

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

**STUDY ON FOOT AND MOUTH DISEASE VIRUS VACCINE STRAIN SELECTION
OF ETHIOPIAN ISOLATES**

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BY
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TABLE OF CONTENTS	PAGES
ACKNOWLEDGEMENTS.....	I
TABLE OF CONTENTS.....	II
LIST OF TABLES.....	IV
LIST OF FIGURES.....	V
LIST OF ANNEXES.....	VI
LIST OF ABBREVIATIONS.....	VII
ABSTRACT.....	IX
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1. Foot-and-mouth disease.....	4
2.2. Foot-and-mouth disease virus	4
2.2.1. Classification	4
2.2.2. Morphology and genomic organization.....	4
2.2.3. Foot-and-mouth disease virus replication	6
2.2.4. Physico-chemical properties.....	7
2.2.5. Genetic and antigenic variation	7
2.3. Epidemiology	8
2.3.1. Geographical distribution of the serotypes.....	8
2.3.2. Sources of infection and modes of transmission	9
2.3.3. Carriers of FMD virus	10
2.4. Pathogenesis and clinical signs.....	11
2.5. Economic importance	13
2.6. Diagnosis	13
2.6.1. Field diagnosis.....	13
2.6.2. Laboratory diagnosis	14
2.7. Immune response to foot-and-mouth disease	16
2.8. Prevention, control, and eradication of foot-and-mouth disease.....	17
2.8.1. Endemic areas.....	17
2.8.2. Disease free areas	19

2.9. Foot-and-mouth disease virus vaccine strain selection..... 20

2.10. Selection of field viruses for vaccine matching..... 20

2.11. Selection of vaccine strains to be matched..... 21

2.12. Foot and mouth disease virus vaccine matching..... 21

 2.12.1. Vaccine matching by two-dimensional virus neutralization test..... 23

 2.12.2. Vaccine matching by liquid phase blocking enzyme linked immunosorbent assay . 24

 2.12.3. Vaccine matching by complement fixation test 25

3. MATERIALS AND METHODS 27

 3.1. Study areas..... 27

 3.2. Study design..... 28

 3.3. Selection and culturing foot and mouth disease virus vaccine strain candidates 28

 3.4. Vaccine preparation..... 29

 3.5. Antiserum preparation in cattle 30

 3.6. Selection of foot and mouth disease virus field isolates for vaccine matching 31

 3.7. Titration of foot and mouth disease virus and antisera collected 33

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

 3.9. ‘r1’value determination and interpretation..... 36

 3.10. Data management and analysis..... 36

4. RESULTS 37

 4.1. Results of two-dimensional virus neutralization test (Observation) 37

 4.2. Results of antigenic relationship ‘r1’ value by two-dimensional virus neutralization test..... 38

5. DISCUSSION 42

6. CONCLUSIONS AND RECOMMENDATIONS..... 45

7. REFERENCES..... 46

8. ANNEXES 57

9. CURRICULUM VITAE..... 65

10. SIGNED STATEMENT OF DECLARATION 68

LIST OF TABLES

Table 1: Foot and mouth disease virus vaccine strains selected for vaccine matching 29

Table 2: Foot and mouth disease virus serotype O selected field isolates..... 32

Table 3: Foot and mouth disease virus serotype A selected field isolates..... 32

Table 4: Foot and mouth disease virus serotype SAT2 selected field isolates 33

Table 5: ‘r1’ values obtained between serotype O field isolates and vaccine strains..... 38

Table 6: ‘r1’ values obtained between serotype A field isolates and vaccine strains..... 39

Table 7: ‘r1’ values obtained between serotype SAT2 field isolates and vaccine strains in two-dimensional virus neutralization test 40

Table 8: ‘r1’ values obtained by match between candidate vaccine strains 41

LIST OF FIGURES

Figure1: Genomic of foot and mouth disease virus.....6

Figure 2: A map showing the origin of the vaccine strains and field isolates used for the study.....27

Figure 3: BHK-21 Cell before virus inoculation (A), Cytopathic effect observed within 24 hours of virus inoculation (B).....35

Figure 4: Protected BHK-cell observed after 48 hours (A), infected BHK-cell observe after 48 hours of incubation in microtiter plate (B).....37

LIST OF ANNEXES

Annex 1:	3ABC-ELISA test procedure and plate layout.....	57
Annex 2:	Procedure for two-dimensional virus neutralization Test (VNT).....	58
Annex 3:	Serum sample collection procedure.....	64
Annex 4:	Vaccine preparation procedure.....	64

LIST OF ABBREVIATIONS

BEI	Binary Ethyleneimine
BHK	Baby Hamster Kidney
BSS	Borate Saline Solution
cDNA	Complementary Deoxyribonucleic Acid
CFT	Complement Fixation Test
CFU	Complement Fixing Units
CPE	Cytopathic Effect
CSA	Central Statistical Authority
DNA	Deoxyribonucleic Acid
EA-3	East African Lineage
ELISA	Enzyme Linked Immunosorbant Assay
ETH	Ethiopia
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GDP	Gross Domestic Product
IB-RS-2	Porcine Kidney Cells
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LPB-ELISA	Liquid Phase Blocking-Enzyme Linked Immunosorbent Assay
MEM	Minimum Essential Medium
ml	Mililiter
MOA	Ministry of Agriculture
nm	Nanometer
NSP	Nonstructural Protein
nt	Nuclotide
NVI	National Veterinary Institute
OD	Optical Density
OIE	Office International des Epizooties

ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PP	Percentage Positivity
RNA	Ribonucleic Acid
RT-PCR	Reveres Transcriptase Polymerase Chain Reaction
SAT	Southern African Territories
SPSS	Statistical Package for Social Sciences
SRBC	Standardized Sheep Red Blood Cells
ssRNA	Single stranded Ribonucleic Acid
TCID50	Tissue Culture Infective Dose
UK	United Kingdom
UTR	Untranslated Region
VBD	Veronal Buffer Diluents
VNT	Viral Neutralization Test
VP	Viral Capsid Protein
Vpg	Viral Genomic Protein
WRLFMD	World Reference Laboratory for Foot and Mouth Disease

ABSTRACT

This study was conducted at National Veterinary Institute (NVI) on foot and mouth disease viruses of known serotypes and topotypes identified and characterized from samples collected from cattle and swine, at different times from different parts of Ethiopia. The study was designed with the aim of selecting appropriate vaccine strains that can provide a broad spectrum cross protection against the circulating serotypes or strains by assessing the serological or antigenic match between the candidate vaccine strains and field isolates of O, A, and SAT2. The study was done by two-dimensional virus neutralization assay to determine the serological or antigenic relationship 'r1' value between the candidate vaccine strains and field isolates. A total of 21 serotype O, 7 serotype A, and 8 serotype SAT2 FMD viruses were used for the study including candidate vaccine strains. The study findings revealed that out of 19 serotype O field isolates tested for vaccine match with antisera raised against candidate vaccine strains, O-ETH/58/2005 and O-ETH/38/2005, all (100%) were found to be antigenically similar with vaccine viruses with 'r1' values above the cut-off point (0.3)(with mean 'r1' value of 0.83 and 0.84, respectively), indicating that both these vaccine strain candidates can confer protection for serotype O field viruses circulating in the country. Of the 5 serotype A field strains tested, 4 (80%) match serologically with antisera raised against candidate vaccine strains, A-ETH/6/2000 and A-ETH/7/2008 with 'r1' value greater than the cut-off point (mean 'r1' value of 0.58 and 0.70, respectively). But 1 serotype A field virus, A-ETH/13/1981 showed significant antigenic variation with vaccine strains with 'r1' value below the cut-off point (r1 value: 0.14 and 0.25 for A-ETH/6/2000 and A-ETH/7/2008, respectively). Furthermore, the 'r1' value obtained by serological matching between 6 SAT2 serotype field isolates and two vaccine strain candidates, SAT2-ETH/64/2009 and SAT2-ETH/76/2009, revealed that the vaccine strains can protect field viruses circulating in the country (Mean 'r1' value: 0.70 and 0.76, respectively). In general, the overall assessment of antigenic relations between the candidate vaccine strains and circulating field isolates tested showed better matching. The protection level of the selected vaccine strains against the challenge by field viruses should be supported by potency testing or animal challenge.

Keywords: 'r1' value, Ethiopia, field isolates, FMD virus, vaccine matching, vaccine strains.

1. INTRODUCTION

Ethiopia is one of the richest countries in its livestock population. It has approximately 50.8 million cattle, 25.9 million sheep, 21.9 million goats, 21.7 million horses, 5.57 million donkeys, 380 thousand mules, 1 million camels, 39.6 million chickens, and 4.7 million beehives. This makes the country the first in Africa and tenth in the world (CSA, 2010). Livestock plays a critical role for majority of Ethiopian population in terms of income, savings, food security, employment, traction, fertilizer and fuel. This sector is also important to the national economy, contributing 16% of the total Gross Domestic Product (GDP), one-third of agricultural GDP, and 8% of export earnings (MOA, 1997). An improvement in this sector, therefore, has the potential to contribute significantly to the national income and the welfare of majority of rural families. However, due to higher prevalence of different diseases and/or poor management system the country is not utilizing these huge livestock resources (Abebe, 1993). Among these livestock health problems, foot and mouth disease is one of the major livestock diseases of socio-economic importance in the country (Sahle, 2004).

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed domestic and wild animal species. It is characterized by fever, salivation and vesicular eruptions on the feet and mouth. Morbidity is up to 100% in susceptible animal populations but mortality is low in adults. Infected animals show a spectrum of responses to FMD ranging from inapparent infection to severe disease and death. Foot and mouth disease (FMD) is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus, namely, O, A, C, SAT 1 (South African Territories), SAT 2, SAT 3 and Asia 1. Within serotypes, many subtypes can be identified by biochemical and immunological tests (OIE, 2009). The presence of the seven serotypes, multiple subtypes, and variants has added difficulty in laboratory diagnosis and control of the disease (Domingo *et al.*, 2003).

Foot-and-mouth disease (FMD) is endemic to most of sub-Saharan Africa, except in a few countries in southern Africa, where the disease is controlled by the separation of infected wildlife from susceptible livestock as well as by vaccination. A number of countries nowadays realise

that FMD is one of the transboundary diseases that should be controlled to ensure economic stability and access to lucrative international export markets for animal and animal products (Sahle, 2004). Furthermore, they recognise that a regional approach would be needed to succeed. Knowledge about the epidemiology of FMD can greatly assist in developing control policies for such a regional approach (OIE, 2009).

Foot-and-mouth disease has considerable economic consequences which can be attributed to both direct and indirect costs. The direct effects of the disease are loss of milk production, loss of draught power, retardation of growth, abortion in pregnant animals, death in calves and lambs while indirect losses can be attributed to the disruption in trade of animals and derivative products (Paton *et al.*, 2005). Its sequelae are found to be more important than the acute illness. A striking example is the 2001 outbreak of serotype O (the Pan Asian strain) in Great Britain, a country which had been free of FMD since 1981. This devastating epidemic spread to Ireland, France and the Netherlands where the United Kingdom alone was forced to slaughter about 4 million infected and in contact animals. The cost of this epidemic in the UK was estimated to be more than US \$29 billion (Samuel and Knowles, 2001a; Thompson *et al.*, 2003).

Antibody generated by 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).

Studies in Ethiopia have shown that five of the seven serotypes of FMDV were recorded as a cause of FMD outbreaks at different times. The presence of serotypes A, C, O and SAT2 were reported by Roeder *et al.* (1994) and Sahle (2004). Serotypes A, O and SAT2 were reported by Gelaye *et al.* (2005). Furthermore, serotypes A, C, O, SAT1 and SAT2 were reported by Gelagay *et al.* (2009). In all cases serotype O was the most predominant strain circulating in the country.

Such a complex epidemiological situation of FMD in Ethiopia due to the circulation of multi-serotypes of the virus and involvement of different host-species as well as additional factors such as the presence of high numbers of wildlife (especially African buffalo), roaming free cross

borders between the neighboring countries, lack of control of animal movements and lack of cross-protection among different serotypes and even strains within same serotype urges the need of having appropriate vaccine strains for vaccine based animal disease control strategies of the country.

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions (OIE, 2009).

Eventhough many serotypes and/or strains are circulating in the country, without any other sound control strategies in place, currently Ethiopia is producing and using bivalent FMD vaccine by incorporating local strains of only A and O serotypes. Moreover, SAT2 is the third most prevalent serotype in the country according to the research findings of Gelagay *et al.* (2009) from samples collected between 1981 to 2007 but there is no vaccine produced against this strain.

Therefore, it is crucial to select appropriate vaccine strains that can provide a broad spectrum cross-protection against the circulating serotypes or strains by checking the match between the vaccine strains and field isolates.

Thus, the objectives of this study were:

- To select appropriate FMD virus vaccine strains for serotypes A, O, and SAT2 of Ethiopian isolates
- To determine antigenic relationship between field isolates and candidate vaccine viruses by identifying ' r_1 ' value

2. LITERATURE REVIEW

2.1. Foot-and-mouth disease

Foot-and-mouth disease (FMD) is highly contagious viral infection of cloven-hoofed animals, causing considerable production losses in farm animals (Grubman and Baxt, 2004). The disease is characterized by fever, lameness and vesicular lesions on the tongue, feet, snout and teats. It severely constrains international trade in animals and animal products (Paton *et al.*, 2005). Features of FMD that contribute to its highly contagious nature include the very small quantities of virus that can initiate infection, the short generation time of virus in infected host, the large quantities of virus excreted by the infected animals and the multiplicity of the routes by which virus can infect its host (Alexanderson *et al.*, 2001).

2.2. Foot-and-mouth disease virus

2.2.1. Classification

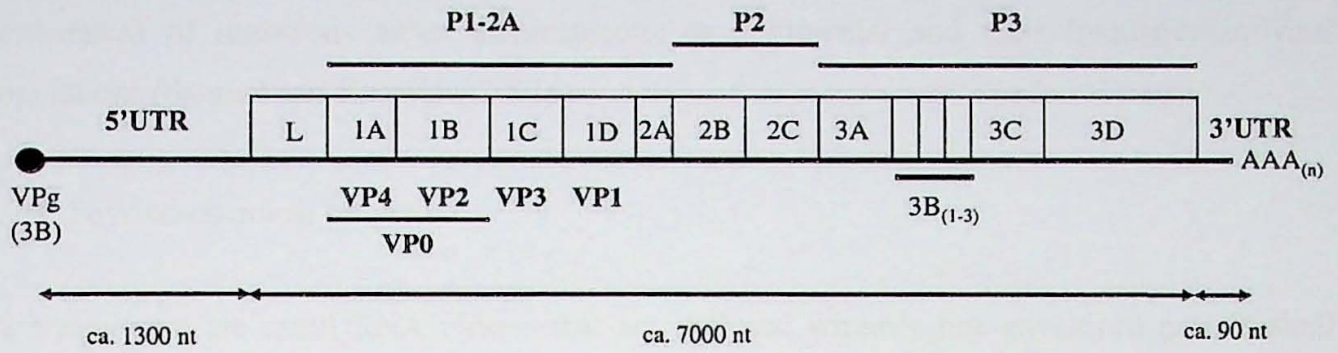
Foot and mouth disease virus belongs to the family *Picornaviridae* which is composed of nine genera: *aphthovirus*, *cordiovirus*, *enterovirus*, *erbovirus*, *hepatovirus*, *kobuvirus*, *parechovirus*, *rhinovirus*, and *teschovirus* (Grubman and Baxt, 2004; David and Petter, 2007).

2.2.2. Morphology and genomic organization

Members of *Picornaviridae* are non enveloped viruses with single-stranded RNA (ssRNA) genomes of positive polarity. The names of the virus family intended to convey the small size of the virus ('pico', stands for small and 'rna' stands for RNA virus) which is the type of nucleic acid that constitute the viral genome (David and Petter, 2007). The viral particle or virion is approximately 8500 nucleotides long, with an icosahedral particle of a smooth surface and a diameter of about 30nm (Jackson *et al.*, 2003).

Figure 1. Shows genomic structural organization of FMD virus (Balinda *et al.*, 2010). It consists of a single open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (5' and 3' UTRs), which are approximately 1300 and 100 nucleotides respectively (Barco *et al.*, 2000). The 5' UTR consists of a 350-380 nucleotide short fragment (S), a 100-420 nucleotides poly C tract (90%) and approximately 700 nucleotides 5' terminus of the genomic long (L) fragment which contains three repeated pseudo knots, a stem loop *cis*-acting replication element (*cre*) and internal ribosomal entry Site. The FMDV 5' UTR plays an important role in cap independent translation initiation of viral polyprotein and viral genome replication (Martinez-Salas and Fenandez-Miragall, 2004). The 3' UTR is about 90 nucleotides long and is thought to contain *cis*-acting elements required for efficient genome replication (Carrillo *et al.*, 2005). The single ORF of *picornaviruses* is organised into three regions, designated as P1, P2 and P3. The P1 region encodes the four structural viral proteins (VP) VP4, VP3, VP2 and VP1, whereas the P2 region encodes the three non-structural proteins 2A, 2B and 2C. Furthermore, the P3 region encodes the non-structural proteins 3A, 3B, 3C protease (3Cpro) and the 3D polymerase (Balinda *et al.*, 2010).

The most important antigenic site of FMDV is in the VP1 (Quian *et al.*, 2003). It has multiple roles including receptor recognition, neutralization and antigenic diversity (Bastos *et al.*, 2003). The VP1 coding sequence analysis can be used to determine the relatedness of FMDVs and thus evaluate the likelihood that a vaccine will provide protective immunity to the vaccinated animals since most FMDV vaccines are developed targeting the VP1 (Chenwen *et al.*, 2007). The Surface with a cell binding motif consisting of a prominent structure, the so called G-H loop, originating from capsid protein VP1. Early work established that the immunogenicity of FMDV was largely associated with the capsid protein VP1 and that the hypervariable G-H loop was immunodominant and an important region for the generation of virus neutralizing and protective antibodies. However, the VP1 G-H loop region and its role toward protective immunity in target species is still unclear and there is increasing evidence that other sites on the capsid are also important for protection (Aggarwal and Barnett, 2002). Another study which experimentally substituted the VP1 G-H loop with glycine residues showed that the removal of this region could dramatically enhance the immune response to less "dominant" regions leading to broader cross reactivity within and between serotypes (Frimann *et al.*, 2007).



Source: (Balinda *et al.*, 2010).

Figure 1: Genomic structure of foot and mouth disease virus

The 5'untranslated region (ca. 1300 nt.), polyprotein region (ca. 7000 nt.) and the 3'untranslated region (ca 90 nt.) are shown. P1-2A, P2 and P3 indicate the precursors or intermediates for the capsid region proteins, 2BC proteins and the 3ABCD proteins, respectively.

2.2.3. Foot-and-mouth disease virus replication

FMDV uses its highly conserved triplet sequence (Arg-Gly-Asp) motif on the G-H loop to attach to specific receptors of the cell membrane. These receptors mediate the release of the viral genome from the protein shell into the cytoplasm. The incoming RNA uses the host cell protein synthesizing machinery causing shut down of the host cell replication. Complementary negative RNA strand synthesis of the positive RNA strand is initiated by a virally encoded RNA polymerase. Further synthesis of positive RNA strands leads to the formation of multi-stranded replicative intermediates. These replicative intermediates generate a pool of positive RNA strands for translation and some for synthesis of additional negative RNA strands. As protein level increases, some positive RNAs are packaged into virions (Mateu *et al.*, 1996).

The negative strands are found in hundred folds in lower concentration than positive strands in infected cells, indicating that each negative strand may serve as a template for the synthesis of many positive strands. The kinetics of RNA synthesis is not well understood and the number of rounds of template copying in infected cells is unknown. This is true for most RNA viruses and

it is of relevance to interpret viral evolution. Particularly it is difficult to relate a rate of occurrences of mutations either advantageous or detrimental and their frequency in viral populations (Samuel and Knowles, 2001a).

2.2.4. Physico-chemical properties

Picornaviruses are small RNA viruses that are enclosed within a non-enveloped protein shell (capsid). The capsid consists of polypeptides, which are devoid of lipo-protein, and hence is stable to lipid solvents like ether and chloroform (Cooper *et al.*, 1978). The virus is PH sensitive; and is inactivated when exposed to PH below 6.5 or above 11. However, in milk and milk products, the virion is protected, and can survive at 70°C for 15 seconds and PH 4.6. In meat, the virus can survive for long periods in chilled or frozen bone marrow and lymphnodes. The FMDV can be inactivated by a number of chemical substances; 2% percent solutions of NaOH or KOH and 4% Na₂CO₃ are effective disinfectants for FMD contaminated objects, but the virus is resistant to alcohol, phenolic and quaternary ammonium disinfectants (Sahle, 2004). The sizes of droplet aerosol also play an important role in the survival or drying out of the virus; droplet aerosol size of 0.5 - 0.7µm is optimal for longer survival of the virus in the air, while smaller aerosols dry out. In dry conditions the virus also survives longer in proteins like in epithelial fragments (Donaldson, 1987).

2.2.5. Genetic and antigenic variation

RNA viruses are characterized by an error-prone RNA replication without proof reading, which gives them great potential for variation. Antigenic variation is one of the striking characters of FMD virus. It is a process by which an infectious organism alters its surface proteins in order to evade a host immune response and is associated with mutation leading to amino acid replacement. These changes may result either in the field or in the laboratory leading to the development of a new strain of virus which totally differs from the circulating field strain challenging the vaccine strains use for controlling the diseases. The high sequence variability found in VP1 region of this virus accounts for the low cross-reactivity observed among different serotypes of FMDV (Neeta and Tilling, 2011).

FMDV RNA polymerase has a high error rate, estimated to be in the order of 10^{-3} to 10^{-5} misincorporations per nucleotide per genome replication, which is not mitigated by a proofreading mechanism (Domingo *et al.*, 2001; Haydon *et al.*, 2001). Thus, nucleotide changes during a single replication event are likely to be common since the virus genome is 8,500 bases long and replicates via a negative-strand intermediate. The high mutation rate of this virus, coupled with a fast replication rate and extensive population size, results in the rapid evolution of FMDV (Domingo *et al.*, 2003). Similar to other RNA virus, it shows a high degree of genetic and structural variability in its capsid proteins, evidenced by the existence of seven different serotypes with a large number of subtypes (Knowles and Samuel, 2003; Carrillo *et al.*, 2005). The structural protein coding region, particularly the sequence for VP1, has been shown to vary significantly between strains and serotypes hence it is used extensively for evolutionary relationship inference (Knowles and Samuel, 2003). Not only mutation but persistent infection, recombination, and quasi-species dynamics have also been suggested as contributing to the genetic variation (Carrillo *et al.*, 2005; Domingo *et al.*, 2005; Jackson *et al.*, 2007).

2.3. Epidemiology

2.3.1. Geographical distribution of the serotypes

There are seven serotypes of foot and mouth disease virus (FMDV), namely O, A, C, Southern African Territories (SAT1, SAT2, and SAT3) and Asia1. Within these serotypes, over 60 subtypes have also been described using biochemical and immunological tests; and new subtypes arise spontaneously (Domingo *et al.*, 2003; Knowles and Samuel, 2003; Mason *et al.*, 2003). FMDV serotypes are not uniformly distributed in the regions of the world where the disease occurs. Six of the seven serotypes (O, A, C, Southern African Territories SAT 1, SAT 2, SAT 3) are endemic in most sub-Saharan African countries with differences in the distribution and prevalence of serotypes (Vosloo *et al.*, 2002) and four serotypes (O, A, C, Asia 1) in Asia and three in South America (O, A, C) (Rweyemamu *et al.*, 2008). Serotypes A, C and O have a global distribution. They have been isolated in most parts around the world (Grubman and Baxt, 2004; Quian *et al.*, 2004). Outbreaks due to Asia 1 have been restricted to Asia (Gelagay *et al.*,

2009). FMD outbreaks due to SAT serotypes are known to occur in South African region spreading to the Eastern and Western regions of the continent (Bruckner *et al.*, 2002; Knowles and Samuel, 2003).

Studies in Ethiopia have shown that serotypes A, C, O and SAT 2 were recorded as a cause of FMD outbreaks between 1974 to 2003 (Sahle, 2004). Serotypes A, C and O were responsible for the FMD outbreaks in cattle from 1957 to 1979 in the country. Antibodies to SAT2 were also detected in 1971 from cattle in North Omo, Southwestern Ethiopia (Roeder *et al.*, 1994). According to Gelaye *et al.* (2005), serotypes A, O, and SAT2 were identified from samples collected from different parts of the country between 1982 to 2000. The recent study conducted by Gelagay *et al.* (2009) on FMD samples collected between 1981 to 2007 throughout the country from different species of animals showed that serotype A, C, O, SAT1 and SAT2 were recorded with various rates. Type O was the most dominant serotype identified with the degree of 73.3% and widely distributed throughout the country, while the rate for serotype A was (19.5%), SAT2 (4.1%), SAT1 (1.8 %) and C (1.4%).

2.3.2. Sources of infection and modes of transmission

FMD is a highly contagious disease and can be transmitted in many ways leading to very rapid spread of the disease. Infected animal is the main source of infection (Singh, 2011). The primary transmission modes are: direct contact between infected animals and susceptible animals, mechanical transmission by indirect means such as contaminated human clothing animal feeds, and aerosol spread (Alexandersen *et al.*, 2002; Singh, 2011).

Direct contact between infected and susceptible animals is the major way of virus transmission and spread of the disease. Infected animals release virus in exhaled air, all excretions and secretions, and from ruptured vesicles. As excretion may commence up to four days before the appearance of clinical signs, the movement of animals that are incubating the disease is of great epidemiological significance. The disease may spread extremely rapidly in intensive farming areas because of high stocking density and the level of challenge both from infected animals and the environment (Bannet and Cox, 1999). Conversely, disease spread in extensive grazing areas

in hotter climates can be more insidious. Congregation of animals, for example, at common grazing and watering points, at gatherings for vaccination, dipping, shearing, or nomadism, favors the spread of the disease to new herds and areas. The disease can also be disseminated very rapidly by movement of infected animals through livestock markets and shows. The movement of infected sheep, that may show few, if any, clinical signs of the disease, can play a role in the spread of FMD (Donaldson and Alexandersen, 2002).

Aerosol transmission normally occurs when there is close contact between animals. When there is massive contamination of the environment, aerosols may also transmit infection to animals in neighboring farms. Long-range windborne transmission of virus is an uncommon but important route of infection in certain environmental and epidemiological conditions like the case of temperate regions and of no significance in the dry regions such as East Africa (Gloster *et al.*, 2007).

Sexual transmission could be a significant route of spread for the SAT type viruses in African buffalo populations. Individual African buffalo have been shown to be carriers for at least five years, and the virus can persist in a herd of African buffalo for at least 24 years (Fiebre Aftosa, 2007).

2.3.3. Carriers of FMD virus

Animals in which the FMD virus persists in the oropharyngeal region for more than 28 days post infection are called carriers. Carrier ability of animals depends on the ability of the virus to persist in the pharyngeal area of animals, which signifies a special virus-host relationship while the duration of persistence varies from species to species. The virus can persist in African buffaloes up to 5 years, in cattle 3.5 years, in sheep up to 9 months and in goats 3 to 6 months (Bastos *et al.*, 2000; Alexandersen *et al.*, 2002).

2.4. Pathogenesis and clinical signs

Primary exposure to live virus results in the establishment of infection and accumulation of FMDV in the noncornified epithelium of the pharyngeal area. Virus then spreads through regional lymph nodes and via the bloodstream to cornified epithelial cells of the skin and mouth, resulting in several cycles of viral amplification and spread (Alexandersen *et al.*, 2003). In cattle infected via aerosols, this location appears to be the nasopharynx (Arzt *et al.*, 2010). Replication at the primary site is followed by viremia, usually accompanied by a fever, with dissemination to secondary replication sites (Gay *et al.*, 2007). In cattle infected by aerosols, viremia coincides with the replication of virus in pneumocytes in the lungs and decreased replication in the nasopharyngeal tissues (Arzt *et al.*, 2010). Viremia lasts 2-3 days, and ends when circulating antibodies appear. The secondary replication sites for FMDV are primarily stratified; cornified squamous epithelia (Alexandersen, 2002). Viral replication in skin and mucous membranes at locations such as the mouth, snout, feet and teats causes the formation of vesicles. Although there are some species differences in timing, peak virus production usually occurs around the time the vesicles rupture and most clinical signs appear (Alexandersen, 2002; Kitching and Alexandersen, 2002; Kitching and Hughes, 2002; Cox and Barnett, 2009). In some cases, replication can peak as early as 2-3 days after infection. FMDV is usually eliminated from secondary sites of replication within 10-14 days (Oliver, 1988) cited in Gay *et al.*, 2007).

For official control purposes, the World Organization for Animal Health (OIE) defines the incubation period for FMD as 14 days (OIE, 2010). The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals; vesicles are not always found in these cases (Musser, 2004).

In cattle, clinical signs appear in 2 to 14 days, depending on the dose of the virus and route of infection (Kitching, 2002). Cattle typically become febrile and develop lesions on the tongue, dental pad, gums, soft palate, nostrils or muzzle, and sometimes the teat (Kitching, 2002; Musser, 2004; Torres, 2008). Hoof lesions occur in the area of the coronary band and interdigital

space, and can cause lameness, reluctance to rise, or stamping or shaking of the feet. The loss of condition can cause a drop in milk production, which does not usually recover during that lactation.

In pigs, the incubation period is usually two days or more, but it can be as short as 18-24 hours, and may be as long as 9 days. In pigs, the most severe lesions usually occur on the feet. The snout and udder may also be affected, and lesions may be seen on the hock and elbow if the animals are on rough concrete. Mouth lesions are typically small and less apparent than in cattle, and drooling is rare. Fever may be seen in pigs, but the temperature elevation can be short or inconsistent (Musser, 2004; Torres, 2008).

In contrast to pigs and cattle, the clinical signs in sheep and goats tend to be mild. The signs can vary with the topotype. The most common clinical signs are fever and mild to severe lameness of one or more legs. Vesicles can develop in the interdigital cleft and on the heel bulbs and coronary band, but they may rupture and be hidden by foot lesions from other causes. Mouth lesions are often not noticeable or severe in sheep, and generally appear as shallow erosions (Kitching, 2002; Thomson *et al.*, 2003; Musser, 2004; Torres, 2008).

The clinical signs in wildlife resemble those seen in domesticated livestock. Vesicles and erosions may be found at various sites, particularly on the feet, and in and around the mouth. Infections with SAT-type viruses in African buffalo are often subclinical, although small mouth and/or foot lesions have been reported. Severe illness has been documented in a population of mountain gazelles, as well as in impala, blackbuck, white tailed-deer, pudu, warthogs, a kangaroo and some other species. Young animals of any species can die suddenly of myocarditis (Schaftenaar, 2002; Thomson *et al.*, 2003).

2.5. Economic importance

Foot-and-mouth disease (FMD) is an economically significant disease of cattle, sheep, goats and pigs, and cloven-hoofed wildlife species. It causes production losses, particularly to the dairy and pig industries (loss of productivity in terms of meat and milk, loss of weight, delayed conception) and high mortality in young animals, and is a major constraint to international trade in live animals and their products (James and Rushton, 2002). In addition to disruption of animal trade, FMD outbreaks have widespread economic and social impacts both in the short and long term, including disruptions of animal feed, veterinary pharmaceutical and tourism associated industries. Costs incurred usually associated to application of control measures such as quarantines, slaughter, compensation, vaccination as well as conducting scientific surveillance after an outbreak in order to prove that the disease and the virus have been eliminated (Prempeh and Robert, 2001). For instance, in 1997 FMD virus caused widespread outbreaks in Taiwan, which resulted in the immediate closure of the export trade to Japan and South Korea and a loss of four billion dollars, 90% of which was lost export earnings. In 2001, the Netherlands slaughtered 200,000 animals vaccinated against FMD as part of the control programme during an outbreak that had spread from the UK, in order to re-establish the country's FMD trading position (Pluimers *et al.*, 2002; Perry and Randolph, 2003; Kitching *et al.*, 2007).

2.6. Diagnosis

2.6.1. Field diagnosis

FMD cannot be distinguished clinically from other vesicular diseases including vesicular stomatitis, swine vesicular disease and vesicular exanthema. In domesticated animals, the symptoms may also resemble foot rot, traumatic stomatitis, and chemical and thermal burns. In cattle, oral lesions can resemble rinderpest, infectious bovine rhinotracheitis, bovine viral diarrhea, malignant catarrhal fever and epizootic hemorrhagic disease. In sheep, the lesions can be confused with bluetongue, contagious ecthyma, and lip and leg ulceration (Barnett and Cox,

1999). Therefore, laboratory confirmation is necessary, as all vesicular diseases have almost identical clinical signs (Fiebre Aftosa, 2007)

2.6.2. Laboratory diagnosis

Early detection of the infected animals, if possible even before the appearance of clinical signs, is essential for effective control of the disease and requires a rapid and sensitive method of diagnosis (Chen *et al.*, 2011). Diagnosis of FMD is by virus isolation or by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status (FiebreAftosa, 2007; OIE, 2009).

Virus isolation

The isolation and characterization of the virus is the "golden standard" for the diagnosis of viral diseases. The suspensions of field samples suspected to contain FMD virus are inoculated into cell cultures (primary pig kidney cells), incubated at 37⁰C and examined for cytopathic effect (CPE), 24 to 48 hours post inoculation. Sensitive cell culture systems that can be used for FMDV propagation include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IBRS- 2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The use of IB-RS-2 cells aids the differentiation of swine vesicular disease virus from FMDV as it will only grow in cells of porcine origin (OIE, 2009).

Serological assays

The detection of specific antibodies to structural proteins in non vaccinated animals is indicative of prior infection with foot and mouth disease virus. Tests for antibodies to some nonstructural proteins (NSPs) of foot and mouth disease virus are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural

proteins, are highly conserved and therefore are not serotype specific and as a result, the demonstration of these antibodies is not serotype restricted. In situations of FMD infection the use of serological tests such as 3ABC-ELISA that detect antibodies against FMD viral NSPs is useful in discriminating infected from non-infected animals. NSPs are exclusively produced as a result of infection and therefore the presence of anti-NSP antibodies in animal sera indicates past or present infection (Robiolo *et al.*, 2006). Engel *et al.* (2008) described that such serological test has very high sensitivity and specificity of 95% and 97%, respectively for identifying the infected animals from non infected ones.

For 3 ABC- ELISA, micro plates are supplied pre-coated with recombinant FMDV 3ABC viral antigens. Dilutions of the samples to be tested are incubated in the wells of these plates. Any antibody specific for 3ABC binds to the antigen the wells and forms an antigen antibody complex in the plate well surface. Unbound material is removed from the wells by washing. A peroxidase labelled anti-IgG conjugate is added, which binds to the antibodies of the samples complexes with 3ABC antigen. Unbounded conjugate is removed by washing, and the chromogen containing substrate is added to the wells. The degree of colour which develops (optical density measured at 405 nm, is directly proportional to the amount of antibody specific for 3ABC present in the sample. The diagnostic relevance of the result is obtained by comparing the optical density (OD) which develops in the wells containing the samples with the OD from the wells containing the positive control.

The 3ABC-ELISA is also rapid test for screening of large number of sera. In areas where more than one serotypes exist, the test is also cheaper compared to the conventional liquid phase blocking ELISA, which has the disadvantage that each serum sample must be tested against all existing serotypes (Sangare, 2002).

Nucleic acid detection

The presence of viral genomic material can be detected using RT-PCR assays. RT-PCR can be used to amplify genome fragments of FMD virus in diagnostic materials including epithelium, serum and probang samples. RT combined with real-time PCR has sensitivity comparable to that

of virus isolation and automated procedures enhance sample throughput (Reid *et al.*, 2001; Reid *et al.*, 2003). Specific primers have been designed to distinguish between each of the seven serotypes (Callahan *et al.*, 2002). Nucleic acid tests can identify the serotype and strains involved in FMD outbreaks providing a tool for tracing the spread of the virus, more detailed characterization and vaccine selection.

The polymerase chain reaction (PCR) is a technique which amplifies a specific genome segment of virus in a diagnostic sample. It relies on prior knowledge of the nucleotide sequences flanking the region to be amplified. For the identification of FMD virus genome, a c-DNA segment is first produced from a short DNA oligonucleotide primer which is complementary to the FMD virus genome in the sample. Following this, and using an additional oligonucleotide primer complementary to the distal end of the c-DNA, a further complementary sequence is produced (Ferris and Donaldson, 2004). These two complementary strands of DNA are then amplified through a series of primer annealing, extension and thermal denaturation in the presence of a mixture of nucleotides and thermostable DNA polymerase. Each cycle doubles the quantity of amplified segment to a level that can be detected on an agarose gel. The specificity of the amplified sequence can be confirmed with a labeled DNA probe, by restriction endonuclease digestion or by nucleotide sequencing a region or the entire product (Reid *et al.*, 2001).

2.7. Immune response to foot-and-mouth disease

Immunologically, there is no cross protection between serotypes. Cattle which have recovered from infection with one of the seven serotypes of FMD are not immune to the other serotypes but remain protected against the first serotype for a considerable period of time. Serotype specific immunity is based on the presence of neutralizing antibodies to one of the viral capsid protein, VP1. Serum immunoglobulin M (IgM) may be detected 3 to 5 days after infection, reaching a peak between 5 to 10 days. Serum IgG appear from 4 days onwards and reach maximum levels between 15 to 20 days (Doel, 1996). Immunity against FMD is documented as being primarily mediated by neutralizing anti-FMDV antibodies as reviewed in (Rigden *et al.*, 2003). Eventual destruction of the virus requires the phagocytosis of antibody-opsonised virus by macrophages.

Although serum antibody levels play an important role in host protection against FMD virus infection, the cellular responses mediated by T-helper and T- cytotoxic cells also play a role in the immune response to FMD virus infection (Sanzparra *et al.*, 1998).

2.8. Prevention, control, and eradication of foot-and-mouth disease

The control of foot and mouth disease is dependent on the understanding of the disease epidemiology in specific areas or region. The selection of appropriate vaccine strain, vaccine policies, improved techniques in vaccine production, identification of FMD infected zones and serological surveillance, and improved information technologies are amongst the most important considerations in the control and eradication of FMD (Domingo *et al.*, 2003). A successful disease eradication strategy relies on high levels of vaccination, effectiveness of outbreak responses, and control of animal movement. This strategy must have a regional, not national, focus and must be based on risk analysis methodology. The multilateral administration of vaccination campaigns and field activities to ensure wide and simultaneous vaccine application, along with primary prevention and joint border activities, is the key to eradicating FMD and maintaining areas free of the disease (Correa *et al.*, 2002).

2.8.1. Endemic areas

In FMD endemic regions, the disease is controlled by vaccination and movement restriction of animals. When vaccinating animals, it is important that the vaccine contain the same subtype of virus as is in the area. This necessitates frequent checking of the serotype and subtype during an outbreak because FMD virus frequently changes during natural passage through various species. Intratypic variation of the field strains of FMD viruses must also be considered in the selection of seed virus for vaccine production (Grubman and Mason, 2002.). Inactivated bivalent, trivalent, or polyvalent vaccine, which contains the representative strains of the serotypes that are circulating in the region, must be used. Ring-vaccinations should be performed without delay and should include all susceptible species. Preferably, the vaccination should be carried out from the outside of the “ring” towards the centre.

The conventional foot and mouth vaccines are produced by inoculating vaccine strain virus to infect a suspension or monolayer cell culture of BHK21- cells and the resulting preparations are clarified, inactivated with ethyleneimine and blended with the adjuvant (OIE, 2009). These chemically inactivated whole-virus vaccines play a key role in the control and prevention of FMD. However, the traditional vaccines have several disadvantages, such as the requirement for storage under refrigeration, the need for periodic revaccination, and the difficulty in differentiating infected from vaccinated animals. Furthermore, the immunogenic diversity of the seven distinct serotypes of FMDV necessitates serologic matching for the formulation of efficacious vaccines. Importantly, there is a potential risk of the escape of live virus from biosafety facilities during vaccine production or from residual live virus inside the vaccines. Another problem is that the conventional FMD vaccines do not induce sterile immunity and thus do not prevent a carrier status. For these and other reasons, alternative vaccines that do not require live virus material, such as subunit vaccines, synthetic peptides, DNA vaccines, and recombinant virus vaccines, have been explored extensively (Doel *et al.*, 1990).

Among the alternative vaccines explored the first one is the one that has been developed using a genetically engineered FMD subunit vaccine that only contains the portions of the viral genome required for virus capsid assembly and lacks the coding region for most of the viral nonstructural proteins including the highly immunogenic 3D protein. One inoculation of this subunit vaccine delivered in a replication-defective human adenovirus vector can induce rapid, within 7 days, and relatively long-lasting protection in swine (Grubman, 2005).

The other is potential of using naked DNA as a means of eliciting humoral and cellular immunity to confer protection has been explored using a number of pathogens including FMDV. It has been reported that a neutralizing antibody response was detected in mice following administration of a DNA vaccine expressing empty FMDV capsids (Chinsangaram *et al.*, 1998) and a DNA vaccine incorporating an FMDV attenuated full length infectious clone could induce protection in swine (Cedillo-Barron *et al.*, 2001). The concept of DNA vaccination offers several potential advantages for FMD vaccines. For example, aside from its stability, it would avoid manipulation of the infectious virus reducing production costs and the risk of potential escape of the virus and also by using an incomplete virus genome as the immunogen would potentially

allow discrimination between infected and vaccinated animals. However, this avenue of research is only at its infancy and one of its limitations is the requirement for multiple administrations of this type of vaccine. Therefore, there is a need to examine further DNA vaccines, using alternative routes of administration, either alone or as hybrid molecules using other delivery systems and with the inclusion of other adjuvants. Co-expression of such immunogens with specific cytokines, for example, may promote greater response efficiency (Lai and Bennett, 1998).

2.8.2. Disease free areas

Disease incursions in previously FMD-free countries are controlled by inhibition of susceptible animal movement, slaughter of infected or in-contact animals, and decontamination. The use of vaccination is often a decision of last resort mainly because of the adverse economic consequences of vaccination as compared to slaughter (Grubman, 2005). Countries that slaughter infected or in-contact animals can regain FMD-free status by documenting absence of disease for 3 months after the last case, while, until recently, countries that vaccinate and do not slaughter these animals had to wait a year to regain this status. The choice of the stamping out Option depends on the possibility of tracing dangerous contacts, political will and available resources (Grubman and Baxt, 2004). The United Kingdom, Ireland, Japan, Scandinavia, and the United States of America are some of exemplar countries that were able to control foot and mouth disease by stamping out strategy. In Europe, foot and mouth disease has been successfully controlled for several decades by extensive vaccination of the cattle population. The strategy that used to control the outbreak of foot and mouth disease that occurred in the United Kingdom in 2001 raised a large debate on the policy of disease control by stamping out regarding the issue of animal welfare. This debate provokes the reassessment of the policy and reconsideration of vaccination as part of alternative and more flexible foot and mouth disease control strategies (Rossides, 2002).

2.9. Foot-and-mouth disease virus vaccine strain selection

Foot and mouth disease is highly contagious, affecting all cloven-hoofed animals, and is caused by a virus that exists as antigenically diverse serotypes and intratypic variants (subtypes); thus infection or vaccination with one serotype of foot and mouth disease virus (FMDV) does not cross-protect against other serotypes and may also fail to protect against other subtypes of the same serotype (Mattion *et al.*, 2004; Mumford, 2007).

To select the most appropriate strains of FMDV for use as vaccines to control disease in endemic areas and for incorporation into vaccine reserves, it is necessary to monitor the current variety of prevailing FMDVs and to check the suitability of currently used or stored vaccine strains (Paton *et al.*, 2005). The two most important vaccine-related determinants of the protection that a vaccine will afford are: how well it can stimulate a strong immunity and how closely related it is to the field virus against which protection is sought (antigenic match). Vaccine strains are selected on the basis of a number of characteristics, but good growth characteristics and the ability to elicit an antibody response which is broadly cross-reactive within a subtype are the most important (Doel, 2003). It is essential to maintain an awareness of current virus types and the strains within those types which are circulating and this is a major challenge given the diversity of strains even within one continent (Vosloo *et al.*, 2002). A cornerstone of effective vaccination programmes to control and eradicate the disease in endemic areas and to prevent incursions into normally disease-free areas is the use of vaccines containing strains that are well matched to the outbreak strains (Paton *et al.* 2005).

2.10. Selection of field viruses for vaccine matching

Serological matching of field isolates to vaccine strains requires that isolates have been serotyped and adapted to growth in cell cultures. BHK or IB-RS-2 cell cultures are usually used for *in vitro* virus replication. For vaccine matching, preferably, at least two isolates should be evaluated from any outbreak and inconsistent results should be followed up to determine whether this is because of genuine antigenic differences or not (OIE, 2009).

Viruses for vaccine matching can be selected based on epidemiological information, such as isolation at different stages of an epidemic, from different geographical locations or from different hosts or animal species (Alonso *et al.*, 1993). Field evidence for a suspected lack of vaccine efficacy, related to reduced apparent protection, is an important criterion for vaccine matching. Once the virus has been serotyped and selected for vaccine matching, use can be made of *in vitro* serological methods which compare the ability of sera collected from vaccinated animals to react with the homologous vaccine virus and candidate field isolates (Kitching *et al.*, 1988).

2.11. Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate may give indications as to the vaccine strains most likely to provide an antigenic match. Antigenic characterization has two purposes; first, to choose the most effective vaccine strain for use in a particular circumstance and, second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves (OIE, 2009). Vaccine strains are selected on the basis of a number of characteristics, but good growth characteristics and the ability to elicit an antibody response which is broadly cross-reactive within a subtype are the most important (Doel, 2003).

2.12. Foot and mouth disease virus vaccine matching

Vaccine matching is the procedure used to quantify the antigenic relationships between FMDV strains. It is used to determine whether a given vaccine is likely to provide good protection against a field strain. For vaccine matching purposes, FMDV strain selection is based on indirect serological methods (*r*-values), on sequence data (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). The most reliable method of matching is to conduct vaccination and challenge studies in the target species. Challenge studies can

determine both the potency of the vaccine and its cross-reactivity with the field strain. However, these studies require the use of live virus and facilities for Containment Group 4 pathogens. They are also slow and expensive; it takes approximately 1 month to test existing vaccines against the field strain by this method. Because a decision for emergency vaccination must often be made within days, this is often impractical (OIE, 2009).

In vitro alternatives to animal challenge tests can also be used. Genetic characterization using sequence analysis of the P1 region (the capsid precursor polypeptide) of the FMD genome, and antigenic profiling of the field virus can suggest that a new strain has emerged and needs to be matched with a vaccine, or that the field virus is genetically close to one that already has vaccine matching information (Gay *et al.*, 2007; OIE, 2010).

In vivo protection from FMDV is correlated with antibody titers, and therefore, a variety of *in vitro* serological methods can be used to quantify antigenic differences or relationship between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate for vaccine matching (Brehm *et al.*, 2008; OIE, 2009). Recently, methodologies for FMDV vaccine strain selection mainly rely on serological approaches (Paton *et al.*, 2005). Tests that can be used for vaccine matching include virus neutralization (the two-dimensional neutralization test), enzyme linked immune sorbent assay (ELISA) and complement fixation (CFT). *In vitro* serological tests can generate results rapidly and do not require animal testing. ELISA tests do not require the use of live virus. A disadvantage to *in vitro* serological tests is that variation between the batches of antisera used can cause inconsistent results. There can also be discrepancies between virus neutralization test (VNT) and ELISA results. An additional disadvantage is that serological tests alone cannot account for differences in the potency of each vaccine; more potent vaccines may protect animals from less closely related strains (OIE, 2010).

Because serological assays have low repeatability, all tests should be repeated; confidence in the relatedness is related to the number of times the test is done (Rweyemamu and Hingley, 1984; OIE, 2009). The serological relationship between the field isolate and the vaccine virus is the 'r' value. OIE recommends one way testing (r1) using antiserum raised against the vaccine strain (OIE, 2010). Two-way testing (r2) would also match but requires an antiserum raised against the

field isolates. The most relevant test for protection in the animal may be virus neutralization. However, non-neutralizing antibodies may also be protective, and the ELISA is quick and requires smaller amounts of post-vaccination serum, which is often limited in supply. The OIE suggests using the ELISA and CFT to screen, and VNT method for more definitive results (Mattion *et al.*, 2009).

2.12.1. Vaccine matching by two-dimensional virus neutralization test

Rweyemamu (1984) reviewed that the neutralization reaction is the most appropriate *in-vitro* reference test system for assessing intra-type antigenic variation as it involves the antigenic determinant responsible for virus strain specifically and evoking protective antibody. Antigenic relationships determined in different neutralization test systems are independent of the system used and are assumed to truly reflect antigenic variation. The two-dimensional micro-neutralization test was found to be appropriate for FMD virus strain differentiation. It is used to characterize the relationship between field isolates of a particular serotype and a vaccine strain known to confer protection against a broad spectrum of subtypes of that serotype. This test utilizes a reference serum raised against a vaccine strain (Paton *et al.*, 2005). The titer of this reference serum against 100TCID₅₀ of the homologous (vaccine) strain is known. The titers against the same dose of a heterologous (field) strain can be determined to indicate how antigenically similar the heterologous virus to the vaccine (homologous) virus. Antibody titers of the reference serum against the homologous (reference) and heterologous (field) viruses for each virus dose used is then calculated from regression data as the log₁₀ reciprocal antibody dilution required for 50% neutralization of 100 tissue culture infective units of virus. The relationship between the field strain and the reference strain is then expressed as an 'r₁' value (Barnet and Stham, 2004). Derive the 'r₁' value, the relationship between the field and the vaccine strain, as:

$$r_1 = \frac{\text{Titer of vaccine strain antisera against field or heterologous virus}}{\text{Titer of vaccine strain antisera against homologous virus}}$$

If vaccine matching between the vaccine strain and field isolates is done by two dimensional virus neutralization test, the result of 'r₁' value is interpreted as (Paton *et al.*, 2005; OIE, 2009):

- 'r1' values greater than 0.3 suggest that the vaccine strain is likely to be protective. This means the field isolate is sufficiently similar to the vaccine strain that use of the vaccine is likely to confer protection against challenge with the field isolate.
- 'r1' values less than 0.3 indicate that protection is unlikely. This implies that the field isolate is so different from the vaccine strain that the vaccine is unlikely to protect. In these cases, either the field isolate should be examined against alternative vaccine strains or, rarely, it will be necessary to adapt a suitable field isolate to become a new vaccine strain.

2.12.2. Vaccine matching by liquid phase blocking enzyme linked immunosorbent assay

Similar to that of two-dimensional virus neutralization test, this test also uses an antiserum raised against a vaccine strain. The blocking ELISA titers of the reference serum against antigens prepared from the homologous vaccine strain are compared with the corresponding titers of the serum against a field isolate to determine how antigenically similar the field virus is to the vaccine virus (OIE, 2009).

Field isolates and the vaccine strains are grown in the BHK-21 or IB-RS-2 cells. The vaccine and field viruses are harvested and titrated using a panel of trapping rabbit antisera and detector guinea-pig antisera raised against the same or closely related vaccine strains. If necessary, the virus antigens may be inactivated prior to use using binary ethyleneimine (BEI).

Optimum trapper/detector combination and the working dilution of the field virus are selected. Serum of vaccine strain is titrated against the field isolate and the homologous vaccine strain. To determine the serum titer, the average optical density (OD) of 24 antigen control wells without blocking serum is calculated. This represents the maximum OD value for the test, i.e. the 100% control value. The end-point is defined as the dilution at which half of the wells show 50% inhibition or more (i.e. identify the dilution at which one out of the two duplicate wells has an OD less than 50% of the antigen control). Derive the 'r' value, the relationship between the field and the vaccine strain, as:

$$r1 = \frac{\text{Reciprocal arithmetic titer of the reference sera against the field isolates}}{\text{Reciprocal arithmetic titer of the reference sera against the vaccine strains}}$$

If matching is done by LPB-ELISA then r1 value is determined (Ferris and Donaldson, 1992; OIE, 2010) as:

- 'r1' value 0.4–1.0: The field and vaccine strains are closely related. If the vaccine strain is potent, it is likely to be protective.
- 'r1' value 0.2–0.39: The field isolates and vaccine strain are antigenically related. The vaccine strain might be suitable, if there is no better match and the vaccine is potent. If possible, the animals should be vaccinated more than once.
- 'r1' value <0.2: The field isolates and vaccine strain are distantly related, and the vaccine is unlikely to be protective.

2.12.3. Vaccine matching by complement fixation test

The relationship between a field isolate and a vaccine strain can also be determined by complement fixation test (CFT) using a guinea pig antiserum raised against the relevant vaccine strain. CFT 50% titers of this reference serum against antigens prepared from the homologous vaccine strain and a field isolate are compared to determine how antigenically similar the field virus is to the homologous vaccine virus (Paton *et al.*, 2005; OIE, 2009).

Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, the virus titer that fixes 2.5 CFU50 (50% complement fixing units) is determined. A relationship is established by titration of the guinea-pig antisera through a twofold dilution series against 2.5 CFU50 of the homologous and heterologous antigens in veronal buffer diluents (VBD) or borate saline solution (BSS) placed in separate tubes. Four haemolysis units of complement are then added to each reaction. The test system is incubated at 37°C for 30 minutes prior to the addition of 2% of standardized sheep red blood cells (SRBC) in VBD or BSS sensitized with rabbit anti-SRBC. Reagents are incubated at 37°C for a further 30 minutes and the tubes are subsequently centrifuged and read.

The CFT 50% titers are calculated by the Spearman Karber method and an 'r1' value is derived from the relationship between the reactivity of the field isolate and the vaccine strain, as:

$$r1 = \frac{\text{reciprocal arithmetic titre of hyperimmune serum against field virus}}{\text{reciprocal arithmetic titre of hyperimmune serum against vaccine virus}}$$

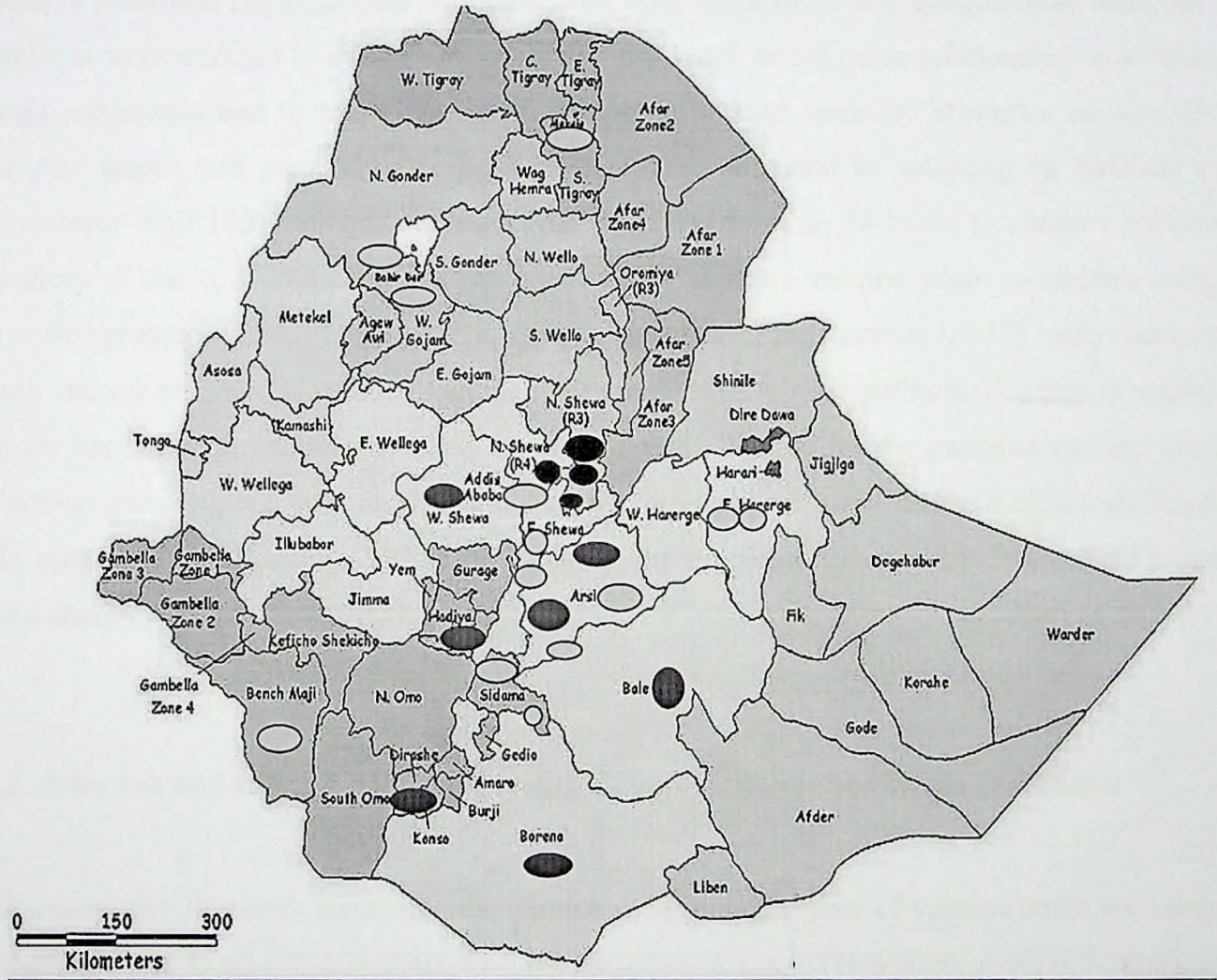
Interpretation of the results:

- 'r1' values greater than 0.25 indicate that the field isolate is sufficiently similar to the vaccine strain and that use of the vaccine is likely to confer protection against challenge with the field strain (OIE, 2009)

3. MATERIALS AND METHODS

3.1. Study areas

The study was conducted on foot-and-mouth disease virus serotypes and/or topotypes collected from different parts of Ethiopia (Figure 2) and stored in National Veterinary Institute (NVI) foot and mouth disease laboratory



Source: Graphic design produced by United Nation emergency unit for Ethiopia (March, 2000)

Figure 2: A map showing the origin of the vaccine strains and field isolates used for the study

○ = Serotype O

● = Serotype A

● = Serotype SAT2

3.2. Study design

In vitro experimental study was conducted to evaluate antigenic or serological relationship between foot and mouth disease virus candidate vaccine strains and representative field isolates. Foot and mouth disease virus (FMDV) vaccine strain candidates, two for each serotype O, A, and SAT2 were selected. A total of nineteen O, five A, and six SAT2 FMDV serotypes field isolates identified from samples collected from different species and geographical locations of Ethiopia were selected to evaluate their serological match or antigenic relationship with vaccine strain candidates and to select the most appropriate vaccine strain of Ethiopian isolates. 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 for the selected vaccine strain candidates using a standard protocol (OIE, 2009). FMDV specific antibody negative cattle (n=30) were vaccinated with vaccine prepared from each vaccine strain candidates to raise antisera. The titer of antibody to the vaccine strain was established for each serum collected for the intended vaccine match. Vaccine matching test was conducted using two-dimensional virus neutralization test. Finally, the serological relationships 'r1' value' between the candidate vaccine strains and field isolates were determined.

3.3. Selection and culturing foot and mouth disease virus vaccine strain candidates

Representative foot-and- mouth disease viruses used as a candidate of vaccine strain for vaccine matching were O (O-ETH/38/2005, O-ETH/58/2005), A (A-ETH/6/2000, A-ETH/7/2008), and SAT2 (SAT2-ETH/64/2009, SAT2-ETH/76/2009) (Table 1). These vaccine strain candidate serotypes and topotypes were selected based on their strong titer, and high prevalence and wide distribution throughout the country. These strains had already been shown to fulfill the other criteria laid down by Rweyemamu and Hingley (1984) and OIE (2009) for a good vaccine strain, in that they grew readily in baby hamster kidney (BHK) suspension cells, high virus yield and stability. All these strains were propagated three times in BHK-21 cell culture as previously described (Alonso *et al.*, 1993), with a slight modification prior to their analysis.

Table 1: Foot and mouth disease virus vaccine strains selected for vaccine matching

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/58/2005	Benchmaji	2005	O	EA-4
A-ETH/7/2008	Sinana	2008	A	Africa
A-ETH/6/2000	Konso	2000	A	Africa
SAT2-ETH/76/2009	Sululta	2009	SAT2	XIII
SAT2-ETH/64/2009	Debre Birhan	2009	SAT2	XIII

The selected known serotypes or topotypes of FMD viruses of both vaccine strain candidates 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 established monolayer of baby hamster kidney (BHK-21) cell lines and incubated at 37°C in an incubator for 1 hour for adsorption of the virus to the cells. After 1 hour, each cell culture was washed with phosphate buffer saline (PBS) containing antibiotic and 10ml of 2% minimum essential medium (MEM) was added into each cell culture flask. After that all cell cultures were incubated at 37°C for 48 hours and examined for cytopathic effects (CPE) using inverted microscope. The viruses were subcultured until 100% CPE (cytopathic effect) observed within 24 hours of the inoculation to obtain a sufficient quantity of the virus for the study.

3.4. Vaccine preparation

Vaccine was prepared for the selected vaccine strain candidates using standard protocol of vaccine production recommended by OIE (2009) manual. The virus strain was inoculated to a suspension or monolayer cell culture of BHK-21 cells and the resulting preparation was clarified, inactivated with ethyleneimine and adjuvanted with aluminium hydroxide and saponin. The

prepared vaccine was checked to be free from residual live virus by subcutaneous administration of 4ml of each prepared vaccine in calves separately, after being screened for the presence of antibody against FMDV and following for clinical signs up to ten days. The vaccines prepared were kept at +4°C until use.

3.5. Antiserum preparation in cattle

A total of thirty young cattle (6-12 months old) were used after being screened for the presence of FMD specific antibodies using 3ABC-ELISA kit (Annex 1). The CHEKIT 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 37°C in a humid chamber. Unbound antibody was washed away, and a horseradish peroxidase-labeled guinea pig anti-bovine immunoglobulin G conjugate was added. Unbound conjugate was removed by washing, and the chromogen substrate was added and incubated for 15 minutes at room temperature or optimally at 25°C for 15 minutes. Finally, 100µl stopping solution was added to stop the reaction, and the result was read using a spectrophotometer at 450nm 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.

Equation 1: Percentage positivity calculation of 3ABC- ELISA.

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

Where: OD sample is optical density of the test sera, OD negative is optical density of negative control, and OD positive is 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.

FMDV specific antibody negative animals were selected, quarantined in a separate area (barn), ear-tagged and vaccinated with 4ml subcutaneously in dewlap region with the vaccine prepared from each vaccine strain candidates on day 0. Booster dose was also given on day 21 post initial vaccination. For each vaccine strain five animals were vaccinated. Sera were collected following booster dose separately for each vaccine candidate strains on day 28. The titer of antibody to the vaccine strain was established for each serum. Sera with strong titer were selected excluding low responders and stored at -20°C for the intended vaccine matching.

3.6. Selection of foot and mouth disease virus field isolates for vaccine matching

Representative FMDV field isolates were selected as per the OIE (2009) recommendation. Accordingly, foot and mouth disease virus field isolates identified, serotyped and characterized to the topotype level at world reference laboratory for foot and mouth disease (WRLFMD), UK, from samples collected from cattle and pigs, from different parts of the country (i.e. Ethiopia) and at different times were used for this study. In addition, active FMD outbreak was assessed and field isolates currently circulating and causing the outbreaks were included in this study. FMDV field isolates selected for this study were indicated in Tables 2, 3, and 4.

Table 2: Foot and mouth disease virus serotype O selected field isolates

Name of the Virus	Site of isolation	Species	Year of isolation	Serotype	Topotype
ETH/1/2005	Sidama, Awassa	Bovine	2005	O	EA-3
ETH/54/2005	Arsi, Robe	Bovine	2005	O	EA-3
ETH/63/2005	Arsi, Robe	Bovine	2005	O	EA-3
ETH/20/2008	Bahirdar Zuria	Bovine	2008	O	EA-3
ETH/34/2008	Addis Ababa	Bovine	2008	O	EA-3
ETH/24/2009	Haromaya	Bovine	2009	O	EA-3
ETH/70/2009	Haromaya	Bovine	2009	O	EA-3
ETH/29/2011	Debre zeit	Swine	2011	O	EA-3
ETH/32/2011	Debre zeit	Swine	2011	O	EA-3
ETH/34/2011	Debre zeit	Bovine	2011	O	EA-3
ETH/38/2011	Sidama	Bovine	2011	O	EA-3
ETH/42/2011	Sidama	Bovine	2011	O	EA-3
ETH/45/2011	Sidama	Bovine	2011	O	EA-3
ETH/46/2011	Sidama	Bovine	2011	O	EA-3
ETH/48/2011	Debre zeit	Bovine	2011	O	EA-3
ETH/49/2011	Debre zeit	Bovine	2011	O	EA-3
ETH/50/2011	Debre zeit	Bovine	2011	O	EA-3
ETH/51/2011	Debre zeit	Bovine	2011	O	EA-3
ETH/59/2011	Mekele	Bovine	2011	O	EA-3

Table 3: Foot and mouth disease virus serotype A selected field isolates

Name of Isolates	Origin	Species	Year of Isolation	Serotype	Topotype
ETH/10/2005	Gobe Arsi	Bovine	2002	A	Africa
ETH/13/2005	Geferssa,	Bovine	1981	A	Africa
ETH/16/2005	Hadiya	Bovine	2000	A	Africa
ETH/9/2008	Yabello	Bovine	2008	A	Africa
ETH/57/2008	Gobe Arsi	Bovine	2008	A	Africa

Table 4: Foot and mouth disease virus serotype SAT2 selected field isolates

Name of the isolates	Site of isolation	Species	Year of isolation	Serotype	Topotype
ETH/67/2009	Debre Birhan	Bovine	2009	SAT2	XIII
ETH/68/2009	Debre Birhan	Bovine	2009	SAT2	XIII
ETH/70/2009	Debre Birhan	Bovine	2009	SAT2	XIII
ETH/72/2009	Debre Birhan	Swine	2009	SAT2	XIII
ETH/77/2009	Sululta	Bovine	2009	SAT2	XIII
ETH/78/2009	Sululta	Bovine	2009	SAT2	XIII

3.7. Titration of foot and mouth disease virus and antisera collected

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 (2% MEM) and subsequent transfer of 1ml of previous virus dilution to the next using sterile micro pipette. Fifty microliter (50 μ l/well) of each virus dilution (10^{-1} to 10^{-8}) was distributed into the wells of their respective rows of microtiter plates containing established cell layers of baby hamster kidney (BHK-21). Then all wells were over flown by dispensing hundred microliter/well (100 μ l/well) of 2% minimum essential medium (MEM) and incubated at 37°C for 24 hours. The titer for each virus was determined by use of Spearman Kaber formula.

Equation 2: The virus titer (log₁₀ TCID₅₀/ml) determination by end-point titration Spearman Karber formula.

$$\text{Log}_{10} = (X_o - \frac{d}{2}) + \frac{d(\sum ri)}{ni}$$

Where: X_o = Log₁₀ of reciprocal of the lowest dilution at which all test monolayers are Positive (protective).

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

ni = Number of test monolayers used at each individual dilution

ri = Number of positive test monolayers out of ni
 $\sum (ri/ni) = \sum (p)$ sum of proportion of the tests beginning at the lowest dilution showing 100% positive result (protection).

The sera raised 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 56°C for 30 minutes before testing.

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 candidates. It was performed by using the two-dimensional virus neutralization test, according to the standard protocol of the OIE (2009) manual.

Nineteen FMDV serotype O field isolates indicated in (Table 2), five FMDV serotype A field isolates (Table 3) and six SAT2 type FMD virus field isolates (Table 4) were assessed for their serological relationships to the reference vaccine strain candidate viruses of O-ETH/38/2005 and O-ETH/58/2005, A-ETH/6/2000 and A-ETH/7/2008, and SAT2-ETH/64/2009 and SAT2-ETH/76/2009, respectively against antisera raised for vaccine strain viruses in bovine. Briefly, both field isolates and vaccine strains were passaged on monolayer of BHK-21 cell cultures until adapted to give 100% CPE in 24 hours (Figure 3). The infected BHK-21 monolayer cells were subjected to three times freeze-thaw cycles to release the viral particles from the cells. Fifty microliter (50µl) of serum raised against the reference vaccine strain was added into top wells (A1 to A10) of row A in microtiter plate containing 50µl/well of 2% minimum essential 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 (50µl) of pretitrated field isolates of 100TCID₅₀ dose was added in each well using two columns for each antigen, sealed and incubated at 37°C. After 1 hour incubation, fifty microliter (50µl) of virus/serum mixture was transferred into their respective microtiter plate wells containing established monolayer

BHK- 21 cells, 50 μ l of 2% MEM added in all wells, sealed with a semi permeable sealer and incubated in a 5% CO₂ 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 candidate 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 candidate vaccine strains. The serological or antigenic relationship between the candidate vaccine strains and field isolates was determined as 'r1' value.

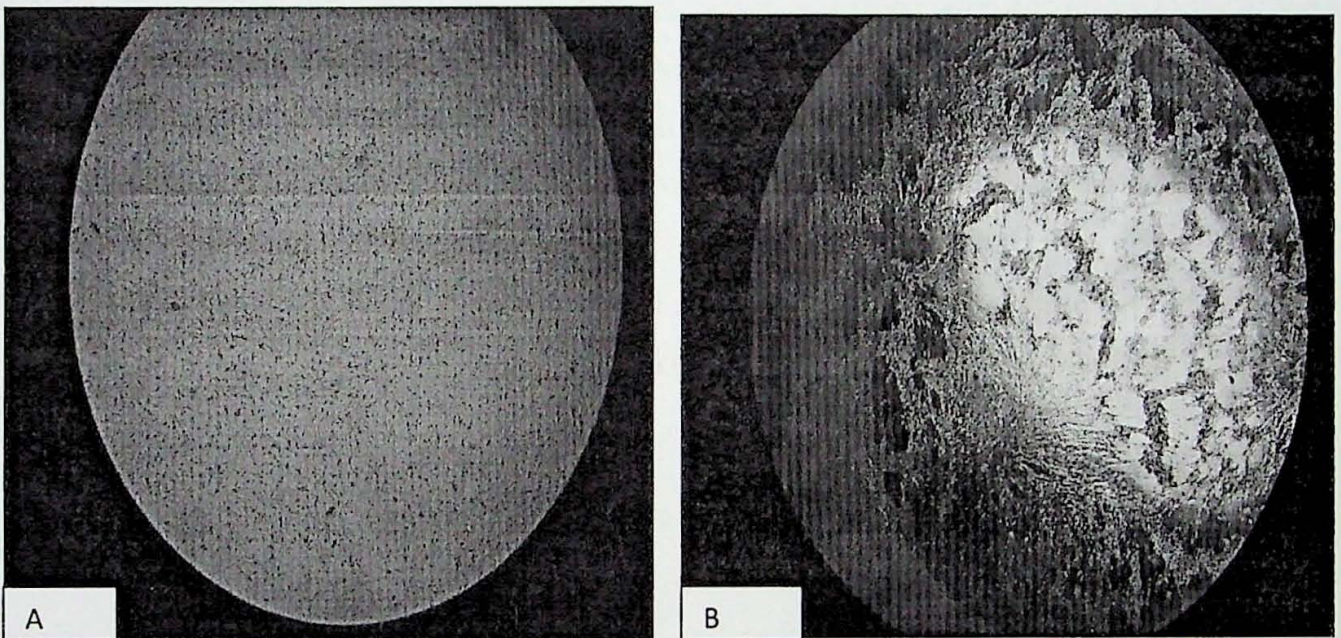


Figure 3: BHK-21 Cell before virus inoculation (A), Cytopathic effect observed within 24 hours of virus inoculation (B)

All tests were repeated three times and average 'r' value was taken for each test virus on different days as per the recommendation of Rweyemamu and Hingley (1984) and OIE (2009) to increase the confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated.

3.9. 'r₁' value determination and interpretation

The relationships of the field virus isolates to the vaccine strains were expressed as an 'r₁' value. The 'r₁' value was calculated by employing the following formula (OIE, 2009).

Equation 3: Formula for calculation of 'r₁' value that derived from the relationship between the reactivity of the field isolates and vaccine strains.

$$r_1 = \frac{\text{Titer of the vaccine antiserum against the field isolate or heterologous virus}}{\text{Titer of the vaccine antiserum against the vaccine virus or homologous}}$$

For two-dimensional virus neutralization test, 'r₁' value was interpreted according to the criteria indicated by Rweyemamu (1984) and Paton *et al.* (2005) as:

- 'r₁' value > 0.3 was considered as the field virus is sufficiently similar to the candidate vaccine strain and vaccine that contain vaccine strain virus is likely to confer protection against challenge with the field isolates.
- 'r₁' value <0.3 was considered as field isolate is different from vaccine strain that the vaccine containing vaccine strain is unlikely to confer protection against field isolates.

3.10. Data management and analysis

The data obtained by two-dimension virus neutralization assay were stored in Microsoft Office Excel spread sheet and SPSS version 16.0 was employed (SPSS, 2007) for statistical analysis to calculate mean and standard deviation of 'r₁' value.

4. RESULTS

4.1. Results of two-dimensional virus neutralization test (Observation)

Prior to determination of 'r1' value in two-dimensional virus neutralization test cytopathic effects were assessed on BHK-cells. Cytopathic effect was observed in wells of the microtiter plates containing the lowest diluted antisera in most microtiter plates (1/64, 1/128 and 1/256) after 48 hours of incubation; indicating that as the antisera diluted more and more antibody available cannot neutralize the infectivity effect of the virus. However, for serotype A field isolate, A-ETH/13/1981, the cytopathic effect was observed starting from $\frac{1}{4}$ dilution. While in wells of the microtiter plates containing the higher antisera concentration ($\frac{1}{2}$ to $\frac{1}{32}$) for all test viruses there was no cytopathic effect except for A-ETH/13/1981 in which there was no cytopathic only at first dilution (1/2). This implies that wells with no cytopathic effect were considered as protected due to the neutralizing effect of antisera raised against the vaccine virus. Whereas, wells with cytopathic effect were as non-protected or infected (Figure 4).

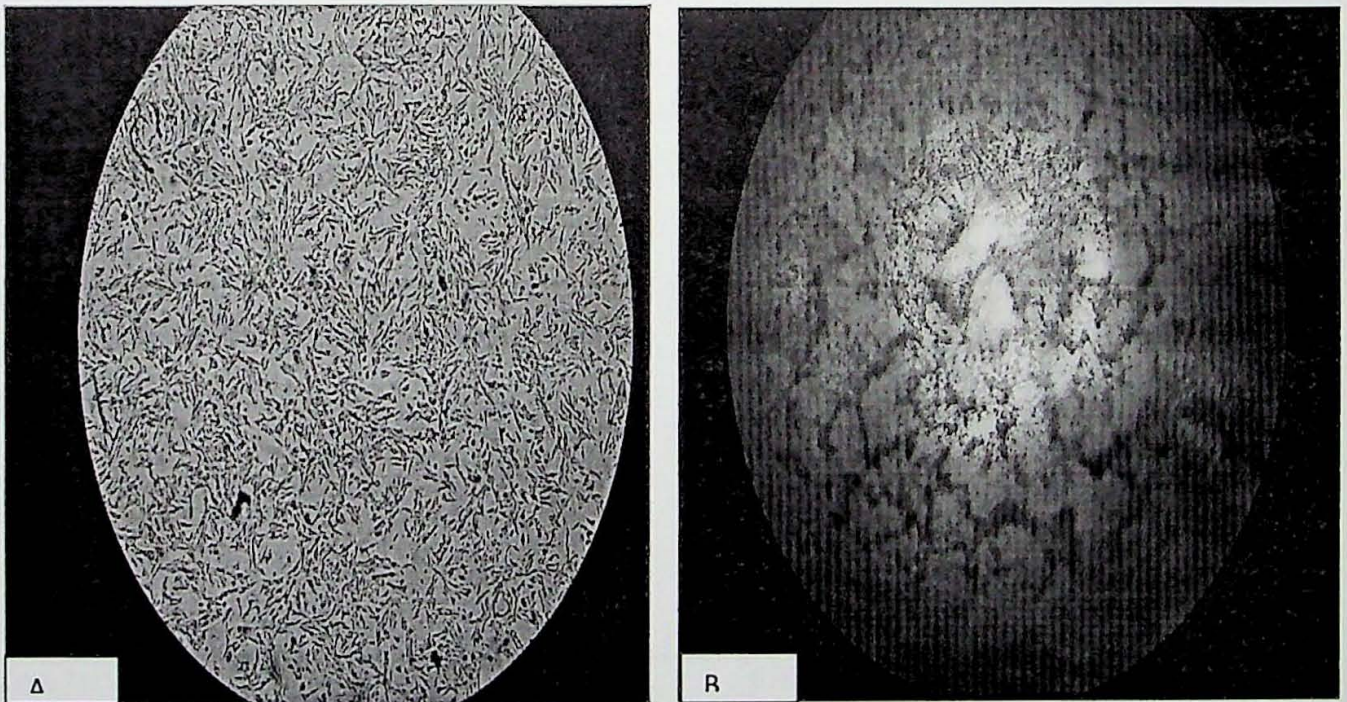


Figure 4: Protected BHK-cell observed after 48 hours (A), infected BHK-cell observe after 48 hours of incubation in microtiter plate (B)

4.2. Results of antigenic relationship 'r1' value by two-dimensional virus neutralization test

The results of serological or antigenic relationship 'r1' values between the field isolates and candidate vaccine strains of serotypes O, A, and SAT 2, detected by two-dimensional virus neutralization test were shown in Tables 5, 6 and 7, respectively.

Table 5: 'r1' values obtained between serotype O field isolates and vaccine strains

Field Virus	Origin	Vaccine Viruses	
		O-ETH/58/2005	O-ETH/38/2005
ETH/01/2005	Sidama, Awassa	0.71	0.75
ETH/54/2005	Arsi, Robe	0.86	0.86
ETH/63/2005	Arsi, Robe	0.82	0.69
ETH/20/2008	Bahirdar Zuria	0.71	0.75
ETH/34/2008	Addis Ababa	0.71	0.63
ETH/24/2009	Haromaya	0.94	0.88
ETH/70/2009	Haromaya	0.76	0.86
ETH/29/2011	Debre zeit	0.82	0.88
ETH/32/2011	Debre zeit	0.88	0.94
ETH/34/2011	Debre zeit	0.82	0.86
ETH/38/2011	Sidama	0.88	0.94
ETH/42/2011	Sidama	0.82	0.86
ETH/45/2011	Sidama	0.94	0.81
ETH/46/2011	Sidama	0.88	0.86
ETH/48/2011	Debre zeit	0.82	0.86
ETH/49/2011	Debre zeit	0.88	0.69
ETH/50/2011	Debre zeit	0.94	0.94
ETH/51/2011	Debre zeit	0.82	0.94
ETH/59/2011	Mekele	0.88	0.94
Mean 'r1' Value		0.83	0.84
Standard deviation		± 0.073	± 0.094

The results presented in Table 5, showed that there is a close antigenic relationship between all the field viruses and the candidate vaccine viruses, because for all viruses tested the 'r1' value was considerably greater than the minimum requirement (>0.3). This realizes that the two vaccine strain candidates can protect the challenge by the field isolates. The mean 'r1' values of the type O field isolates to the candidate vaccine strains O-ETH/58/2005 and O-ETH/38/2005 were 0.83 and 0.84, respectively. The standard deviation of 'r1' values to O-ETH/58/2005 was ± 0.073 while standard deviation of 'r1' values to O-ETH/38/2005 was ± 0.094 .

Table 6: 'r1' values obtained between serotype A field isolates and vaccine strains

Field Isolates	Origin	Vaccine Strains	
		A-ETH/6/2000	A-ETH/7/2008
ETH/10/2002	Gobe Arsi	0.57	0.69
ETH/13/1981	Geferssa	0.14	0.25
ETH/16/2000	Hadiya	0.71	0.81
ETH/9/2008	Yabello	0.86	0.88
ETH/57/2008	Gobe Arsi	0.64	0.88
Mean 'r1' values		0.58	0.70
Standard deviation		± 0.270	± 0.264

The result of two-dimensional virus neutralization tests conducted to assess the serological relationship between two vaccine strain candidates and five field isolates of serotype A FMD virus revealed that all field isolates were antigenically related to both vaccine strain candidates except one field isolate. As shown in Table 6, the calculated 'r1' values with both A-ETH/6/2000 and A-ETH/7/2008 were found to be above the cut-off point (0.3). One virus (A-ETH/13/1981), originated from Geferssa/West Shewa isolated from bovine was clearly distinguishable from the two reference vaccine strains showing 'r1' value below the cut-off point to A-ETH/6/2000 and A-ETH/7/2008 candidate vaccine strains (with 'r1' values 0.14 and 0.25, respectively).

This revealed that this particular field strain has very significant serological variation with A-ETH/6/2000 and A-ETH/7/2008 vaccine strains. The overall 'r1' values indicated that 4 out of 5 of serotype A field isolates tested were serologically or antigenically matched to the candidate vaccine strains. The mean 'r1' value of field isolates for A-ETH/7/2009 is found to be greater than that of A-ETH/6/2000 (Mean 'r1' value: 0.70 and 0.58, respectively) indicating that vaccine strain A-ETH/7/2008 is better choice as compared to vaccine strain A-ETH/6/2000. The standard deviation to A-ETH/6/2000 and A-ETH/7/2008 was ± 0.270 and ± 0.264 , respectively.

Table 7: 'r1' values obtained between serotype SAT2 field isolates and vaccine strains in two-dimensional virus neutralization test

Field Virus	Origin	Vaccine Strains	
		SAT2-ETH/64/2009	SAT2- ETH/76/2009
ETH/67/2009	Debre Birhan	0.71	0.71
ETH/68/2009	Debre Birhan	0.71	0.75
ETH/70/2009	Debre Birhan	0.64	0.75
ETH/72/2009	Debre Birhan	1.00	0.86
ETH/77/2009	Sululta	0.57	0.63
ETH/78/2009	Sululta	0.57	0.86
Mean 'r1' value		0.70	0.76
Standard deviation		± 0.160	± 0.090

For six serotype SAT2 field isolates studied using two dimensional virus neutralization test to assess their serological or antigenic match against two vaccine strains all of them showed that their relationship 'r1' values were found to be above the cut-off point (>0.3) (Table 7).

The mean serological relationship 'r1' value of field isolates tested to vaccine strains SAT2-ETH/64/2009 and SAT2- ETH/76/2009 were 0.70 and 0.76, respectively. This implies that both

candidates can be chosen as vaccine strain to provide broad cross-protection against the circulating field isolates. But vaccine strain SAT2-ETH/76/2009 was found to be the vaccine of choice based on its higher 'r1' value.

Table 8: 'r1' values obtained by match between candidate vaccine strains

Vaccine strains	Antisera Raised Against the Vaccine Strains					
	O-ETH 58/2005	O-ETH 38/2005	A-ETH 6/2000	A-ETH 7/2008	SAT2-ETH 64/2009	SAT2-ETH 76/2009
O-ETH 58/2005	1.00	0.81	-	-	-	-
O-ETH 38/2005	0.76	1.00	-	-	-	-
A-ETH 6/2000	-	-	1.00	0.88	-	-
A-ETH 7/2008	-	-	0.86	1.00	-	-
SAT2-ETH 64/2009	-	-	-	-	1.00	0.75
SAT2-ETH 76/2009	-	-	-	-	0.63	1.00

As shown in Table 8, the candidate vaccine strains were significantly related with their serotypes with 'r1' values greater than the cut-off point. This suggested that O-ETH/38/2005, A-ETH/7/2008, and SAT2-ETH/76/2009 were found to be better vaccine of choice as compared with their alternative vaccine strain candidates.

5. DISCUSSION

The aim of this study was to evaluate the serological match between vaccine strain candidates of FMD virus serotypes O, A, and SAT2 and representative field isolates and thereby estimating the relationship 'r1' value to select the most appropriate vaccine strains with broad spectrum cross-protection to the field strains circulating in the country.

For vaccine matching purposes, FMDV vaccine strain selection is based on indirect serological methods (*r*-values), on sequence data (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). This study was performed by the use of indirect *in vitro* serological method using two-dimensional virus neutralization test to assess serological or antigenic relations of the field isolates to antisera raised against the candidate vaccine strains.

Viruses for vaccine matching can be selected based on epidemiological information, such as isolation at different stages of an epidemic, from different geographical locations or from different hosts or animal species (Alonso *et al.*, 1993) by evaluating at least two isolates from any outbreak (OIE, 2009). The current study also used FMDV serotypes originated from different geographical areas of the country by incorporating more field isolates from each outbreaks in most cases particularly for serotypes O and SAT2. Seventeen serotype O field isolates were from bovine and two were from swine. All serotype A field isolates were obtained from bovine. Five SAT2 serotype field isolates were from bovine while one virus was from swine. However, it was not possible to get virus isolated from other species such as sheep, goats, and others.

Vaccine strains are selected on the basis of a number of characteristics, but good growth characteristics and the ability to elicit an antibody response which is broadly cross-reactive within a subtype are the most important (Doel, 2003). In this study the candidate vaccine strains were selected based on their rapid growth on BHK-21 cell and their very strong titer. In addition, new topotype for serotype O (O-ETH/58/2005 topotype EA-4) was also considered to evaluate the extent of its serological match or antigenic relation with other field isolates.

The findings of the serological matching between serotype O field isolates and candidate vaccine viruses demonstrated that these two vaccine strains O-ETH/2005 and O-ETH/38/2005 can confer protection for the field isolates circulating in the country. The serological match of O-ETH/38/2005 toptotype EA-3 was found to be slightly higher than O-ETH/58/2005 toptotype EA-4 with mean of 0.83 and 0.84, respectively. This could be attributed to the toptotype similarity of all field isolates tested with O-ETH/38/2005.

Previous study showed that the new toptotype O-ETH/58/2005 EA-4 and other field isolates of serotype O toptotype EA-3 of Ethiopian isolates assessed for their serological relationship with antisera raised against vaccine strains of O1 Manisa and O1 Lausanne in WRLFMD, at UK, by virus neutralization test revealed a good serologic match (Gelagay *et al.*, 2009).

The results of 'r1' value of serotype A field strain tested showed that a significant antigenic similarity in which 4(80%) of the tested viruses were serologically matched with the vaccine strain candidates A-ETH/6/2000 and A-ETH/7/2008. This study indicated that, while there was little antigenic or serological variation among field strains tested and vaccine strain candidates, with only one virus with low 'r' value below the cut-off point, the remaining tested field strains were regarded as significantly related to both A-ETH/6/2000 and A-ETH/7/2008 vaccine strains. This shows that these vaccine strain candidates can be incorporated in vaccine formulation to be used in the region. The significant serological or antigenic variation obtained between A-ETH/13/1981 and the candidate serotype A vaccine strains may be associated to the very evolving nature of FMDV. The recent vaccine strain candidates appear through long time evolution which could increase the chance of significant antigenic variation with the viruses isolated many years ago. This result is a very good indicative of vaccine strains of FMD should be continually updated by evaluating their antigenic match with newly evolving field isolates.

The findings of the serological relationship 'r1' values obtained for SAT2 serotype, in two dimensional virus neutralization test indicated that 6 (100%) of the tested viruses were above the cut-off point for both vaccine strains. The 'r1' values ranged from 0.57 to 1.00 and 0.63 to 0.86 for SAT2-ETH/64/2009 and SAT2-ETH/76/2009 vaccine strains, respectively. This result agrees with the interpretation criteria of Rweyemamu (1984) and Paton *et al.* (2005) in which r values

greater than 0.3 is an indicative of serological match between field isolates and vaccine strain viruses. The maximum 'r1' value was observed for serotype SAT2 as compared to other serotypes. This shows strong antigenic relationship between candidate vaccine strains and field isolates.

6. CONCLUSIONS AND RECOMMENDATIONS

The current study was conducted to assess *in vitro* serological matching between vaccine strain candidates and representative FMDV circulating in the country and to select appropriate vaccine strains. 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/7/2009) with their respective serotype field isolates realize strong antigenic match except one serotype A field isolate which showed antigenic or serological variation with the vaccine strains. To conclude, based on the study findings, both serotype O vaccine strain candidates (O-ETH/58/2005 and O-ETH/38/2005) can confer protection for serotype O field viruses circulating in the country with O-ETH/38/2005 better match. Serotype A vaccine strain A-ETH/7/2008 showed better antigenic match with the tested field isolates based on the *r* values as compared to A-ETH/6/2000. The antigenic or serological variation that occurred between A-ETH/13/1981 and the candidate vaccine strains could be good indicative of evolution of the FMDV that the vaccine strains should be assessed for their antigenic match with the evolving field strains. SAT2 serotype also revealed strong matching between candidate vaccine viruses and field isolates. Therefore, most FMDV vaccine strain candidates tested were serologically or antigenically matching with the representative field isolates implying that these vaccine strains could confer protection against the challenge by the field isolates circulating in the country.

Based on the above conclusions the following recommendations are forwarded

- The candidate vaccine strains selected could be included in vaccine formulation so as to effectively control FMD outbreaks in the country.
- The protection level of the selected vaccine strains against the challenge by field viruses should be supported by potency testing or animal challenge.
- The vaccine match should be extensively studied further by increasing the number of field isolates.
- The selected vaccine strains could also be checked for their match with FMD virus topotypes circulating in neighboring countries.

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8. ANNEXES

Annex 1: 3ABC-ELISA test procedure and plate lay out

About 100µl of prediluted samples (1:16 in diluent buffer A) and controls (1:100 in CHEKITFMD 3ABC sample diluents) were dispensed into the appropriate wells of the microtiter plate precoated with recombinant FMDV 3ABC viral antigen. The plates were covered with a lid and incubated for 60 minutes at 37 °C in a humid chamber. After incubation microplates were filled with about 300µl CHEKIT FMD 3ABC washing solution and washed three times. Then 100µl of the ready to use CHEKIT-FMD 3ABC-Anti-Ruminant-IgG-Po-Conjugate was dispensed into each well and incubated for 60minutes at 37 °C in humid chamber. After washing the plate, 100µl of TMB-substrate was dispensed into each 96 wells and incubated at room temperature or optimally at 25 °C for 15 minutes. Finally, adding 100µl, CHEKIT-stopping TMB-solution stopped the reaction, and the result was read using a spectrophotometer at 450nm 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 OD value. The percentage positivity (PP) for test samples in relation to the negative and the positive controls was calculated as follows:

$$Value (\%) = \frac{OD\ sample - OD\ negative}{OD\ positive - OD\ negative} \times 100\%$$

Where, Positive Control: OD positive - OD negative, Sample: OD sample - OD negative

Interpretation of the result: The manufacturer's recommended interpretation was that a final OD value of less than 20% is negative, one of 20% to 30% is ambiguous, and one of greater than 30% is positive.

Plate layout used for FMD 3ABC-ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	13	21	29							
B	N	N	14	22	etc							
C	1	7	15	23								
D	2	8	16	24								
E	3	9	17	25								
F	4	10	18	26								
G	5	11	19	27								
H	6	12	20	28								

P = positive control sample

N = negative control sample

1, 2, 3.etc = test sample

Annex 2: Procedure for two-dimensional virus neutralization test

1. Virus dilution series

The pre-determined virus titer (eg. $10^{5.5}$) 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.

1.1.1 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.

1.1.2 Make up the required media from the appropriate stock solution

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

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

1.1.4 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.

1.1.5 Disinfect and place all virus dilution bottles at $+4^{\circ}\text{C}$ until required.

1.2 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 its titration plate.

1.2.1 Add 100 μl /well of media to well columns 11 and 12 (cell control) and 50 μl /well to columns 1-10

1.2.2 Add 50 μl reference sera to the top well rows A1-A10, making an initial serum dilution of 1/2.

1.2.3* 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).

1.2.4* Sterilise the 50 μl 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.

1.2.5* 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 **1.2.3** to **1.2.5** can be performed manually using a multichannel pipette with appropriate tips. Discard tips after each plate.

Virus neutralization plate layout

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

	1	2	3	4	5	6	7	8	9	10	11	12
A											C	V
B											E	I
C											L	R
D											L	U
E												S
F											C	C
G											O	O
H											N	N
											T	T
											R	R
											O	O
											L	L

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

1.2.6 In the cabinet, add 50µl/well of test virus to each pair of well columns. Discard all tips and bottles into disinfectant. Repeat in similar plates for each virus in the test. Stack the plates and cover the top one(s) and place in the incubator for 30 minutes or 1 hour in the cabinet at room temperature.

1.3 VT plate layout

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

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

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

- 1.3.1 Add 50µl/well of media to well rows 1-10.
- 1.3.2 In the cabinet, add 50µl/well of chosen virus dilution (eg 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 1-10), using the multichannel pipette and tips.
- 1.3.3 Overlay every well with 50µl/well of media. Leave to incubate with the test plates in BHK-21 cell line monolayer.
- 1.4 Observe the plates microscopically for cytopathic effect.
- 1.5 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 plate

2. Virus titer

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 titer 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	$10^{-5.8}$	
<u>Total number of wells exhibiting 100% CPE</u>	<u>20</u>	=2.5
No. of wells/dilution	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 titer is expressed as		$10^{5.5}$ tcid ₅₀ /ml

From this result, the 2 log dilution used in the serum neutralisation 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 titer) 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.

2.1 Antibody titer

In order to calculate this, the antibody titer for each virus dilution must be obtained. Again, the titer 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.)

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

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

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

For the reference serum/**heterologous** test plate, the serum titer is calculated (eg 2.04) and the following equation used:-

$$\text{heterologous titer (2.04) - homologous titer (2.3)} = -0.26$$

$$\text{Reciprocal log. of -0.26} = \mathbf{0.55} \text{ which is the 'r1' value}$$

The serological relationship ('r1' 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 3: Serum sample collection procedure

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

Annex 4: Vaccine preparation procedure

1. Virus propagation for antigen production in BHK-21 cell monolayer or suspension cultures under sterile conditions at 37°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. Centrifugation at 1500-2000 revolution per minute for 15 minutes, and inactivated at 26°C by addition of ethyleneimine, (BEI) for 24 hours.
3. The preparation was blended by adsorbing the inactivated virus on to aluminium 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.

9. CURRICULUM VITAE

PERSONAL INFORMATION

Name: Mekonnen Sorsa Berecha

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Place of birth: Oromia Regional State, South West Shewa Zone, Woliso District, Ethiopia

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EDUCATIONAL BACKGROUND

Year	University/Institute/School	Qualification/Award
2007/08-2010/11	*Adama Science and Technology University, School of Business	*Bachelor of Arts Degree(BA) in Business Administration (CGPA 3.67 Great Distinction)
2004/05-2008/09	*Addis Ababa University, College of Health Sciences, School of Veterinary Medicine	*Bachelor of Science degree (BSc) in Veterinary Laboratory Technology (CGPA 3.88 Very Great Distinction)
2007	*Success Computer and Language Center	*Diploma in Basic Computer Application Software
2001/02-2002/03	*Addis Ababa University, College of Health Sciences, School of Vet. Medicine	*Diploma in Animal Health (cGPA 3.57 Distinction)
1998-2001	*Dejazmach Geresu Duki Comprehensive Secondary school, Woliso (From grades 9-12)	*E.S.L.C.E with result of 3.0
1990-1997	*Dilella Elementary & Junior Secondary School, South Westshoa Zone, Oromia Regional State	*Grade 8 National Examination Certificate (99% percentile)

LANGUAGE SKILLS

- Afan Oromo, English and Amharic: Excellent in hearing, reading, speaking and writing

WORK EXPERIENCE

- As technical assistant staff of AAU, School of Veterinary Medicine (from April, 2004 to December, 2009).
- Teaching experience in the practical classes for the courses of veterinary Surgery, large animal medicine, Small animal medicine and clinical work experience at school of veterinary medicine, AAU.
- Participating in an open-air veterinary clinic, school of veterinary medicine, AAU in diagnosing and treating different cases of different animal species (from 2004 to 2009).
- Academic staff of Ambo University, College of Agriculture and Veterinary Sciences, Department of Veterinary Laboratory Technology, with the rank of Graduate Assistant I (From 2010 to date).

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10. SIGNED STATEMENT OF DECLARATION

I, the undersigned, declare that the thesis is my original work and has not been presented for a Degree in any University and that all sources of materials used for the thesis have been duly acknowledged.

Name:	Mekonnen Sorsa Berecha.
Signature	_____
Date of submission	_____

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

Tesfaye Sisay (DVM, MSc, PhD, Asst. Prof)	_____
Gelagay Ayelet (DVM, MSc)	_____