelaye E. (2011): Study on seroprevalence, risk factors, and economic impact of foot-and-mouth disease in borena pastoral and agro-pastoral system, Southern Ethiopia. *Tropical Animal Health Production*, **43**: 759–766.

Belsham G. J. (1993): Distinctive features of foot-and-mouth disease virus, a member of the picorna virus family; aspects of virus protein synthesis, protein processing, and structure. *Progress in Biophysics Molecular Biology*, **60**: 241-260.

Bewket S., Mesfin S. and Gelagay A. (2012): Presentation on foot and mouth disease surveillance, laboratory and diagnostic activities in Ethiopia. East African Region Laboratory Network 3rd Annual Network Meeting, 5 March, Nairobi, Kenya.

Blood D. C., Radostits O. M. and Henderson J. A. (1994): In: veterinary Medicine. A text book of diseases of cattle, sheep, goats, pigs and horses. 8th edition. The English language book society and Baillier Tindall. Pp: 968-973.

Brehm K. E., Kumar N., Thulke H. H. and Haas B. (2008): High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. *Vaccine*, **26**: 1681–1687.

Brooksby J. B. (1982): "Portraits of viruses: foot-and-mouth disease virus," *Intervirology*, **18** (1-2): 1–23.

Buxton A. and Fraser G. (1977): Animal Microbiology. Blackwell scientific publications Ltd., Edinburge.Volume 2, Pp: 611-619.

Callens M., De Clercq K., Gruia M. and Danes M. (1998): Detection of foot-and-mouth disease by reverse transcription polymerase chain reaction and virus isolation in contact sheep without clinical signs of foot-and-mouth disease. *Veterinary Quarterly*, **20** (2): 537–540.

CSA (2009): Central Statically Authority, Federal Democratic Republic of Ethiopia, Central Statically investigation, statically Abstract.

Condy J. B., Hedger R. S., Hamblin C. and Barnett I. T. R. (1985): the duration of the FMD carrier state in African buffalo. *Comparative Microbiology, Immunology and Infectious Disease*, **8**: 259-265.

Cox S. J., Voyce C., Parida S., Reid S. M., Hamblin P. A., Paton D. J. and Barnett P. V. (2005): Protection against direct contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. *Vaccine*, **23**: 1106–1113

David M. K. and Howley P. M. (2001): Fields virology. Volume l, 4th edition. Lippincott willians and wilkins 530, Walnut Street. Philadlphia, PA 19106, USA. Pp: 15,686.

Doel T. R. (2003): FMD vaccines. *Virus Research*, **91**: 81-99.

Ekboir J. M. (1999): Potential impact of foot and mouth disease in California. The contribution of animal health surveillance and monitoring, California. Agriculture Research Management, Pp: 7-13.

Esterhuysen J. J. (1994): The antigenic variation of foot-and-mouth disease viruses and its significance in the epidemiology of the disease in southern Africa; University of Pretoria. M.Sc. Thesis. Pp: 1 -50.

Ethiopian Herald (2009): The Ethiopian Herald, Location of Debre Berhan. About 42 km Debre Berhan- Ankober road being maintained, 190 (158): Pp 6-8.

FAO (2005): Livestock development in Ethiopia, Concept Note, FAO Representation, Addis Ababa.

Francisco S., Margarita S., Miguel A., Jiménez C., Jose I. N., María F. R., Eric B. and Victoria L. (2001): Foot-and-Mouth disease virus: a long known virus, but a current threat. EDP Sciences. *Veterinary Research,* **32**:1-30.

Gelagay A., Mahapatra M., Essayas G., Berhe G. E., Tesfaye R., Mesfin S., Ferris N. P., Wadsworth J., Hutchings G. H. and Knowles N. J. (2009): Genetic characterization of foot-and-mouth disease viruses, Ethiopia,1981-2007. *Emerging Infectious Diseases,* **15** (9**)**: 1409 -1471.

Gloster J., Sellers R. F. and Donaldson A. I. (1982): Long distance transport of FMDV over the sea. *Veterinary Research,* **110**:47-52**.**

Goel A. C. and Rai A. (1985): Growth curve, plaque assay and inactivation studies of FMD virus subtypes O5, O1 and O6 of Indian origin. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases,* **6** (1): 16–28.

Goris N., Maradei E., D'Aloia R., Fondevila N., Mattion N. and Perez A. (2008): Foot-and - mouth disease vaccine potency testing in cattle using homologous and heterologous challenge strains: precision of the "protection against Podal Generalization" test. *Vaccine*, **26:** 3432–3437.

Gulima D. (2011): Disease reporting, Presentation on VACNADA Project Close out workshop, 5th to 7th December, Debre-Zeit, Ethiopia.

Habtamu M., Desta B., Tesfaye R., Ashenafi F. and Fufa A. (2011): Study on the prevalence of foot-and-mouth disease in Borena and Guji Zones of southern Ethiopia. *Veterinary World,* **4**(7*)*: 293-296.

Haileleul N., Gelagay A., Shiferaw J., Sintayehu M. and Liyuwork T. (2013): Molecular epidemiology and vaccine matching study on foot-and-mouth disease virus circulating in Ethiopia. *African journal of microbiology Research*, **7** (44):5101 – 5106.

Hailu M., Mengistie T., Negussie H., Alemu S. and Asaminew T. (2010): Incidence of foot and mouth disease and its effect on milk yield in dairy cattle at Andassa dairy farm, Northwest Ethiopia. *Journal of Agricultural Biology*, **1**: 969-973.

Haydon D. T., Samuel A. R. and Knowles N. J. (2001): The generation and persistence of genetic variation in foot-and-mouth disease virus. *Preventive Veterinary Medicine*, **51**: 111-124.

Huang X., Li Y., Fang H. and Zheng C. (2011): Establishment of persistent infection with foot and mouth disease virus in BHK-21 cells. *Journal of Virology*, **8**: 169.

Iyer A.V., Ghosh S., Singh S. N. and Deshmukh R. A. (2001): Evaluation of three 'ready to formulate' oil adjuvants form foot-and-mouth disease vaccine production. *Vaccine,* **19**: 1097-1105.

James A. D. and Rushton J. (2002): The economics of foot-and-Mouth disease. *Review Science Technology Office International Epizootic*s, **21**: 637–644.

King A. M. Q., McCahon D., Slade W. R. and Newman J. I .W. (1982): Recombination in RNA. *Cell*, **29**: 921-928.

Kitching R. P. (1999): Foot and mouth disease: Current world situation. Elsevier science Ltd. *Vaccine,* **17**: 1772-1774.

Kitching R. P., Rendle R. and Ferris N. P. (1988): Rapid correlation between field isolates and vaccine strains of foot-and-mouth disease virus. *Vaccine*, **6**: 403–8.

Knowles N. J., Samuel A. R., Davies P. R., Midgley R. J. and Valarcher J. F. (2005): Pandemic strain of foot-and-mouth disease virus serotype O. *Emerging Infectious Diseases,* **11**: 1887–93.

Larska M., Wernery U., Kinne J., Schuster R.., Alexandersen G. and Alexandersen S. (2009): Differences in the susceptibility of dromedary and Bactrian camels to foot-and-mouth disease virus. *Epidemiology Infection*, **137**: 549–554.

Leforban Y. (2005): Report of a Mission on FMD in Ethiopia, proposals for strategic plan for control program oriented to exports. Addis Ababa, Ethiopia. Pp: 1- 43.

Marquardt O., Straub O.C., Ahl R. and Hass B. (1995): Detection of foot-and-mouth disease virus in nasal swabs of asymptomatic cattle by RT-PCR within 24 hours. *Journal of Virological Methods*, **53**(2-3): 255–261.

Martel J. L. (1974): Foot and mouth disease in Ethiopia. Distribution of viral serotypes. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, **27**(2): 169-175.

Meeser J. N. (1962)**:** Foot-and-mouth disease in game animals with special reference to the impala (Aepyceros melampus). *Journals of Science Africa Veterinary Medicines Associations*, **33**: 351–355.

Megersa B., Beyene B., Abunna F., Regassa A., Amenu K. and Rufael T. (2009): Risk factors for foot and mouth disease seroprevalence in indigenous cattle in southern Ethiopia: the effect of production system. *Tropical Animal Health Production*, **41**: 891–8.

MoARD (2007): Training of personnel from regional laboratories on sample collection, handling, preservation and shipment. Training manual. National Animal Health Research Center, Pp: 1-6.

Mullis K. B. and Faloona F. A. (1987): Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, **155**: 335–350.

Mumford J. A. (2007): Vaccines and viral antigenic diversity. *Revision Science Technology Office International Epizootic*, **26**: 69–90.

Negusssie H., Moses K., Yami M., Ayelet G. and Jenberie S. (2010): Outbreak investigations and genetic characterization of foot-and-mouth disease virus in Ethiopia in 2008/2009. *Tropical Animal Health Production*, **43**: 235–243.

NMSA (2010): National Metrological Agency, Addis Ababa, Ethiopia.

OIE (2004): Manual of Diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees): 5th edition, volume I. Office international des Epizooties (OIE), Paris, France. Pp. 111-128.

OIE (2008): Foot-and-mouth disease, Manual of Standard for Diagnostic Tests and Vaccine for Terrestrial Animals, 6th ed. Paris. Pp. 156–212.

OIE (2009): Foot-and-mouth disease, Manual of Standard for Diagnostic Tests and Vaccine for Terrestrial Animals, 6th ed. Paris. Pp. 156–212.

OIE/FAO (2011): FMD Reference Laboratory Network Annual Report. pp. 14-25.

Paton D. J., Valarcher J. F., Bergmann I., Matlho O. G., Zakharov V. M., Palma E. L. and Thomson G. R. (2005): Selection of foot-and-mouth disease vaccine strains review. *Science Technology Office International Epizootic Review,* **24** (3): 981–993.

Quinn P. J., Markey B. K., Carter M. E., Donnelly W. J. C. and Leonard F. C. (2005): Veterinary microbiology and microbial disease. Blackwell Science Ltd, A Blackwell publishing company. Pp. 402-407.

Radostits O. M., Blood D. C and Gay C. C (1997): Veterinary medicine, a text book of the disease of cattle, sheep, pigs, goats and horses. 8th edition, the English Language Book society and W.B. Saunders Co. Ltd. Pp. 965-973.

Robert P. J., and Bruce A. P. (1981): Picornaviral structure and assembly. *Microbiological Review*, **45**: 287-315.

Rodriguez A., Nunez J. I., Nolasco G., Ponz F., Sobrino F. and De Blas C. (1994): Direct PCR detection of foot-and-mouth disease virus. *Journal of Virological Methods*, **47**: 345–349.

Roeder P. L., Abraham G., Mebratu G. Y. and Kitching R. P. (1994): Foot and mouth disease in Ethiopia from 1988 to 1991. *Tropical Animal Health and Production,* **26** (3): 163-167.

Rufael T., Catley A., Bogale A., Sahle M. and Shiferaw Y. (2008): Foot and Mouth Disease in the Borana pastoral system, southern Ethiopia and implications for livelihoods and international trade. *Tropical Animal Health Production*, **40**: 29–38.

Rweyemamu M. M. (1984): Antigenic variation in foot-and-mouth disease: studies based on the virus neutralization reaction. *Journal of Biology Standard*, **12**: 323–337.

Rweyemamu M. M. and Hingley P. J. (1984): Foot-and-mouth disease virus strain differentiation: analysis of the serological data. *Journal of Biological Standard*, **12**: 225–229.

Rweyemamu M. M., Sylla D., Palya V. and Prandota J. (1994): FAO Animal Production and Health paper 118, Rome, Italy, Pp. 48-51.

Sahle M., Venter E. H., Dwarka R. M. and Vsloo W. (2004): Molecular epidemiology of serotype O FMDV isolated from cattle in Ethiopia between 1979-2001. *Understepoort Journal of Veterinary Research*, **71**:129-138.

Salt J. S. (1993): The carrier state of foot-and-mouth disease - an immunological review. *British Veterinary J*ournal, **149**: 207–223.

Samuel A. R and Knowles N. J. (2001): FMD type O viruses exhibit genetically and geographically distinct evolutionary lineage (topotypes). *Journal of Virology*, **82**: 609-621.

Samuel A. R., Knowles N. J. and Mackay D. J. (1999): Genetic analysis of type O viruses responsible for epidemics of foot-and-mouth disease in North Africa. *Epidemiology of Infection*, **122**: 529-538.

Sangar D. V., Clark R. P., Carroll A. R., Rowlands D. J. and Clarke B. E. (1988): Modification of the leader protein (Lb) of foot-and-mouth disease virus. *Journal of General Virology*, **69**: 2327-2333.

Sanitary and Phytosanitary Standards and Livestock and Meat Marketing Program in Ethiopia (SPS-LMM) (2008): Texas Agricultural Experiment Station (TAES)/Texas Agriculture and Marketing University System, Foot and Mouth Disease surveillance and Sero-epidemiological Situation in Ethiopia, Pp: 1-30.

Thomson G. R. (1994) Foot and mouth disease. In: Infectious diseases of livestock with special reference to Southern Africa, edited by J.A.W. Coetzer, G.R. Thomson. Cape Town, London, New York: Oxford University Press. Pp: 825-992.

Uppal P. K. (2004): Foot-and-mouth disease in small ruminants: *An issue of concern. Indian Journal of Veterinary*, **89**:190-193.

Viral zone. http://viralzone expasy.org/all_by_species/98.html. Accessed on 29 May 2014.

Wondwossen A. and Tariku S. (2000): the status of FMD in Ethiopia: a growing concern. *Ethiopia Veterinary Epidemiology Newsletter*, **1** (2):1-5.

Woodbury E. L. (1995): A review of the possible mechanisms for the persistence of foot and mouth disease virus. *Epidemiology and Infection*, **114**: 1-13.

8. ANNEXES

Annex I: FMD samples identified, sequenced and stored at NVI

Table 1: FMD samples identified, sequenced and stored at National veterinary Institute were involved in the vaccine match.

Name of Virus	Site of isolation	Species	Year of isolation	Serotype	Topotype
ETH/2/2011	Debre zeit	Swine	15/07/2011	O	EA -3
ETH/6/2011	Debre zeit	Bovine	29/07/2011	O	EA - 3
ETH/27/2011	Sidama	Bovine	8/10/2011	O	EA - 3
ETH/31/2011	Sidama	Bovine	8/10/2011	O	EA - 3
ETH/8/2011	Debre zeit	Bovine	14/10/2011	O	EA - 3
ETH/11/2011	Debre zeit	Bovine	14/10/2011	O	EA - 3
ETH/17/2011	Mekele	Bovine	29/11/2011	O	EA – 3 related to Sudan 1999, 2004 and 2008.

Annex II: Procedure for CHEKIT- FMD-3ABC BO-OV ELISA

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

A. Preparation of reagent

1. Allow all reagents to equilibrate to the required incubation temperature.

2. If only a portion of a microtiter stripped plate is required, it is possible to cut the foil sealing the plate and remove the strips which are not needed for the assay.

3. Determine the amount of CHEKIT-Washing and dilution-solution needed for washing the microtiter plates, and diluting conjugate. Dilute the CHEKIT-10x-Concentration 1:10 with water (1part concentrate with 9 part water, e.g. 100ml 10x concentrate + 900ml distilled

water). When prepare under sterile conditions, the CHEKIT-Washing and Dilution Solution can be stored during one week at +4^{O}C to +8^{O}C.

B. Dilution, distribution and incubation of bovine/ovine samples and controls

1. Pre-dilute each sample and control 1:100 in tube using CHEKIT-FMD-3ABC-Sample-Diluent. For example, add 5µl of sample or control to 495µl CHEKIT-FMD-3ABC-Sample-Diluent. Dispense 100µl of pre-diluted samples and controls into the appropriate wells of the microtiter plate. Final dilution =1:100.

2. Cover the microtiter plate with a lid and incubate for 60 minutes (± 10minutes) at 37^{O}C (± 2^{O}C) in a humid chamber.

Format of the microtiter plate and one example of sample distribution

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	N	13									
B	P	P	14									
C	1	7	15									
D	2	8	16									
E	3	9	17									
F	4	10	18									
G	5	11	etc.									
H	6	12										

N = negative control, P = positive control and 1, 2, 3, etc = sample number

Microwell plate uniformly coated with antigen

C. Washing the microtiter plates

After the incubation, wash each microtiter plate as follows. Thoroughly shake it to empty the wells, then fill each well with at least 300µl CHEKIT-FMD-3ABC-Sample-Diluent, avoiding the formation of air bubbles. Repeat this step twice (total number of wash cycles =3). Shake the microtiter plate thoroughly to empty the wells then tap firmly on absorbent paper. Do not leave

the dried plate for a longer period of time on the bench, before dispensing the next solution in the wells.

D. Distribution and incubation of the readly to use CHEKIT-FMD-3ABC-Anti-Ruminant-IgG-PO-Conjugate

Dispense 100µl of the ready to use CHEKIT-FMD-3ABC-Anti-Ruminant-IgG-PO-Conjugate into each well, cover and incubate the covered microtiter plate for 60minutes (± 10 minutes) at $37^{O}C$ (± $2^{O}C$) in a humid chamber.

E. Washing the microtiter plate

As described under C.

F. Addition of the CHEKIT-TMB-Substrate

Dispense 100µl CHEKIT-TMB-Substrate, pre-warmed to $25^{O}C$ into each well. Incubate the substrate at room temperature or optimally at $25^{O}C$ during 15 minutes (± 5 minutes).

G. Reading of the result

Stop the color reaction by adding 100µl CHEKIT-Stopping-TMP-Solution, pre-warmed to room temperature (18ºC – 25ºC) per well. The stopping solution should be dispensed in the same order and at the same speed as was employed with the substrate. Read the results using a photometer at a wavelength of 450nm. In order to validate the assay the optical density (OD) of the positive control should not exceed 2.0 and the OD of the negative control 0.5, respectively. The difference between the positive and the negative control must be ≥ 0.4. Make sure to read the plates within 2hrs after the addition of the stopper solution.

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 (OD_{pos}) as well as the OD of the samples (OD_{sample}) is corrected by subtracting the OD of the negative control (OD_{neg}):

Positive control: $OD_{pos} - OD_{neg}$

Sample: OD_{sample} - OD_{neg}

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

$$\text{Value (\%)} = \frac{\text{ODsample} - \text{ODneg}}{\text{ODpos} - \text{ODneg}} \text{X100\%}$$

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 III: Procedure for Two Dimensional Virus Neutralization Test (VNT)

Procedure for Two Dimensional Virus Neutralization Test (2dmVNT)

1. Virus dilution series

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

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

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

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

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

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

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

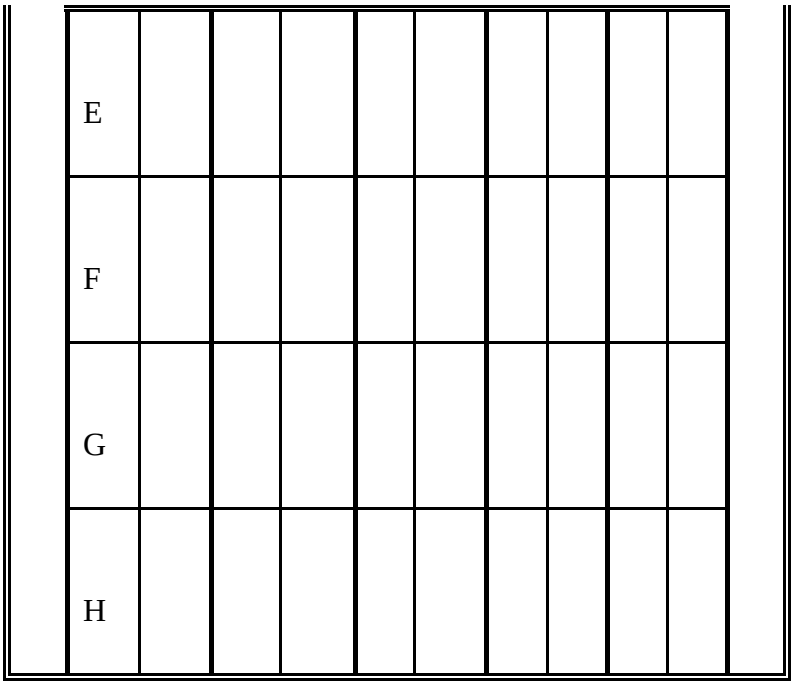
2. Prepare plates as shown.

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

VN plate layout

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

cell ct											virus ct
	A										
	B										
	C										
	D										



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

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

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

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

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

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

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

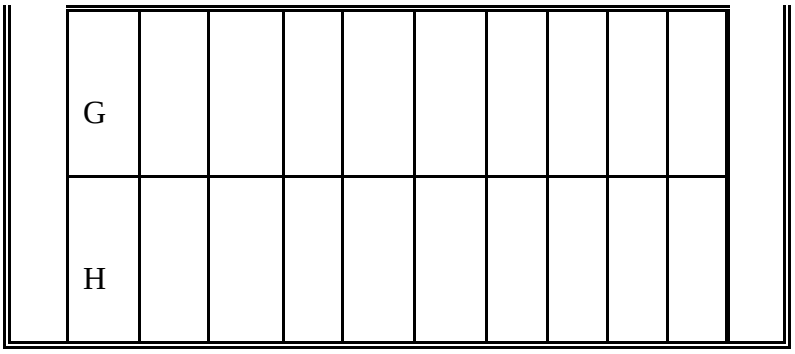
2.6. In the cabinet, add 50µl/well of test virus (starting with the weakest dilution) to each pair of well columns, i.e. $10^{-4.5}$ in well columns 10, 11 and 12 (virus control), $10^{-4.0}$ in well columns 8 and 9 and so on. Discard all tips and bottles into disinfectant. Repeat in similar plates for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

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

3. VT plate layout

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

virus dilln	A										cell ct
	B										
	C										
	D										
	E										
	F										



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

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

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

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

4. After incubation, add 50µl/well of IBRS2 or BHK21 cells at a seeded rate of between 0.7 - $1x10^6$ 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 napthalene 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.

Interpretation

2.1. Virus titre

Each dilution step has a maximum of eight wells where the cells can form into a monolayer. If each individual well contains sufficient infectious virus particles, cell death will occur and no cell monolayer will form. Virus titre is calculated as follows.

8 wells exhibiting 100% CPE at virus log. dilution $10^{-4.9}$

7 wells exhibiting 100% CPE at virus log. dilution $10^{-5.2}$

4 wells exhibiting 100% CPE at virus log. dilution $10^{-5.5}$

1 well exhibiting 100% CPE at virus log. dilution $10^{-5.8}$

$$2.5 = \frac{\text{Total number of wells exhibiting 100\% cpe (20)}}{\text{no. of}\frac{\text{wells}}{\text{dilution}} \quad (8)}$$

Subtract 0.5 (correction factor) =2.0

Multiply by 0.3 (dilution interval) =0.60

Add the highest dilution step with 100% cpe in all wells ($10^{-4.9}$) $=10^{-5.5}$

The virus titre is expressed as $10^{5.5}$ tcid50/ml

From this result, the 2 log dilution used in the serum neutralization plates can be extrapolated. For example the 'strongest' dilution of virus used was $10^{-2.5}$ which would be determined as 5.5 log/ml (virus titre) minus 2.5 (virus dilution factor) resulting in the log. dose of 3 (i.e. 1000 tissue culture infective units). Similarly the rest of the dilution steps used in the neutralization plate can be calculated.

2.2. Antibody titre

In order to calculate this, the antibody titre for each virus dilution must be obtained. Again, the titre is at the point where 50% of the cell monolayers remain. The original 'strongest' serum

dilution was 1/2 (or 0.3 log.) However, this was again diluted 1/2 by the addition of 50µl/well of virus dilution, therefore making the actual serum dilution 1/4 (or 0.6 log.)

Eg.

complete well/s	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
serum log.titre	0.3	0.6	0.75	0.9	1.05	1.2	1.35	1.5	1.65	1.8	1.95	2.1	2.25	2.4	2.55	2.7

The antibody titres are calculated from regression data as the $\log_{10}$ reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infective units of virus.

For the reference serum/homologous test plate, the serum titre is calculated (e.g. 2.3 log.) and is expressed as an 'r' (regression) value of 1.0

For the reference serum/heterologous test plate, the serum titre is calculated (e.g. 2.04) and the following equation used:-

Heterologous titre (2.04) - homologous titre (2.3) = -0.26

Reciprocal log. of -0.26 = 0.55 which is the 'r' value

The serological relationship ('r' value) between the homologous and heterologous virus which fall in the range of 0.3 - 1.0 is indicative of a reasonable level of cross protection.

Annex IV: Test procedure for RT-PCR

Test procedure for RT-PCR

1. Extraction of RNA from FMD Virus Containing Samples

Protocol 1: Viral RNA is extracted from each sample using RNease Spin-columon according to the manufacturer's instructions as follow:

i. Put volume of 400µl of the sample is added to 1500µl in an eppendorf tube and adds equal Volume of lysis buffer RLT (Containing 2 – mercaptoethanol) to the Sample and the mixture is shaken.

ii. Vortex until the cell pellet is dispersed and the cells appear lysed then proceed to Homogenization at room temperature by transferring the lysate into a clean homogenization tube, and perform manual homogenization. Centrifuge the homogenate at 12500 rpm for 3 minutes.

iii. Proceed to RNA Purification as follow

a. Add one volume 70% ethanol to each volume of cell homogenate i.e 400µl 70% ethanol to each volume of cell homogenate

b. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.

c. Transfer up to 700µl of sample (including any remaining precipitate) to the spin cartridge (with collecting tube).

d. Centrifuge in a Mini spine at 12500rpm for 90 seconds at room temperature. Discard flow through, and reinsert the spin cartridge into the same collection tube and reuse collection tube. Repeat with remaining volume.

e. Repeat Steps c – d until the entire sample has been processed.

f. Add 700µl wash buffer I to the spin cartridge.

g. Centrifuge at 12500 rpm for 3 minutes at room temperature. Discard the flow – through and the collection tube. Place the spin cartridge into a new collection tube

h. Add 500µl wash buffer II with ethanol to the spine Cartridge.

i. Centrifuge at 12500 rpm for 3 minutes at room temperature. Discard the flow – through.

j. Repeat Steps i and j once

k. Centrifuge the spin cartridge at 13400 rpm for 3 minutes to dry the membrane with bound RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.

l. Add 40µl RNase – free water (DEPC-H_2O) to the center of the spin cartridge.

m. Incubate at room temperature for 3 minutes.

n. Centrifuge the spin Cartridge for 3 minutes at 13400 rpm at room temperature to elute the RNA from the membrane into the recovery tube.

o. Store in clean tube at -20^{O}C. Label the tube clearly with “RNA”, the virus name, today’s date and your name.

2. Reverse Transcription of extracted RNA into cDNA

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

a. On the first tube, First – Strand cDNA Synthesis

1. Mix and briefly centrifuge each component before use.
2. Combine the following in 0.2 – or 0.5-ml tube:

Component	Amount
Up to 5 µg total RNA	n µl
Primer*	1 µl
*50 µM oligo(dt)20, or 2 µM gene – specific primer (GSP), or 50 ng/ µl random hexamers	
10 mM dNTP mix	1 µl
DEPC – treated water (RNase free water)	3 µl

3. Distribute 5 µl of the above mix to PCR tube, add 5 µl templates (RNA extract) and incubate the tube at 65^{O}C for 5 minutes, then place on ice for at least 1 minute.
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

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

5. Add 10 µl cDNA Synthesis mix to step 3 Mix, gently mix, and collect by brief centrifugation. Incubate as follow:
   Oligo(dt)$_{20}$ or GSP primed: 50 minutes at 50^{O}C
   Random hexamer primed: 10 minutes at 25^{O}C, followed by 50 minutes at 50^{O}C.

6. Terminate the reaction at 85^{O}C for 5 minutes. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate the tubes for 20 minutes at 37^{O}C.
8. cDNA synthesis reaction can be stored at – 30^{O}C to – 10^{O}C or used for PCR immediately.

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

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

Component	Volume
RNase free water	2 µl
Primers*	2 µl for each primers
a. FMDV7 – For and Rev for screen the sample as positive or negative	
b. FMDV O – For and Rev for Serotye O	
c. FMDV SAT – 2 – For and Rev for Serotype SAT – 2	
d. FMDV A – For and Rev for Serotype A	
Taq DNA polymerase (1Q supermix) which contain 5 Unit/ µl	10 µl
PCR buffer minus Mg^{++},50mM $MgCl_2$ and 10mM dNTP mix	
Template (cDNA)	4 µl

2. Mix 16 µl of PCR master Mix and 4 µl template of the contents of the tube. Centrifuge briefly to collect the reaction components.
3. For universal primer, Place reaction mixture in preheated (94^{O}C) thermal cycler.
   Perform an initial denaturation step: 94^{O}C for 4 minutes,
   Denaturation 94^{O}C for 1minute,
   Annealing 54^{O}C for 1 minute 30 *cycles*
   Extension 72^{O}C for 1 minute
   Final Extension 72^{O}C for 10 minutes
4. Upon completion, maintain reactions at 4^{o}C

Protocol 3: Agarose Gel Electrophoresis of PCR products

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

1. Prepare 100ml of 1.5 % agarose in 1x TAE buffer

2. Heat in microwave for 3min on full power 800 watt.

3. Add 5µl GelRed™ per 100ml 1.5% agarose gel (for post – staining Protocol: dilute the gelRed™ 10,000X stock solution 3,300 fold to make a 3X staining solution in water. Generally 50ml staining solution is adequate volume for one mini gel. Note: including 0.1 M NaCl in the staining solution enhances sensitivity, but may promote dye precipitation if gel stain is reused. For precast protocol for agarose Gel: dilute the GelRed™ 10,000X stock reagent into the molten agarose gel solution at 1: 10,000 and mix thoroughly. GelRed™ can be added while the gel solution is still hot).

4. Pour gel and insert well former (Comb). Remove the air bubble and allow to set on a flat surface for about 30 min.

5. Pour buffer 1x TAE (50X TAE buffer diluted into 1X as add 20ml of 50X TAE buffer into 980ml distilled water to get 1X 1 liter TAE buffer) into the tank and remove comb from gel

6. Prepare Samples in tubes, multiwall plate or on parafilm

   1µl loading buffer

   5µl PCR Product

7. Prepare molecular weight marker 0.5µl molecular weight marker VI (Boehringer), 1µl loading buffer and 4µl H_2O

8. Loading samples into the wells formed in the gel. It is often useful to load the molecular weight markers in both the 1st and last lanes.

9. Electrophorese at 120 volts for 1 hour and 20 minutes.

10. View and photograph the gel on an UV-transilluminator. Use UV-safety spectacles.

Annex V: Oligonucleotide primers used for reverse transcription–PCR

Table 2: Location and sequences of oligonucleotide primers used for reverse transcription–PCR and sequencing

Oligonucleotide	Sequence (5'→3')	Sense	Location	Serotype	Method used
EUR-2B52R (Reverese)	GACATGTCCTCCTGCATCTGGTTGAT	–	2B	O, A, C	RT-PCR
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	+	VP3	O	RT-PCR and sequencing
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	+	VP3	O	RT-PCR
O-1C283F	GCCCAGTACTACACACAGTACAG	+	VP3	O	RT-PCR
A-1C562F	TACCAAATTACACACGGGAA	+	VP3	A	RT-PCR
A-1C612F	TAGCGCCGGCAAAGACTTTGA	+	VP3	A	RT-PCR
SAT2-P1-1223F	TGAACTACCACTTCATGTACACAG	+	VP3	SAT 2	RT-PCR
		+	5' UTR	Universal	
FMDV7 - for	GCCTGGTCTTTCCAGGTCT				RT – PCR
FMDV7 - rev	CCAGTCCCCTTCTCAGATC	-	5' UTR	Universal	RT – PCR

FMDVO	CTGCCACCGTCGAGAACTAC	+	VP1	O	RT - PCR
FMDVO	CAGGCGCCACTATCTTCTGT	-	VP1	O	RT-PCR
FMDV SAT2	CCACATACTACTTTTGTGACCTGGA	+	VP1	SAT - 2	RT- PCR
FMDV SAT2	ACAGCGGCCATGCACGACAG	-	2B	SAT - 2	RT- PCR
FMD A - for	TACCAAATTACACACGGGAA	+	VP3	A	RT- PCR
FMD A - rev	GACATGTCCTCCTGCATCTG	-	2B	A	RT- PCR
SAT 2B208R	ACAGCGGCCATGCACGACAG	-	VP3, 2B	SAT 1, SAT 2	RT-PCR

Annex VI: BHK-21cell growth and maintenance medium preparation protocol

BHK-21 maintenance medium (Glasgow modification) preparation protocol

(Composition for making 2 liters of medium): NaCl - 12.8 g, KCl - 0.800 g, $CaCl_2.2H_2O$ - 0.530 g ,$MgSO_4.7H_2O$ - 0.400 g, $NaH_2PO_4.2H_2O$ - 0.280 g , Glucose - 9.0 g, L-glutamine - 1.170 g (Part of distilled water is added to dissolve these salts) Phenol red (Sodium salt) - 0.034 g , $NaHCO_3$ - 5.5 g, Penicillin - 2 Lakh I.U., Streptomycin - 0.2 g, Amino acid stock - 100 ml, Vitamin stock - 8 ml, Tryptose phosphate broth - 6.0 g, Distilled water - Up to 2000 ml and pH adjusted to 7.4

BHK-21 growth medium (Glasgow modification) preparation protocol

BHK-21 maintenance medium - 900 ml (pH 7.4), Healthy calf serum - 100 ml Sterilized by positive pressure seitz filtration and Incubated for overnight at 37^0C before use. Stored at 4^oC.

Trypsin-versene solution preparation protocol

NaCl - 5.0 g KCl - 0.125 g $Na_2HPO_4.2H_2O$ - 0.950 g KH_2PO_4 - 0.125 g Trypsin - 0.850 g Versene (EDTA) - 0.700 g Phenol red (0.5%) - 0.5 ml Distilled water - To make 500 ml pH adjusted to 7.4 then Mixe by stirring on a magnetic stirrer and sterilize by positive pressure seitz filtration. Incubate overnight at 37^{o}C before use. Stored at +4^{0}C.

Annex VII: Serum sample collection procedure

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 -20^{O}c until laboratory investigation.

Annex VIII: Vaccine preparation procedure

1. Virus for antigen production in BHK-21 cell monolayer or suspension cultures under sterile conditions at +37^{O}c 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 30^{O}C 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 IX: Sample collection format

Table 3: Sample collection format

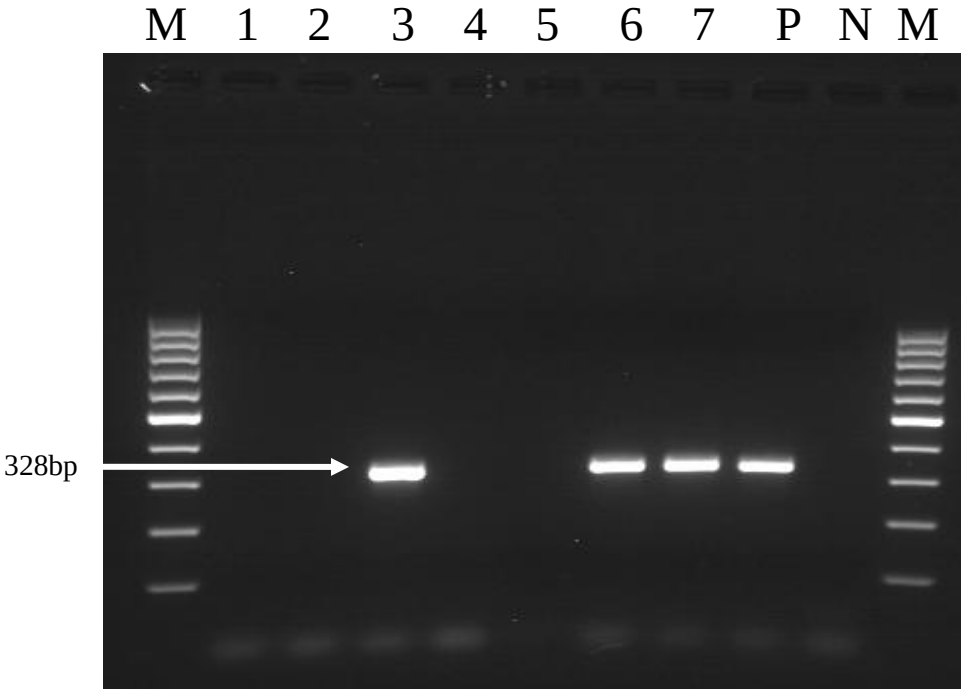
No	Code No.	Area			age	sex	breed	Types of sample		Date of sample collection	Remark
		Region	Zone/woreda	PA				lesion	Vesicular		

fluid

Annex X: Figures: Results of gel electrophoresis

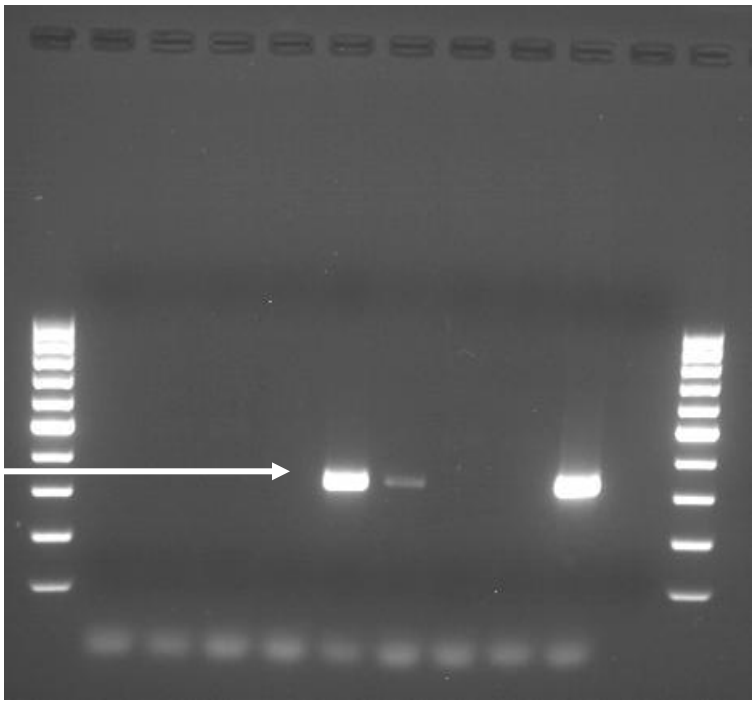
Figures: Results of gel electrophoresis

Figure 5. Detection of FMDV genome by RT – PCR. Primer mixture FMDV7F/ FMDV7R was used for targeting around 328 bp 5' UTR coding region of the virus.



M = Marker started with 100bp, N. Negative control, P=Positive control
1. ETH01/13=No amplification
2. ETH 02/13= No amplification
3. ETH03/13=Positive around 328bp
4. ETH04/13= No amplification
5. ETH05/13= No amplification
6. ETH06/13=Positive around 328bp
7. ETH07/13=Positive around 328bp

M 1 2 3 4 5 6 7 8 P N M



M=Marker started with 100bp

Positive control N. Negative control without template No amplification

1..ETH13/13Noamplification

2.ETH14/13 No amplification

3.ETH15/13 No amplification

4.ETH01/12 No amplification

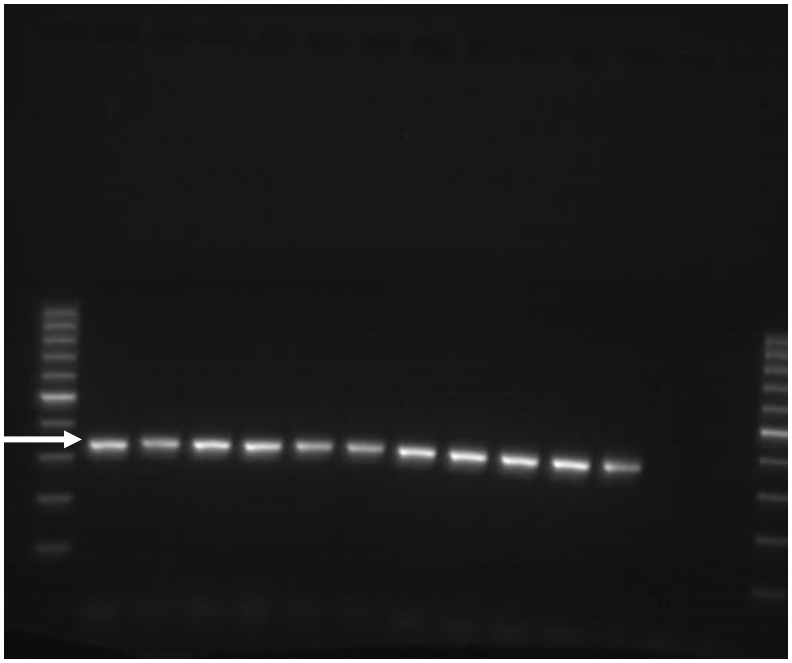
5.ETH17/13 Positive around 328bp

6. ETH18/13 Positive around 328bp

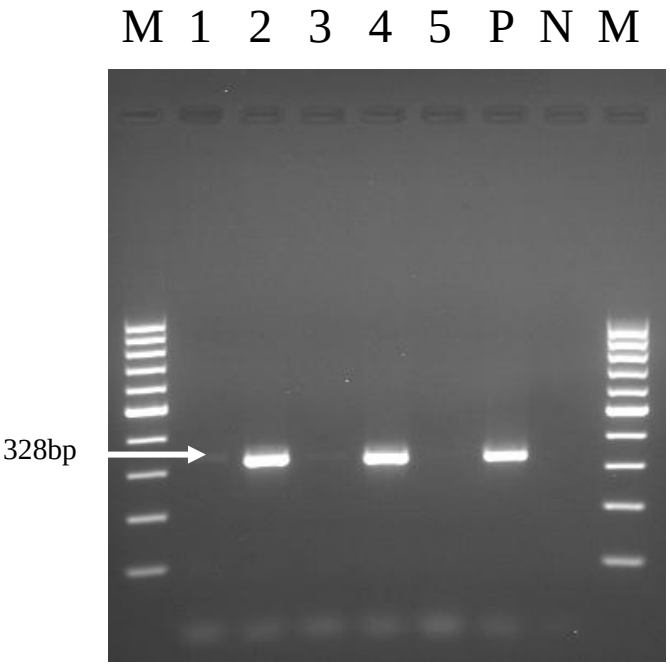
7. ETH19/13 No amplification

8.ETH20/13 No amplification

M 1 2 3 4 5 6 7 8 9 10 P N M

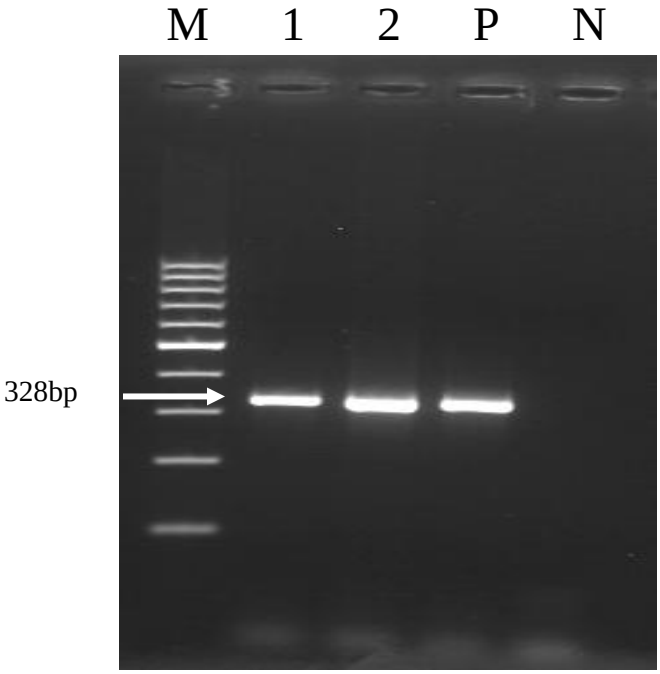


M=marker started with 100bp, and P (positive control) were Positive around 328bp and N negative control without template no amplification.1 (ETH 21/13) – 10 (ETH30/13) were positive around 328 bp.



M = Marker started with 100bp, P. Positive control= positive around 328bp and N. Negative control without template= No amplification

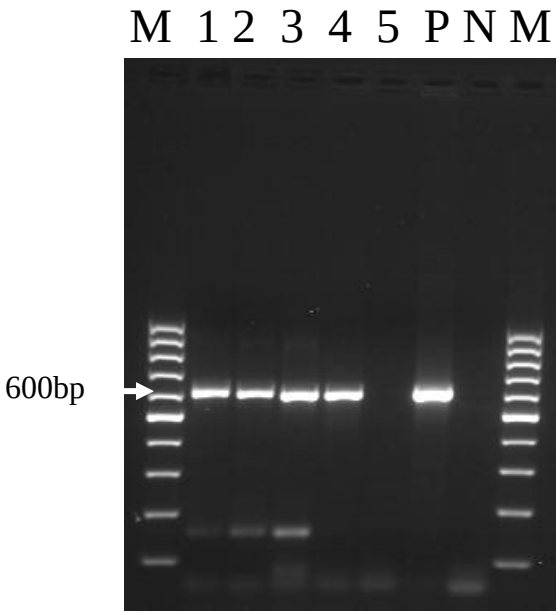
1.ETH16/13 Weakly Positive around 328bp
2. ETH02/12 Positive around 328bp
3. ETH03/12 weakly Positive around 328bp
4. ETH04/12 Positive around 328bp
5. ETH01/14 No amplification



M= Marker started with 100bp, P. Positive control= positive around 328bp N. Negative control without template= No amplification

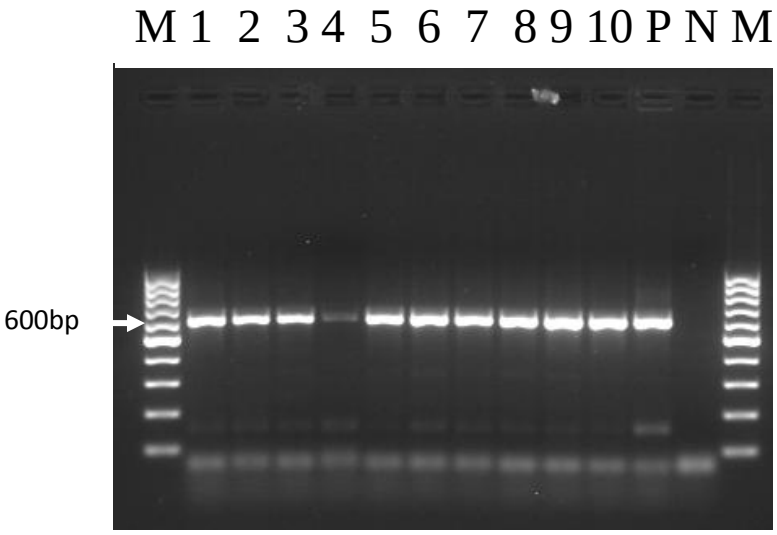
1. ETH 08/13 Positive around 328bp
2. ETH 09/13 Positive around 328bp

Figure 5.1. Detection of FMDV genome by RT – PCR. Primer mixture FMDVOF/ FMDVOR was used for targeting around 600 bp VP1 coding region of the virus.

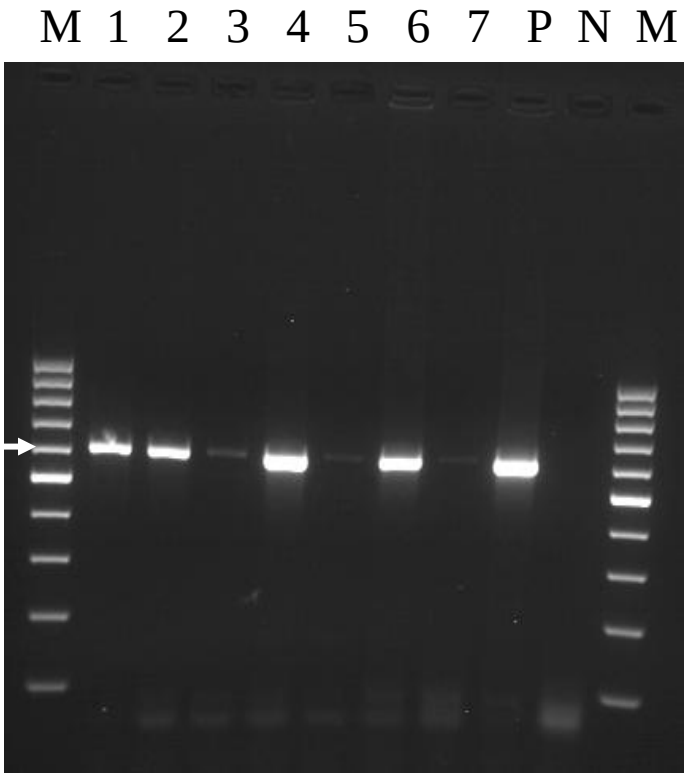


M= Marker started with 100bp, P. Positive control= positive around 600bp N. Negative control without template= No amplification

1. ETH03/13 Positive around 600bp
2. ETH06/13 Positive around 600bp
3. ETH07/13 Positive around 600bp
4. ETH17/13 Positive around 600bp
5. ETH10/13 No amplification

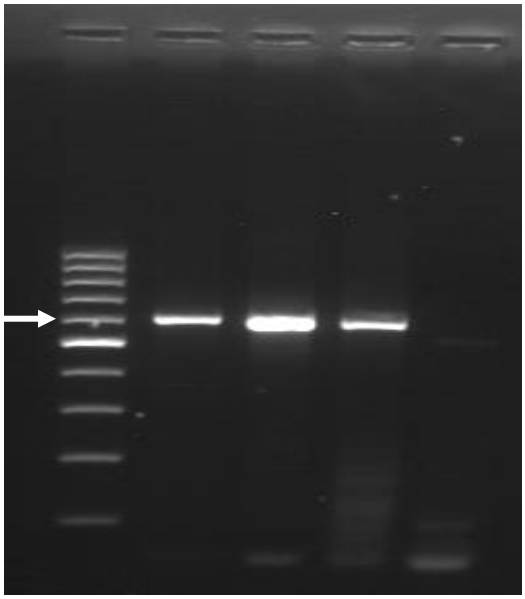


M=marker started with 100bp, P (positive control) were positive around 600bp and N = negative control without template there was no amplification 1 (ETH 21/13) – 10 (ETH30/13)



M= Marker started with 100bp P. Positive control= positive around 600bp and N. Negative control without template No amplification

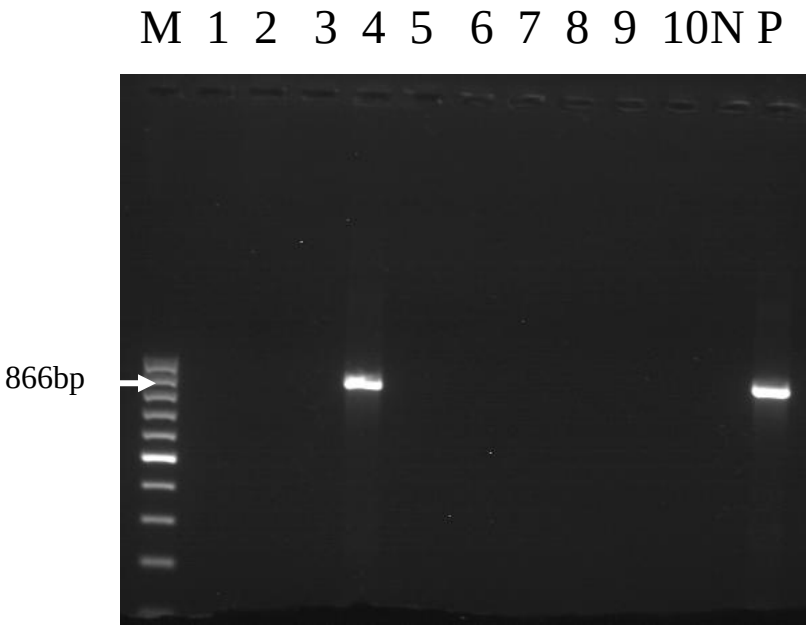
1. ETH08/13 Positive around 600bp
2. ETH09/13 Positive around 600bp
3. ETH18/13 weakly Positive around 600bp
4. ETH02/12 Positive around 600bp
5. ETH03/12 weakly Positive
6. ETH04/12 Positive around 600bp
7. ETH16/13 weakly positive 600bp



M= Marker started with 100bp, P. Positive control= positive around 600bp and N. Negative control without template No amplification

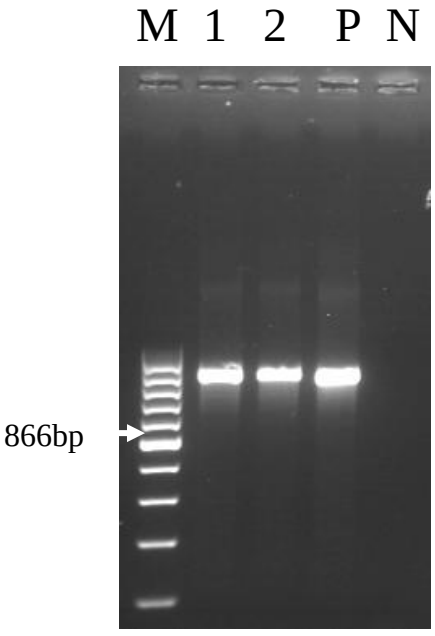
1. ETH16/13 Positive around 600bp
2. ETH11/13 Positive around 600bp

Figure 5.2. Detection of FMDV genome by RT – PCR. Primer mixture FMDVAF/ FMDVAR was used for targeting around 866 bp VP3 coding region of the virus.



M= Marker started with 100bp, and P. Positive control= positive around 866bp

2. ETH06/13 No amplification
3. ETH08/13 No amplification
4. ETH10/13 No amplification
5. ETH11/13 Positive around 866bp
6. ETH18/13 No amplification
7. ETH24/13 No amplification
8. ETH03/13 No amplification
9. ETH12/13 No amplification
10. ETH03/12 No amplification 10. ETH04/12 No amplification, 11. No amplification

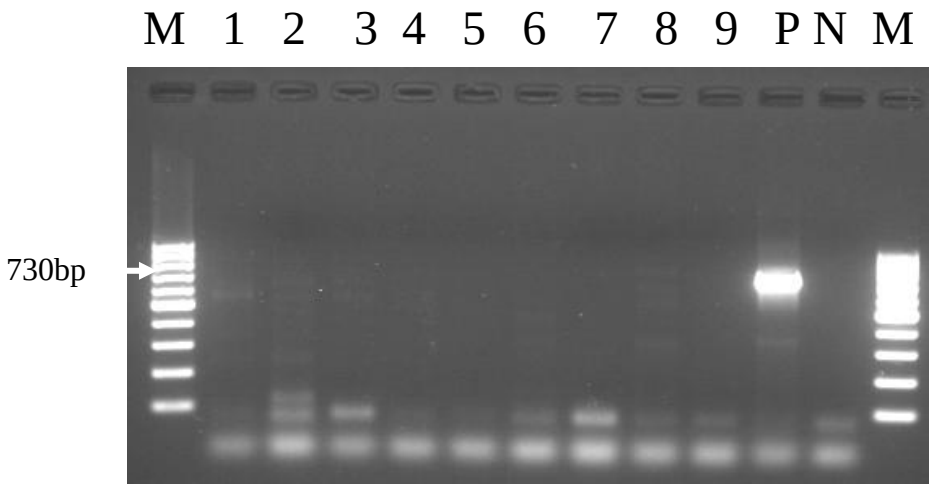


M= Marker started with 100bp P. Positive control= positive around 866bp and N. Negative control without template No amplification

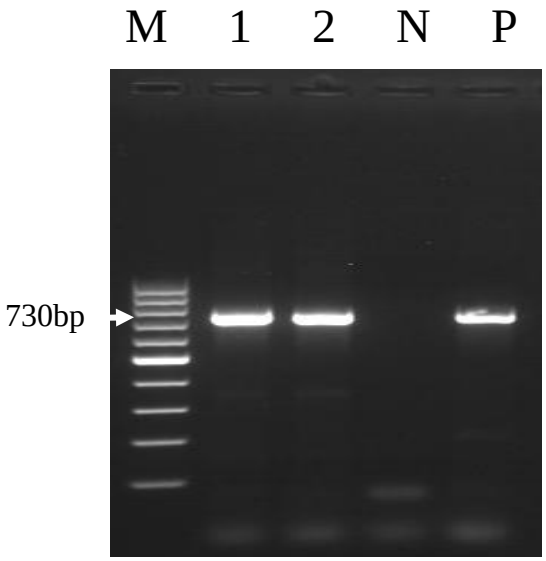
1. ETH10/13 (vaccine strain) positive around 866bp

2. ETH10/13 (vaccine strain) positive around 866bp

Figure 5.3. Detection of FMDV genome by RT – PCR. Primer mixture FMDVSAT2F/ FMDVSAT2R was used for targeting around 730 bp VP1 coding region of the virus.



M= Marker started with 100bp, P= Positive control ETH12/13 were positive around 730 bp and N= negative 1. ETH03/13, 2. ETH06/13, 3. ETH09/13, 4. ETH18/13, 5. ETH16/13, 6. ETH22/13, 7. ETH04/12, 8. ETH10/13 and 9.ETH11/13 were negative



M- Marker started with 100bp, N. Negative control = No amplification P. Positive control positive around 730bp.

1. ETH12/13(vaccine strain) -Positive around 730bp
2. ETH 12/13 (Vaccine strain) - Positive around 730bp