THESIS Ref. No._____

ISOLATION AND MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUSES IN CATTLE FROM OUTBREAKS OCCURRED IN DIFFERENT PARTS OF ETHIOPIA

MVSc THESIS

BY

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SEPTEMBER, 2020

BISHOFTU, ETHIOPIA

ISOLATION AMD MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE VIRUSES IN CATTLE FROM OUTBREAKS OCCURRED IN DIFFERENT PARTS OF ETHIOPIA

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

By

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September, 2020

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As member of the Examining Board of the final MSc defense, we certify that we have read and evaluated the thesis prepared by: **Bayush Wondimeneh Ayenew**, titled: **Isolation and Molecular Characterization of Foot-And Mouth Disease Viruses in cattle from outbreaks occurred in different parts of Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the degree of master of Science in Veterinary Microbiology.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made.

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ACKNOWLEDGMENTS

First and foremost, praises and thanks to the God, the Almighty, for His showers of blessings throughout my research work to complete the research successfully.

I am sincerely thankful to my advisor Mr. Hika Waktole (Assistant professor) for his incredible contribution in shaping the foundation of my research career and constructive comments. I am extremely grateful for what he has offered me and great sense of humor. I would also extend my sincere gratitude to my co advisors Dr Esayas Gelaye and Dr. Fanos Tadesse for their advice, encouragement and insightful comments.

I want to express my deepest gratitude and appreciation to National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta and National Veterinary Institute (NVI), Ethiopia for providing me valuable support and assistance during research work. I am grateful to the staff members of the center, especially to Dr. Belayneh Getachew, Dr. Dereje Shegu, Hawa Mohammed, Getaw Derese, Ayelech Muluneh, Membere Kidane, for their positive cooperation during my laboratory work.

I am extremely grateful to my mom, emahoy Tewabech Fikadu for her love and prayers.

It fills my heart with joy unspeakable to express my gratitude to everybody who contributed in the successful accomplishment of this thesis.

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LIST OF ABREBRATIONS

BHK	Baby hamster kidney
CFT	Complement fixation test
CPE	Cytopathic effect
CSA	Central statistical agency
EA	East Africa
ELISA	Enzyme linked immunosorbent assay
Euro/SA	Europe/ South America
FAO	Food and agriculture organization of united states
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
GDP	Gross domestic product
IRES	Internal ribosomal entry sites
ISA	Indonesia
MAb	Monoclonal Antibody
ME/SA	Middle East/ South Asia
NAHDIC	National animal health diagnostic and investigation center
NSP	Non-structural proteins
NVI	National veterinary institute
OIE	World organization for animal health
ORF	Open reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SAT	South African territories
SEA	South East Asia
SVD	Swine vesicular disease
TAD	Trans animal boundary disease
UTR	Untranslated regions
VI	Virus isolation
VNT	Virus neutralization test

Vp	Viral protein
WA	West Africa

ABSTRACT

FMD is a highly contagious list A disease affecting many host species ad posing a high economic loss in different countries of the world. A cross sectional study was conducted with the aim of isolation and molecular characterization of foot and mouth disease viruses in cattle from outbreaks occurred in different parts of Ethiopia from March to April, 2020. Outbreak samples were collected for virus isolation, serotyping and molecular characterization. A total of 23 samples were collected during the outbreak and 19 were inoculated on BHK21 cell culture. Among the 19 tested samples, five (26.3%) showed cytopathic effect (CPE) after 24-48 hr of incubation. The samples were identified by using the antigen detection sandwich ELISA, only three of the samples were serotyped as serotype O and SAT1. Up on molecular characterization by RT PCR using universal primers, only two samples were positive. The two positive samples were further identified for their serotype by RT-PCR using serotype specific primers. The result revealed one of the positive sample was identified as serotype O while the other was negative for all serotype specific primers for O, A, SAT2, and SAT1. This may happen due to the higher antigenic variability of the SAT 1 serotypes which causes the failure of the primer to attach to the nucleotide. Thus, further detailed molecular analysis and serotyping of outbreak FMD viruses using sequence data for the samples which are negative by different serotype specific primers is needed. Also the detection of SAT 1 serotype circulating in the country should be an alarm to consider this serotype as vaccinal strain.

Keywords- *Ethiopia, FMD, molecular characterization, outbreak, serotype*

1. INTRODUCTION

Livestock production is hampered by many factors mainly of diseases. Their impact can vary from reduced productivity and restricted market access to the elimination of entire flocks or herds, with the resultant loss of biodiversity and valuable genetic resources (OIE, 2012). Foot and mouth disease (FMD) is one of the Trans-boundary Animal Disease (TAD), that cause significant loss in many species of livestock and livestock products (OIE, 2009).

The disease is caused by Foot and Mouth disease virus (FMDV), in the genus *Aphthovirus* and the family *Picornaviridae*. The virus has seven immunologically distinct serotypes, namely O, A, C, SAT1, SAT2, SAT3 and Asia 1 as well as over 60 subtypes (OIE, 2016). All of the seven serotypes produce clinically indistinguishable disease but immunity to one serotype does not confer protection against another due to the antigenic diversity (Bari *et al*., 2014). It is a contagious animal disease affecting cloven hoofed animals of domesticated and wildlife, where cattle, pig, sheep, goat and buffaloes are among the susceptible ones (Paton *et al*., 2018). The disease is characterized by fever, loss of appetite and weight, blister on the mucous membrane especially of the mouth, udder and teats (Admassu *et al*., 2015).

Even if the disease has a low mortality rate, it is one of the most economically important infectious diseases of animals according to the World Organization for Animal Health (OIE). This is due to the impact of the disease on the international trade in livestock and animal products and also the effects on the livelihoods of local farmers as a result of impacts upon productivity, food security, and losses of income (Maree *et al*., 2014). Although, the disease has been eradicated in some of developed countries, it remains endemic in developing countries. In these developing regions, livestock farming is their major economic input for more than half of the rural economies (Maree *et al*., 2014). Hence, this disease is one of the most destructing phenomena in these regions of the world affecting their socio economic sector of their country.

In Ethiopia, one of the country that possess a huge number of livestock populations in Africa and where 16.5% of National Gross Domestic Product and 35.5% of the Agricultural GDP is covered by the livestock sector (CSA, 2017), FMD is causing several outbreaks every year. In the country, FMD is endemic with four of the seven serotypes of FMDV (O, A, SAT 1 and SAT 2)

(Mitiku *et al*., 2016). The disease impact has been increasing from time to time. In 2005/06 Ethiopia has lost about 14 million USD in consequence of the Egyptian trade ban (Leforban, 2005). Also a trade ban in 2011 loss estimated to be 3,322,269 USD as a result of bull rejection from the market. Economic loses recorded in terms of losses arising from milk loss, mortality and draft power loss was estimated to be 76 USD per affected herd, 9.8 USD per head in crop-livestock mixed system, and 174 USD per affected herd and 5.3 USD per head in the pastoral system (Yalew, 2019).

Despite the above impacts of the disease, there is no government strategy to control the disease. This is evidenced by the increasing occurrence of outbreaks since 1990 (Ayelet *et al*., 2009) throughout the country due to the presence of high number of susceptible animals, wild and domestic animals sharing common grazing pastures and watering points in areas where wild life occur as well as lack of control of animal movement (Admassu *et al*., 2015). Currently vaccination by a trivalent vaccine with serotypes O, A and SAT2, produced National Veterinary Institute (NVI), is used as a prevention method in the country.

In Ethiopia, the circulation of different serotypes makes the selection of sufficiently cross-protective FMD vaccines a challenge. As a result, local and regional programs of surveillance such as isolation, molecular characterization and serotyping of FMDVs circulating in animal populations are an important component of FMD control, in order to aid the vaccine matching data and access to appropriate viral strains that should be used in the development of new vaccines (Maree *et al*., 2014). However there is no molecular data and serotyping of the circulating FMDV in the current study areas.

Therefore, the objectives of this study were:

General objective

- To determine the molecular characterization of FMDV in different parts of Ethiopia.

Specific objectives

- To isolate the virus from clinical samples collected from different areas.
- To determine the serotype of the virus circulating the study areas.
- To detect and characterize the virus at the molecular level.

2. LITERATURE REVIEW

2.1. Definition

FMD is a highly contagious disease predominantly affecting the animals of the order artiodactyla, with the primary domestic hosts being cattle, buffalo, sheep, pigs and goats, although the virus circulates in wildlife, in particular in the African buffalo (syncerus caffer) (Reeve *et al*., 2016). It was first described in Venice in 1546 (Obrinoa *et al*., 2001). The impact of the disease outbreak not only affects the international trade of livestock and animal product but it causes significant effect on the livelihoods of local farmers due to reduced productivity, food insecurity, and losses of income (Maree *et al*., 2014).

2.2. Etiology

2.2.1. Taxonomy

FMDV, the causative agent of FMD, is the first animal virus to be identified as a filterable agent (Loeffler and Frosch, 1897). It is a prototype member of the genus *Apthovirus* in the family *picornaviridae,* in which 'pico' means small and 'RNA' indicates the genome of the virus (OIE, 2011).

2.2.2. Virus structure

Even if it's the first animal virus, the elucidation of its general morphology became possible after the event of electron microscope which revealed smooth, round particle of 30 nm diameter (Wild *et al*. 1969). A detailed understanding of the virus structure comes later with the advancement of technologies. The virus particle has 60 copies of four structural proteins (VP1-4) which associate to the icosahedral shape or capsid (Klein, 2009). The arrangement of the viral proteins are similar when compared to other *picornaviruses* in which the VP1 proteins are located around the icosahedral five-fold axes, whereas VP2 ad 3 alternate around the three fold axes and the VP4 is located entirely at the inner surface of the viral capsid. The outer proteins have a fold almost ubiquitous to RNA viruses known as the RNA virus fold (RVF) that of wedge shaped eight stranded b-barrel. Loops connecting the strands (N- and C- termini) form the exterior surfaces

and identified by the strands they join; thus hypervariable sequence known as the FMDV loop, spanning residues 140-160 of VP1, is also known as the GH loop (Beard and Mason, 2000).

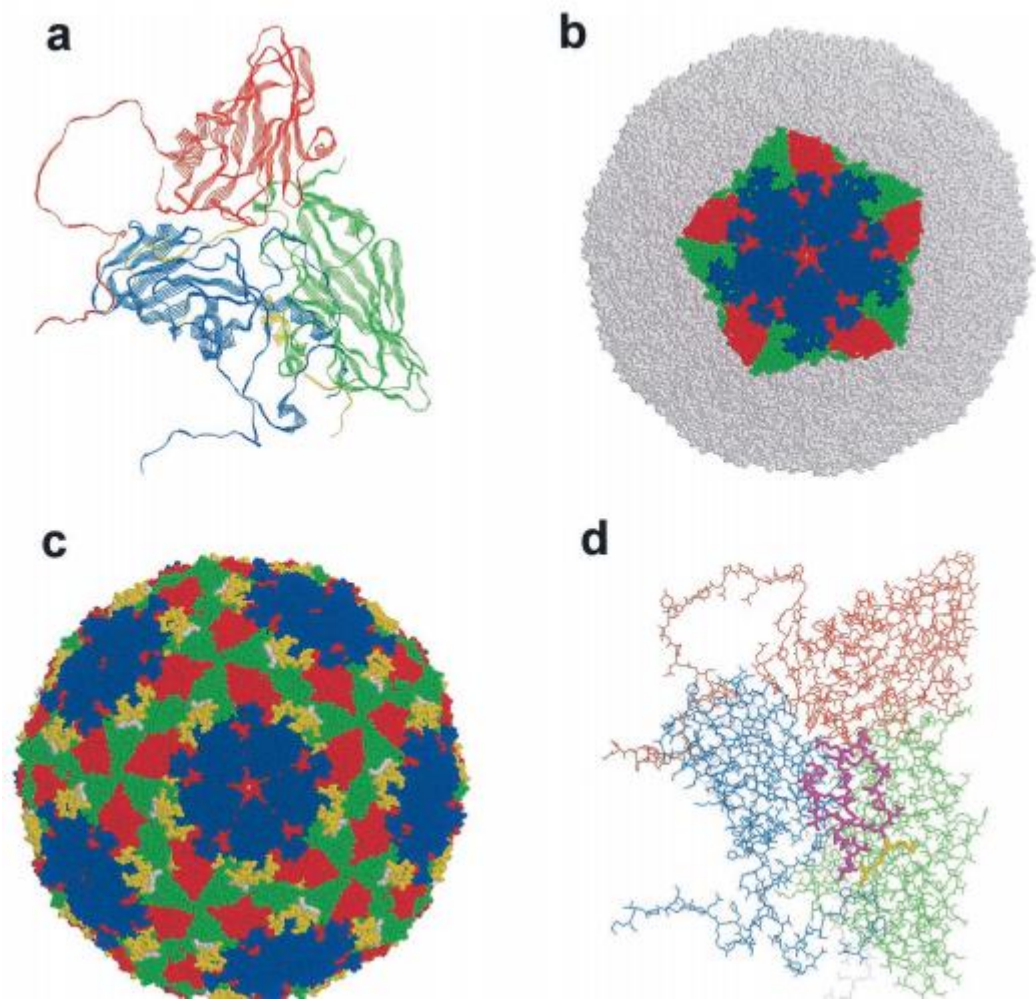

Figure 1: Structure of mature FMD virion based on X-ray crystallographic data. (a) A viral (b) the pentamer position of the virus (c) general Organization of the entire virion, (d) G-H loop with purple colour. Adapted from (Grubman and Baxt, 2004).

Despite structural similarities among *picornavirus* genera, FMDV exhibits distinctive structural features. In most of the *picornaviruses*, there is a prominent depression on the surface, termed as "canyon", which is involved in the interaction with the host cell receptor (Obrinoa *et al*., 2001). But in case of the FMDV capsid, it is relatively smooth with no obvious canyons or pits. This occurs due to the traverse of the C-terminus of the VP1 protein in a clockwise direction finishing adjacent to the VP1 GH loop of the 5-fold related protomer and in doing so fills the depression

which would be analogous to the canyon/pits (Rowlands, 2005). The other unique feature of FMDV among the *picornaviruses* is the highly hydrophobic hole at the 5-fold axis that allows the penetration of molecules such as intercalcating dyes and caesium ions. This feature makes the inactivation of virus infectivity by photoreactive dyes and the high buoyant density of their virions, makes them the highest among the *picornaviruses* (Obrinoa *et al*., 2001).

2.2.3. Genome organization

The genome of FMDV comprises a single strand positive sense RNA virus which is over 8000 bases in length. The viral genome contains one open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (UTR) both predicted to display complex secondary structures. The *picornavirus* internal ribosomal entry sites (IRES) element provides the cap independent translation function, as do those which are present in other viral RNAs and some eukaryotic RNAs. The translation initiation of the FMDV RNA starts at two AUG codons separated by 84 nt. Replication and translation of FMDV RNA occur in the cytoplasm of infected cells, and these biochemical processes are associated with cell membranes (Sangar *et al*., 1977). The single viral ORF encodes only one polyprotein that is cleaved by viral proteases (Ryan *et al*., 1989) to yield the different viral products (Figure 2). The P1-2A region encodes the structural proteins VP1, VP2, VP3, and VP4. The regions L, P2 and P3 encode eight different mature non-structural proteins (NSP). All of them and some of the processing intermediates, are involved in functions relevant to the virus life cycle in infected cells (Obrinoa *et al*., 2001).

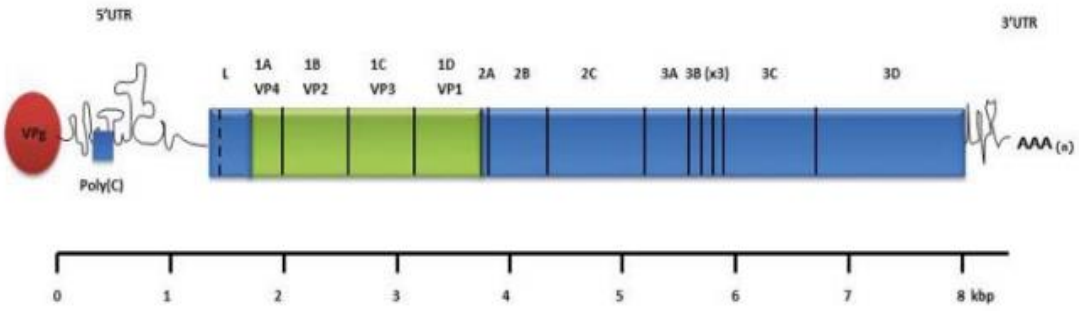

Figure 2: A cartoon of FMDV genome. Adapted from (Freimanis *et al*., 2016).

2.2.4. Antigenic variation

Antigenic variation results when genes encoding the capsid proteins are mutated which also results the evolvement of new subtypes (Haydon *et al*., 2001). More frequently, the impacts of this variation derive from the three major surface exposed proteins of the virus (VP1-VP3) (Maree *et al*., 2014). These proteins contribute to the formation of five known antigenic sites of FMDV (Kitson et al., 1990 ; Crowther et al., 1993). The βG–βH loop and carboxy terminus of VP1 provide to site 1, the critical residues being 144, 148 and 154 and 208. Amino acids at positions 31, 70–73, 75 and 77 of VP2 contribute to site 2, and site 3 is formed in part by residues 43 and 44 of the βB–βC loop of VP1. Only one critical residue, at position 58 of VP3, has so far been identified for site 4. The fifth site, characterized by an amino acid at position 149 of VP1, is mainly formed by interaction of the VP1 loop region with other surface amino acids (figure 3). Site 1 is linear and trypsin sensitive, whereas all the other identified sites is conformational and trypsin resistant (Bari *et al*., 2014).

Even though, the sites appear to be necessary for a complete immunologic response to either infection or vaccination, the major antigenic site, to which most of the immune response is directed and which is common to all of the serotypes, is located within the G-H loop of VP1 (Maree *et al*., 2014). Also mutations are not only occurs within the structural proteins only, it may occur within the nonstructural protein coding regions of the genome. But they are probably less tolerated, since proteins encoded by these regions are necessary for viral replication and changes are more likely to be lethal (Grubman and Baxt, 2004).

Because of this antigenic variation, seven serotypes and multiple subtypes and variants of FMDV has been appeared. This variation leads to the continuous emergence of new variants which makes the selection of FMD vaccine candidates complicated (Verma and Kumar, 2012). Hence, it has a great importance for the epidemiological point of view and for formulating suitable control strategy. In addition to this, the observation that antigenic variation can also occur in tissue culture, has implications for vaccine production, since a number of tissue culture passages are required to produce vaccine for a new variant, leading to the possibility that the virus eventually utilized as antigen may not provide the antigenic coverage needed (Grubman and Baxt, 2004).

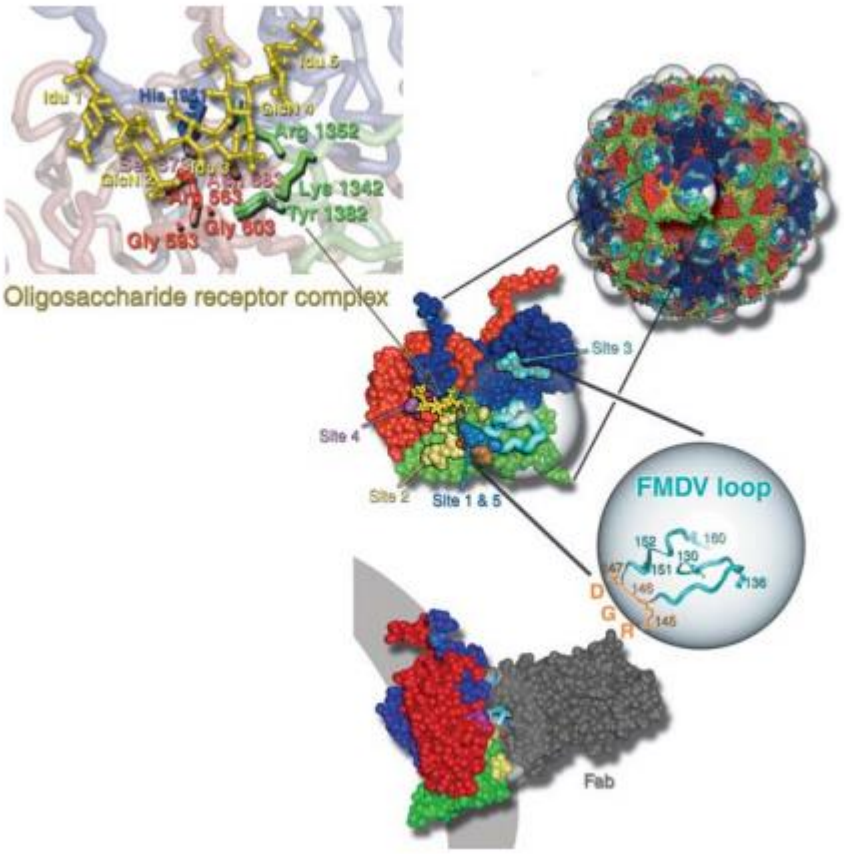

Figure 3: Antigenic sites of FMDV. Adapted from (Rowlands, 2005).

2.2.5. Genetic variation

The high genetic variability of FMDV is a common feature of RNA viruses. Genetic variation in foot-and-mouth disease virus (FMDV) is of interest for 2 reasons. First, changes to the genes encoding capsid proteins results in antigenic variation, and affects vaccine efficiency and effectiveness of vaccination programs; second, genetic changes can lead to important insights into the transport of virus between countries, regions, herds, and individuals (Verma and Kumar, 2012). These changes mainly occurred due to different reasons. The first cause of this variation is mutation. Due to the lack of error correction mechanisms during replication, RNA viruses are prone to high substitution rates that ranges from 103 to 105 per nucleotide site per genome replication. This high rate leads to the differences of FMDV replicates from the original genomes by 0.1 to 10 base positions; hence, a quasispecies concept is developed. Quasispecies are pools of variant genomes statistically defined but individually indeterminate. In such cases all genome sequences are not the same, and that selection occurs at the population level instead of individual

level. Hence, there is not a “wild type” as such but rather an observed “average” phenotype which has adapted to and replicates “best” within any given environment (Grubman and Baxt, 2004).

In addition to mutation, viral recombination is one of the determinants of genetic diversity in FMDV. This recombination occurs due to the polymerase jumping which results the recombination of genetic material between two non-segmented RNA genomes (Metages, 2018). This occurs between viruses of the same serotype as well as between different serotypes. Recombination rates were found to be higher in the acute phase of infection as compared to the carrier phase. Although recombination rates occurred more likely in the NS region, the overall amount of recombination in the capsid was still very high (Ferretti *et al*., 2018).Mutations through recombination could result in the exchange of genetic material that could lead to the generation of new antigenic variants that may escape immune pressure. Therefore, recombination is an important factor in the creation of genetic diversity (Mwanandota, 2013).

2.2.6. Serotypes and subtypes

After the demonstration of FMDV plurality by Valle’e and Carre’ in 1922, there has been a need to differentiate the viruses causing outbreaks of FMD. They have been using the antigenic relationships to show the genetic relatedness, but it leads to the misidentification of the origin of outbreaks. In order to overcome such problems, SDS-polyacrylamide gel electrophoresis and electro focusing were used to examine the migration of viral capsid proteins according to size and or charge. But, a small genetic changes causes large changes in the migration of proteins, hence, genetically closely related proteins seems to be different (King *et al*., 1983).

However, the determination of the nucleotide sequence of viral RNA allowed the characterization of genetic relationships between strains and gave precise evidence for the epidemiological studies. The construction of phylogenetic trees allowed the comparison of nucleotide sequence similarity between virus strains which helped to visualize relationships of FMDV (Crowther *et al*., 1993). The significance of differences in percentage identity values is not clearly understood because; some amino acid substitutions are antigenically conservative, whereas, others are liable to have significant effects on the ability of antibodies to bind. Based on the differences on the VP1 gene, FMDV is classified in to seven serotypes, O, A, C, SAT 1-3 and Asia 1. All of the

serotypes grouped into distinct genetic lineages with approximately 30%-50% differences in the VP1 gene. They are also grouped into geographically restricted clusters also known as topotypes . Topotypes classification is based on the genetic differences which is <15% genetic variations for serotypes O, A and C and <20% for SAT serotypes (Yalew, 2019). Specific characteristics of FMDV serotypes are discussed below.

FMDV type O

Valle'e and Carre' showed the existence of serotype O in 1922 and named it as Valle'e O by the regions they originated, department of Oise in France but it then shortened to O. It is the most widely distributed serotype in the world but there is no precise information for its higher prevalence (Vallée and Carré, 1922). This serotype has low antigenic variation which is not as extensive as the other serotypes which able the few vaccine strains to protect against most field outbreaks. But it has a greater genetic diversity that allows the classification of many distinct lineages. As a result it has eight topotypes with genetically and geographically distinct evolutionary lineages. This topotypes are named as Cathay, Middle East/South Asia (ME/SA), South East Asia (SEA), Europe/South America (Euro/SA), East Africa (EA), Indonesia-1 (ISA-1), Indonesia-2 (ISA-2), and West Africa (WA). Among the eight topotypes, two of them are thought to be extinct (ISA-1 and ISA-2). Nowadays, many of the current virus isolates belong to the ME-SA topotype (Samuel and Knowles, 2001).

FMDV type A

Serotype A was also demonstrated by Valle'e and carre' in 1922 and named as valle'e A by the place of discovery Allemagne in France. But latter it's changed to A. It is the most genetically and antigenically diverse as compared to the seven FMDV serotypes. This may be due to the high recombination rate in this serotype than the others. Due to this high variability, there are about more than 32 subtypes and 26 genotypes (Bari *et al*., 2014). Comparison of complete VP1 sequences has shown that type A viruses can be grouped in to three major geographically restricted genotypes, as Euro/SA, Asia and Africa. However there is occasional spread between these continents may take place (Knowles and Samuel, 2003).

FMDV type SAT 1-3

The SAT 1 and 2 were identified in Bechuanaland (Botswana) and Rhodesia (Zambia) in 1948. Then after, a retrospective testing of samples from Southern Rhodesia from 1930s found the three SAT serptypes (Brooksby, 1958). African buffalos (Syncerus caffer), the natural hosts for the SAT viruses, show overt clinical signs of the disease. This serotypes are confined to sub-Saharan Africa, however a number of outbreaks of SAT 1 have been occurred in the Middle East (1962/1965 and 1969/1970) and SAT2 spread in to Saudi Arabia and Kuwait has been recorded. It is important to know that SAT 1 and 2 has shown greater antigenic variability among the Euroasian serotypes. For SAT-1 eight topotypes were identified throughout the region of which most had localized geographic distribution. However, SAT-2 showed higher genetic diversity with a total of 14 topotypes with five of these possibly extinct (Vosloo *et al*., 2004). Although SAT-3 has the most restricted distribution and is the type least frequently recovered from African buffalo, 6 topotypes were found with 25 genotypes of which four occurred in southern Africa and two were unique to East Africa (Bastos *et al*., 2003).

2.3. Epidemiology of the disease

2.3.1. Global distribution of the disease

FMD is one of widely distributed disease worldwide mainly of South America, Asia, Middle East and sub-Saharan African countries. Among 178 member states of World Organization for Animal Health, only 66 countries are FMD free (65 without vaccination, 1 with vaccination), 10 countries have FMD free zones. North America, Majority of South America, Western Europe, Australia, New Zealand and most Island countries in pacific are free of the disease (Depa *et al*., 2012). The seven serotypes of the disease are not equally distributed around the world. Types O and A have the broadest distribution and are continuously circulating in many parts of FMD endemic countries like Africa, southern Asia, the Far East (Gorna *et al*., 2014) and South America. Type C appears greatly in the Indian sub-continent and Asia 1 normally only occurs in southern Asia. In Africa all the different serotypes of the virus are present except Asia1. The SAT serotypes are normally confined to sub-Saharan Africa (Saeed *et al*., 2015).

The global FMDV population can be roughly divided into seven regional pools. Pool 1 covers south-east Asia with spillover into eastern Asia. Pool 2 represents southern Asia. Pool 3 covers Euro-Asia (including the Middle East). In these three pool serotype O, A and Asia 1 are circulating virus. Pools 4, 5 and 6 cover eastern, western and southern Africa respectively. In pool 4 serotype O, A, SAT 1, 2 and 3 are circulating. In pool 5 serotype O, A, SAT 1 and 2 and in pool 6, only the SATs are tends to circulate. Pool 7 covers South America and has only type A and type O circulating (Logan, 2017).

2.3.2. Distribution of the disease in African countries

FMD is widely distributed in Africa, where 75% of the livestock populations are raised under traditional systems that sustain livelihoods of women and children. The epidemiology of FMD in Africa is affected by two major different patterns i.e. the cycle involving wild life in particular the African buffalo and the independent cycle maintained within domestic animals (Thomson *et al*., 2003). Also the existence of three SAT serotypes which are maintained within the African buffalo population makes the control of FMD challenging. In addition to this lack of veterinary centers, human resources, movement controls and appropriate vaccines pose the developing countries exposed to the disease (Ayebazibwe *et al*., 2010).

Currently, FMD is maintained within the three continents Asia, Africa and South America with different pattern of serotype distribution. In order to make the study easier, the distribution of FMD is divided in to seven major pools of infection. Each pool has at least three serotypes of virus which are specific to the region and needs tailored diagnostics and vaccines for control (Rweyemamu *et al*., 2008). In Africa, FMDV serotypes are not evenly distributed which results different epidemiological patterns. The cumulative incidence of FMDV serotypes demonstr that six of the seven serotypes of FMD (O, A, C, SAT1, SAT2, and SAT3) have occurred in Africa (Paton *et al*., 2009).The distribution of five serotypes and the different topotypes are shown in Figure 4A–E.

Based on the genetic characterization of the virus and antigenic relationship of FMDV in Africa, the virus distribution is divided into three virus pools: namely, pool 4 covering East and North Africa, with predominance of serotypes A, O, SAT1, and SAT2; pool 5 restricted to West and northern Africa, with serotypes O, A, SAT1, and SAT2; and pool 6 restricted mainly to South

Africa, with SAT1, SAT2, and SAT3 serotypes (Donaldson, 1999). Periodically, there have been incursions of types SAT1 and SAT2 from Africa into the Middle East, probably as a result of animal movement (Valarcher *et al*., 2004).

In order to clearly understand the complex epidemiology of FMD and to assist in decision making to control the disease in Africa, the virus pools are further divided into epidemiological clusters. Therefore, eight epidemiological clusters for Africa (Figure 4F) is proposed based on the distribution of serotypes and topotypes in different regions of Africa (Figure 4A–E), animal movement patterns, impact of wildlife, and farming systems (Rweyemamu *et al*., 2008).

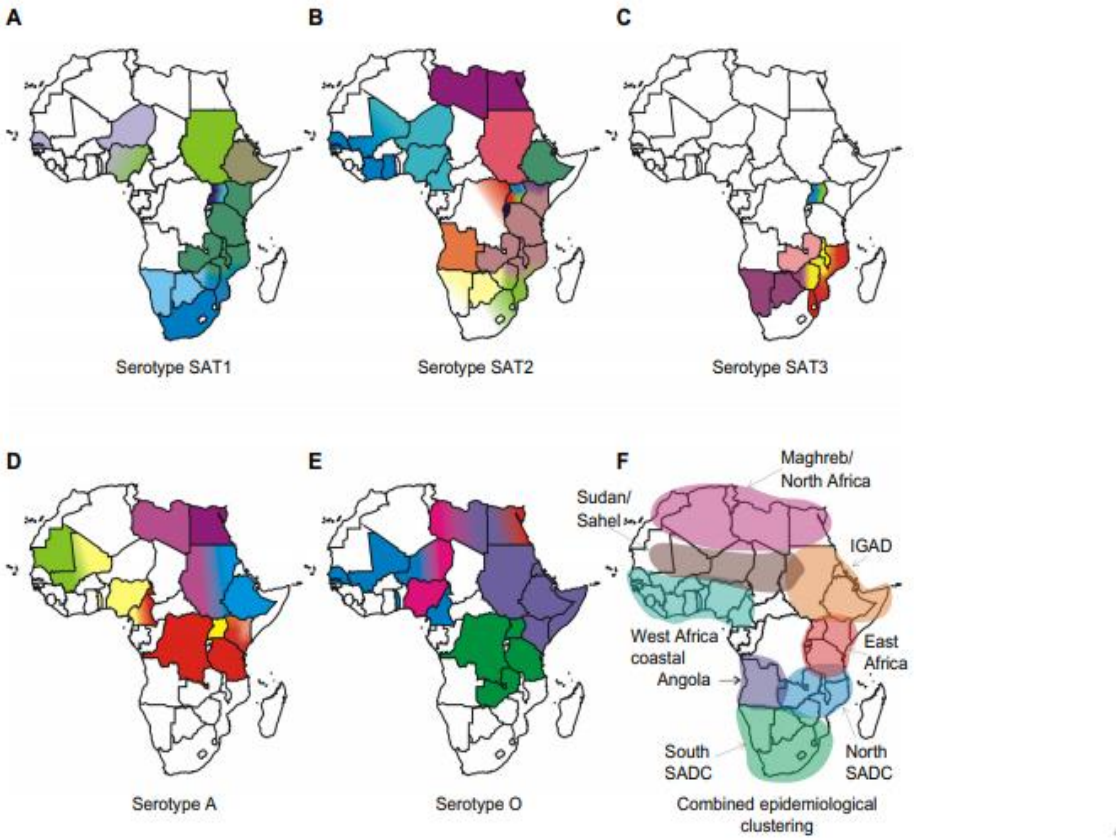

Figure 4: Maps of Africa showing the serotype and topotype distribution. Adapted from (Maree *et al*., 2014).

Notes: The topotypes are color coded. The epidemiological clustering is indicated. The epidemiological clusters shown in the maps (A–F) do not necessarily indicate political borders of the countries.

2.3.3. Molecular epidemiology of the disease

FMD endemic areas in the world are high-risk zones for introducing FMD to countries free of the disease and for the origin of new FMDV lineages. Therefore, a continuing surveillance of FMDV is needed for the early recognition and understanding of emerging risks or changes in the global FMD situation. The detection and characterization of new FMDV lineages and control of possible conformation changes of circulating immune relevant epitopes, is of special interest, as it may indicate that a change in the current used vaccine strains is needed (Klein, 2009). As a result, Molecular epidemiologic studies have contributed more in planning FMD control strategies by elucidating historical and current disease transmission patterns within and between countries and it provides information that should be needed when planning FMD vaccination strategies (Mwanandota, 2013).

The molecular epidemiology of FMDV is studied on the comparison of genetic differences between viruses (Knowles and Samuel, 2003). The VP1 coding region was used to study the molecular epidemiology of the virus. Accordingly, the Phylogenetic analysis of VP1 will be applied to group field strains into discrete topotypes and lineages (Carrillo, 2012). The VP1 gene which is the most highly variable capsid protein that had a major immunogenic site of the virus, is used to genotype the seven serotypes of FMDV into geographically distinct groups called topotypes. In VP1 gene, 26% of proteins are only conserved between serotypes. In addition, comparison of VP1 coding sequences from isolates obtained from FMD outbreaks gives evidence of relatedness between individual FMDV strains. This provides the tracing of the spread and transmission of the virus from one region to another or across national borders (Di Nardo *et al*., 2011). The evolutionary changes of virus are determined by comparing genomic material from more than one virus with each other. At present, sequencing and phylogenetic trees are widely used to illustrate the genetic relationship between viruses (Yalew, 2019).

2.3.4. Transmission

The transmission of the disease can occur both in direct and indirect ways through contact with infected animals, contaminated fomites, virus spread through inhalation of aerosolized virus, contaminated feed and the virus enters through skin abrasions or mucous membranes. The direct

contact transmission occurred when susceptible animals directly contacted with infected animals which leads to the virus enters the host through damaged epithelium, cuts or abrasions and mucous membrane or by deposition of droplets in the respiratory tract as shown in figure 4 (Paton, 2018). In comparison to aerosol transmission, pigs are infected with the virus through physical contact with infected secretions containing large amount of virus. Besides, as pigs are commonly kept on concrete floor, pre-existing damage to the integument may increase the chance of being infected (Muhammad *et al*., 2012).

The indirect transmission of the disease occurred through contaminated vehicles; persons that are working in diseased area and through all other activities like shearing, deworming, blood sampling. In favorable climatic and geographic factors, FMDV can also be transmitted over a long distance in airborne manner. Conditions that prompt the long distance transmission of FMDV include a high relative humidity, usually 55 % or higher, minimal mixing of air by turbulence and convection. Although pigs are relatively resistant to aerosol exposure, infected pigs release largest quantities of air-borne virus and act as an important source of FMDV for long distance aerosol spread (Alexandersen and Donaldson, 2002).

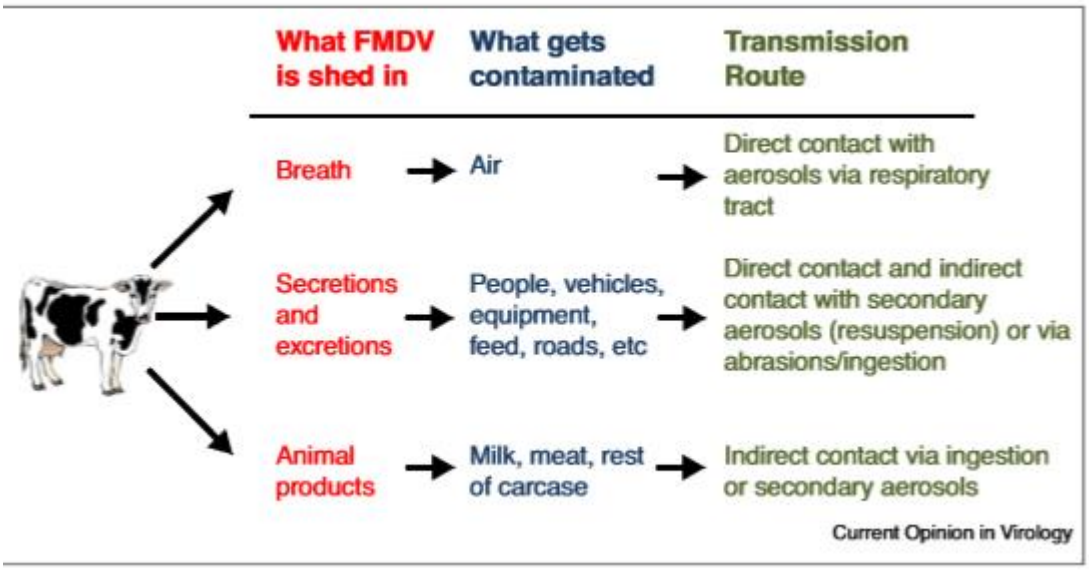

Figure 5: Routes of FMDV transmission. Adapted from (Paton, 2018).

2.3.5. Clinical signs

Virus shedding starts during the incubation period, about 24 hours before the appearance of clinical signs. Based up on the infecting dose, susceptibility of the host and the strain of the virus get in to the animal, the incubation period ranges from 2-14 days (Mitiku *et al*., 2016). The disease is characterized by pyrexia, drooling of saliva and development of vesicles in and around mouth, on tongue, gum, feet, mammary gland and teats in adults. Lesions are often observed initially as blanched areas, followed by development of vesicles and these fluid-filled vesicles are readily seen in cattle. However, these vesicles are rarely observed in the mouth of sheep and goats, may be due to of the thinness of the lingual epithelium causes superficial lesions to rupture early, leaving shallow erosions which usually heal within a few days (Ranjan *et al*., 2016).

While the disease has low mortality in adult animals, it may be high in young animals, including calves, lambs and piglets, due to acute myocarditis. Up on post mortem examination of the heart, it reveals a soft, flaccid heart with white or greyish stripes i.e. “tiger heart” or spots, seen mainly in the left ventricle and interventricular septum (Alexandersen *et al*., 2003). In lactating cows, Vesicles may develop in the skin of teats and udders which causes dramatically decrease in milk yield resulting in mastitis. Secondary bacterial invasion of the ruptured lesions may interfere with healing and may lead to severe involvement of the deep structure of foot and mouth (Mitiku *et al*., 2016).

2.3.6. Pathogenesis

In cattle, the epithelial cells of the dorsal soft palate, the roof of the pharynx just above the soft palate and part of the tonsil are thought to play a significant role in the prime infection. After inhalation of the virus by susceptible animal, the proportions of the particles are deposited in respiratory systems. But the sites of deposition mainly depend on diameter and mass of the virus (Quinn *et al*., 2002). As a result, large particles are deposited in the upper respiratory tract (nares); medium sized particles are deposited at middle to upper respiratory tract (pharynx, trachea, bronchi) and small particles in the lower regions (small bronchioles and alveoli) (Pugh, 2002). Subsequent to the deposition of the virus in the respiratory system, FMDV infection started at the epithelia of mucosal associated lymphoid tissue of the nasopharynx. The virus then attaches to the cells to penetrate into the cytoplasm and replicate until the cells disintegrate. The

disintegration of the cells leads to the release of more viral particles to infect other cells. The released viral particles move from the blood stream during the viremia to infect the epithelium of the oral cavity and feet, where lesions develop (Hirsh *et al*., 2004).

Once infection gains access to the blood stream, the virus is widely disseminated to many epidermal sites, probably in macrophages, but gross lesions develop only in areas subjected to mechanical trauma or unusual physiological condition, such as epithelium of the mouth and feet, the dorsum of the snout of pigs and teats (Lefevere *et al*., 2010). Bacterial complication generally aggravates the lesions, particularly those of the feet and the teat, leading to sever lameness and mastitis, respectively. In young animals, especially neonates, the virus frequently causes necrotizing myocarditis and this lesion may also be seen in adults infected with some strains of the virus particularly type O (Ranjan *et al*., 2016).

2.4. Diagnosis

A pivotal component of any disease control strategy is based on the diagnostic assays to rapidly confirm the initial clinical determination of infection. The accurate and fast diagnosis of FMD is very important for both control and eradication programs in FMD endemic areas and as a supportive measure to the stamping out policy in FMD free areas (Longjam *et al*., 2011). The diagnosis of FMD depends on clinical signs, including high temperature, excessive salivation and formation of vesicles on the oral mucosa, on the nose plus the inter-digital spaces and coronary bands on the feet (Yalew, 2019). However, the clinical signs can be confused with other diseases such as swine vesicular disease (SVD), vesicular stomatitis, and vesicular exanthema of swine that cause the vesicular lesion on the mouth of swine and cattle which is identical to those caused by FMD. In addition, FMDV infection of sheep and goats can be difficult to detect clinically. Also, in a highly susceptible animal the clinical signs are pathognomic however in the endemic region, due to partial natural immunity or vaccinal immunity, clinical signs may be mild or confusing(Verma and Kumar, 2012). Hence laboratory based diagnosis is necessary.

The research and diagnosis of FMD starts by using experimental laboratory animals by Waldmann and Pape followed by Skinner, using the guinea pig and suckling mouse, in 1951. The use of tongue epithelium by Frenkel in 1947, for large scale production of the virus formed the basis for the initiation of FMD vaccine programs in Europe in the 1950’s. The subsequent

development of cell lines brought a remarkable degree of sophistication to the study of virus growth. As the technology advances through time, the recognition of more than one serotype has led to the development of various techniques for serotyping of the virus. Earlier typing of FMDV was done by cross-immunity test in guinea pigs and less frequently in cattle (Waldmann and Trautwein, 2009). As this test was time consuming, expensive, and imprecise, different serological tests like complement fixation test (CFT), virus neutralization test (VNT), and enzyme-linked immunosorbent assay (ELISA) were developed and the most recent is the development of molecular techniques, the polymerase chain reaction (PCR) method making diagnosis more rapid and precise (Rweyemamu *et al*., 1982). For the diagnosis, epithelial tissue or vesicular fluid are used. But in case of advanced or convalescent cases, or in case of subclinical cases, oropharyngeal fluid is collected by means of a probang cup (Yalew, 2019).

Virus isolation

Of the established diagnostic approaches, virus isolation (VI) in cell culture is considered as the ‘‘gold standard’’ as described in OIE Terrestrial Manual 2012. Although primary cell culture of bovine, ovine and porcine origin has exhibited susceptibility to FMDV, the most sensitive culture system is primary thyroid cells. But cryopreservation of bovine thyroid cells directly after trypsinization results in the loss of susceptibility to FMDV (House, 1989). Even if cell lines are less susceptible than primary cells, they are more desirable for diagnostic system. Among the cell lines that are susceptible to FMDV, Baby hamster kidney (BHK21) cell line is the preferred one due to the well growth of the virus and large scale production. The virus is cytocidal, and infected cells shows morphological changes, known as cytopathic effects (CPE), which include cell rounding and alteration and redistribution of internal cellular membranes. The virus also causes biochemical alterations, including inhibition of host translation and transcription (Verma and Kumar, 2012). CPE is detected within 48 hrs. If there is no CPE, the cells would be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hrs. In the absence of CPE after 3 blind passages, the sample can be declared negative for the presence of live virus. However, the cell culture system is laborious, time consuming, and relatively low sensitive. It also requires careful handling of specimens and a biosafety laboratory. In particular, virus isolation requires a laboratory cell culture facility, which can be difficult and expensive to maintain, besides requiring 4 to 6 days for test completion (Jae *et al*., 2009).

Serological tests

The virus infection can be diagnosed by the detection of specific antibody response (Ryan *et al*., 1989). Serological tests are mainly used to monitor the immune status of animals exposed to FMDV or FMDV vaccines. Approaches used include Enzyme Linked Immunosorbent Assays (ELISAs) and VNTs, although CFT are still used in a limited number of countries (Yalew, 2019). As compared to VNT, ELISA results were much more reproducible and are not influenced by variations in tissue culture susceptibility. At the FAO/WRL for FMD, the best procedure for the detection of FMDV antigen and identification of viral serotypes is ELISA (Paiba *et al*., 2004). There are different types of ELISA either for detection of FMDV antigen or antibodies. The antigen detection sandwich ELISA was shown to be rapid and simpler to perform. It is based on the detection of FMDV structural proteins and has 100% specificity for heterologous FMDV and 80% sensitivity for detection of complete virus particles in clinical samples (Ranjan *et al*., 2016).

Although ELISA is far finer to CFT, a large number of samples failed to give positive results and such negative sample has to be confirmed by inoculation of sample into BHK21 cell cultures followed by confirmation of the virus serotype by ELISA lasts 4 more days, which is difficult to rapidly detect disease and in order to take appropriate control measure. As a consequence, there is a need of an alternative assay system that allows more rapid confirmation of clinical diagnosis with more sensitivity and this has resulted in development of PCR (Verma and Kumar, 2012).

Molecular methods

It needs to have a diagnostic test that is sensitive, accurate, rapid and easy to use, because of the rapid spread and the devastating economic consequences of the disease. The polymerase chain reaction is a technique which amplifies a specific genome segment of virus in a diagnostic sample. It needs prior knowledge about the nucleotide sequencing flanking regions which would be amplified. Several groups have been developing quick diagnostic methods based on amplification of specific sequences of viral genome by reverse transcription polymerase chain reaction (RT-PCR), which can be applied to different kinds of biological specimens such as nasal swabs (Marquardt *et al*., 1995), vesicular epithelium (Verma, 2008), milk, serum and probang samples (Yadav, 2009). This approach was used to rapidly detect and type FMDV, to differentiate with other vesicular diseases such as SVD or BVD and to detect virus infection in

animals even before the development of clinical signs (Marquardt *et al*. 1995) as well as for identification of positive cattle at the end of the course of infection when virus isolation may be negative.

Differentiation of vaccinated from infected animals

Vaccination can increase the resistance against the viruses but cannot prevent infection. Vaccination with inactivated virus leads to generation of immune responses similar to that of infection, hence based on serology, identification of infected in vaccinated population is difficult. There is challenge to identify the infected animals among the vaccinated animals for appropriate implementation of the control programme. Differentiation of these two categories of animals is important during serological surveys to detect evidence of infection, as a follow up to ring vaccination in FMD free countries and for import/ export serology (Sharma *et al*., 2012).

In order to alleviate this challenge, a standard established by the OIE, differentiation between vaccinated and infected animals (DIVA), through post-vaccination serological monitoring and analysis of virus circulation, would depend upon improved quality control of FMD vaccines to ensure the elimination of NSP during vaccine production and formulation (Lubroth *et al*., 2007).It is done on the basis of detecting serum antibodies to the viral NSP that are generated during infection but not after vaccination with a vaccine from which the NSP had been removed. However, even purified vaccine preparations are sometimes contaminated with traces of NSP, and after several immunizations with these vaccines, animals that have never been infected with FMDV can also possess anti-non-structural protein antibodies. The occurrence of diseases in vaccinated animal shows the problem of mismatching of the field and vaccine strains according to DIVA test (Robinson *et a*l., 2016).

The term DIVA was coined in 1999 by Jan T van Oirchot. Now the term DIVA has been broadly accepted over earlier term “marker vaccines” as originally marker vaccines were deletion mutants of wild type micro-organisms which could be differentiated easily from wild type. The same principle has been extended to include subunit and killed whole virus vaccines and is broadly termed as DIVA. Different methods have been proposed for DIVA for FMD which includes virus isolation, reverse transcriptase- polymerase chain reaction (RT-PCR), VNT, IgA ELISA and non-structural protein (NSP) immunoassay (Lubroth *et al*., 2007).

NSP based ELISAs are now been considered to be most sensitive method to detect infected in a vaccinated population. The NSP based immunoassays are based on the general DIVA principal that the inactivated FMD vaccine (may be called as DIVA vaccine) induces an antibody response that is different from the antibodies produced by infection with wild type of virus. What is required is just to have an assay that can discriminate the two immune responses. Most of the serological assays for FMD detect antibody to the capsid proteins (VP1, VP2, VP3 and VP4) of the virus. These antibodies are common to both vaccinated and infected animals hence cannot be utilized for DIVA. On the other hand viral replication during infection results in the production of a number of non-structural proteins of which some are immunogenic. Among the eight FMDV NSPs, antibodies against Lpro, 2C, 3A, 3B, 3C and 3D are produced in infected animals but not in vaccinated animals. The NSPs 3A, 3B and 3AB, 3ABC, 2C have been proven for their ability to detect infection specific antibodies in cattle and other species regardless of vaccination status (Sharma *et al*., 2012).

2.5. Treatment

Currently, there is no specific curative drug, which would be recommended to treat foot and mouth disease (Mitiku *et al*., 2016). But in place of specific treatment, symptomatic treatments have been applied depending on the clinical signs observed. Sodium carbonate, boric acid and glycerin are applied on the lesion and the affected animals feet may be washed with 2% copper sulphate solution. Also, washing of the wounds with soda ash solution and topical application of honey is found suitable in foot lesions. Antiviral approaches such as 2'-C-Methylcytidine and ribavirin are used for the purpose of prophylaxis in susceptible animals (Gakuya *et al*., 2011).

But, treatment of secondary bacterial infection and dressing of lesions with proper animal husbandry practices is recommended in FMD endemic countries in which slaughter policy is hard to hard to apply. Also sick animals may be treated by applying broad-spectrum antibiotics parentally, tetracycline in particular, in order to control the consequences of secondary bacterial infections (Radostits *et al*., 2007). Affected animals will recover however with loss of production based on the infection state of the disease. Infected animals are usually killed depending on economy (Hirsh and Zee, 2002).

2.6. Prevention and control

Control of foot and mouth disease is difficult due to its highly contagious nature, multiple hosts, viral stability, multiple antigenic types and sub types and short term immunity. The type of control strategies applied in a country depends on the goal of the control programme. The control strategies varies from country to country based on their epidemiological condition, importance of livestock sector in the national economy and economic capability of the country to invest in control strategies (Radostits *et al*., 2007).

In developing countries, control by eradication is too costly, hence, in most of African countries FMD control is mainly applied by using vaccination and with the control of animal movement (Mekonen *et al*., 2011). Many countries free of the foot and mouth disease have a policy of slaughter of all affected and in contact susceptible animals (economically affordable countries) and strict restriction on movement of live animals, animal products and vehicles around infected premises (Quinn *et al*., 2002). After slaughter, the carcasses must be disposed of safely by incineration, rendering, burial, the building are thoroughly washed and disinfected with mild acid or alkali and by fumigation (Ayelet *et al*., 2009). Rodents and other vectors may be killed to prevent them from mechanical dissemination of the virus. In areas or countries free of FMD in which this is not possible, control is by movement restriction, quarantine of infected premises and vaccination around (and possibly within) the affected premises (Hirsh and Zee, 2002). The commonly used procedures for control of FMD are vaccination and eradication.

Vaccination-Vaccination is instrumental in the control of FMD in endemic countries. Foot and mouth disease vaccines commonly contain more than one serotype of the virus depending on the epidemiological condition of the particular country. Mass vaccination campaigns usually involve a bi-annual or annual schedule (Radostits *et al*., 2007). The current foot and mouth disease vaccine confers protection for 6 months and hence at least two vaccinations are recommended for prophylactive protection in endemic areas. In vaccinated animals the peak antibody response is attained in 21-28 days and protection can be achieved within one to two weeks post vaccination. Vaccination can be used to reduce the spread of foot and mouth disease or protect specific animals (Quinn *et al*., 2002).

Eradication- Eradication is policies and actions designed to eliminate completely FMD virus following an outbreak of disease. This includes both 'stamping out', defined by OIE as the slaughter of all infected and incontact animals, together with cleaning and disinfection, and all the other measures that are necessary in the event of an outbreak in an FMD-free country, region or zone. Stamping out involves: slaughter and disposal, cleaning and disinfection, movement controls, zoo sanitary measures and epidemiological monitoring (Vosloo *et al*., 2002)

2.7. Economic importance

FMD pose significant threats to the livestock sector throughout the world, both from the economic impacts of the disease themselves and the measures taken to control and prevent the risk of disease introduction and spread (Perry and Randolph, 2003). These impacts are multidimensional and not always understood which makes the policy response ineffective. This is particularly the case in developing countries where livestock plays an important role in the household livelihoods and as a pathway out of poverty (Perry and Grace, 2009). At the same time, because of its potential for rapid international spread and effects on animal productivity, international markets for beef are segmented on the basis of FMD status, with higher price premiums available for supplying countries that are both FMD-free and which do not vaccinate their herds (Knight-jones and Rushton, 2013).

Hence, the impact of disease is not equal across all countries and livestock populations due to differences in not only FMD status, incidence and risk of incursion but also (a) the genetics of the national herd; (b) prevailing livestock management practices; (c) prevailing prices of livestock production inputs and outputs and (d) their ability to supply livestock for export markets. This is easier to appreciate when one considers specific countries which differ in these characteristics. When FMD outbreaks occur in disease free countries and zones that produce livestock for export the economic impact is clear to see; however, the impact of the disease in endemic countries is more controversial, particularly when compared to diseases that cause greater mortality (James and Rushton, 2018). The impacts of the disease are direct and indirect, which are discussed below.

The direct losses of the disease are production losses due to reduced milk production (Bayissa *et al*., 2011), affecting both the humans and calves that depend on it. This can account for 33% of

losses in endemic settings (Ellis and Putt, 1981). Not only crucial to commercial dairy operations, milk is an important source of nutrition for many pastoralists, particularly for children (Barasa et al., 2008). Livestock growth rates are also suppressed and mortality amongst young stock is typically 2–3% (Rufael *et al*., 2008) although occasionally much higher. Loss of traction power where draught animals are used is particularly damaging if it occurs during harvest. FMD can result in abortion, the cost of which is high as the farmer will have to pay to keep the cow without it producing anything for another year or more, or cull the animal. Visible production losses are most prominent in pigs in intensive production systems and dairy cattle. These two systems are key sources of animal protein in poor countries and their importance continues to grow (Delgado *et al*., 1999).

The indirect costs are carried out by the state veterinary services (e.g. vaccination, outbreak control, culling and compensation). These costs are enormous with an estimated 2.35 billion doses of FMD vaccine administered in the world every year (Hamond, 2011) at a cost of $0.4–3 or occasionally $9 per dose including delivery and application (Forman *et al*., 2009). Due to the short duration of immunity induced by FMD vaccines, ongoing control programmes vaccinate cattle one to five times a year and sheep and goats once a year; limiting resources available to combat other diseases. Wildlife is sometimes kept out of FMD free zones with fencing which is both costly and affects wildlife ecology (Gadd, 2011). Even if a country is FMD free there are ongoing costs due to efforts to prevent disease introduction, including import controls and sometimes vaccination. In addition, maintaining FMD early detection and control capability, including vaccine banks, is costly. Other costs include FMD related research and permanent restrictions on the livestock sector (such as post-movement standstills and bans on feeding swill). The cost of surveillance are significant, including proving disease freedom after an outbreak; >3 million serum samples were tested after the UK 2001 outbreak (Paton *et al*., 2006) in addition to approximately 3.5 million sera tested during the outbreak.

Also countries infected with FMD cannot trade live animals with FMD free countries. Typically the countries with the best meat prices are FMD free (i.e. EU, USA and Japan) (James and Rushton, 2002) where prices are typically 50% higher (Jarvis *et al*., 2005). The trade of livestock products is also restricted. If regular outbreaks occur only processed, tinned products can be exported to free countries; if FMD is effectively controlled with vaccination by a competent

veterinary service able to detect outbreaks then deboned meat can be exported. Even if a country is FMD free, if it trades with FMD infected countries it will experience trade restrictions (James and Rushton, 2002). Lack of access to lucrative markets has further consequences; it restricts the development of commercial farming. Restrictions limit the supply of livestock and livestock products to free countries; although this is good for domestic producers it leads to increased market prices for consumers. If FMD free status is lost livestock are dumped on the domestic market, reducing prices for consumers at the cost of producers. Even within an endemic country livestock trade is limited; those affected by FMD receive lower prices for their stock and those wishing to purchase animals from FMD free herds face a restricted supply. Furthermore, investment in the livestock sector is limited if there is a perceived risk that FMD may occur. High productivity breeds are typically more susceptible to FMD. The threat of FMD therefore restricts (a) the use of these breeds and (b) prevents the development of more intensive production (Knight-jones and Rushton, 2013).

2.8. FMD in Ethiopia

2.8.1. Disease status

In Ethiopia the disease is widely distributed throughout the country. Previously the disease occurs mostly in the pastoral herds of the marginal lowland areas of the country. But now the disease has been frequently noted in the highlands of the country. Also the disease has shown significant variation across different farming systems and agro ecological zones of the country (Tefera, 2010). FMD outbreaks are reported frequently with increasing occurrence of outbreaks since 1990 (Ayelet *et al*., 2009). According to data from the MoLF (2018) 884 outbreaks were reported from Addis Ababa, Oromia, Amhara, Tigray, SNNPRS, Gambela, Benishangul Gumuz , Somali Afar and Harari. The higher number of outbreaks occurs in Oromia with 438 outbreaks. But these numbers didn't refelect the exact epidemiplogical situation of the disease in the country due to the nature of the disease and the unreported cases by the farmers. According to the seasonal variation in a year, the disease occurs at any season of the year with highest outbreak recorded in the dry season. This is because during the dry season, pastoralists are obliged to move their herds long distance in search of pasture and water. Hence, the transmission exacerbated at herd gathering sites or communal points (Molla *et al*., 2010).

2.8.2. FMD serotypes identified

In Ethiopia foot and mouth disease was first recorded in 1957 when serotypes O and C were found while serotype A was identified in 1969 (Marte, 1975). SAT2 was first isolated from in Awassa, Sidamo and Negelli Borena in 1989 (Roeder *et al*., 1994). But, serotype SAT1 was first isolated and reported in 2008 from three different species of animals; cattle, sheep and goats (Legesse *et al*., 2013). Nowadays, FMD is endemic and widely distributed in all regions of the country. Among the seven serotypes five of them were reported as a cause of different outbreaks in the country during 1981-2018 as shown in the figure. From the report, serotype O was the most predominant serotype circulating in the country. The serotype C has not been reported in the country since 1983 (Ayelet *et al*., 2009).

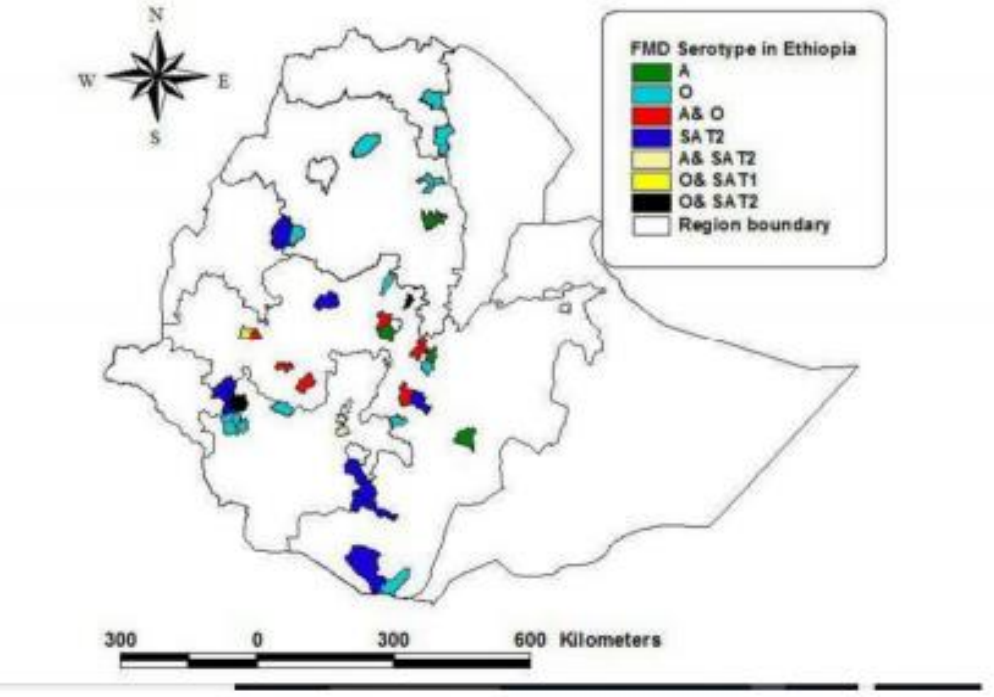

Figure 6: distribution of FMDV serotypes in Ethiopia. Adapted from (wubshet *et al*., 2019).

2.8.3. Control and prevention in Ethiopia

FMD controlling methods includes animal movement restrictions, a vaccination program, animal quarantine, environmental sanitary controls, outbreak investigation, serological surveillance, and slaughtering of sick animals. But due to the inaccessibility of the vaccine in particular, and lack of awareness about the vaccination program, the disease outbreak remains an economical burden to farmers of Ethiopia (Rufael, 2006). Prophylactive vaccination is very limitedly practiced and ring vaccination is carried out in outbreak areas to limit further spread of the virus (Ayelet *et al*., 2009). Trivalent FMD vaccines against FMD strain O, A, and SAT2, particularly topotypes of EA 3, Africa 3, and XIII (OIE, 2017), respectively, are commonly produced and distributed in the country, regardless of the quantity and quality. Most often, the vaccines are insufficient relative to the livestock population in the country, only centrally coordinated vaccination activities have been carried out in some market-oriented dairy farms and feedlots in urban and peri-urban areas (Zewudie *et al*., 2006). In addition, livestock producers in the country use palliative antibiotics or traditional treatments to monitor the clinical signs of FMD in cattle (Jemberu *et al*., 2016). Despite several efforts and attempts to design an FMD control strategy at the national level, an officially endorsed control plan for FMD has not been established. Recently, Ethiopia has joined the progressive control pathway (PCP-FMD) network, launched by FAO and OIE in Bangkok in June 2012, and has started implementing this since 2017 with the progress reaching stage one (Wubshet *et al*., 2019).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in areas where FMD outbreak occurs in Ethiopia from March 2020 to May 2020 to in two regions of the country; Oromia and Tigray regions to isolate and characterize the virus responsible for the outbreak. Outbreaks have been reported in Assela and Woliso from Oromia and Humera from Tigray region.

The first study area was Humera from Tigray region, northern Ethiopia. It is about 560 km to the west of Mekelle, the capital city of Tigray regional state, with an altitude ranging from 580-1820 meters above sea level. The area receives a rainfall of 400-650nm annualy. In this district, there are 15 rural and two urban kebelles (Haileselassie and Ali, 2005).

The second study area was Arsi zone, Asella town, Oromia regional state. Asella is located at 175 kilometers south-east of Addis Ababa with a latitude 7^{o} 57' N and longitude of 39^{o} 7'E with an elevation of 2430 meters above sea level. The total number of cattle found in Arsi Zone is 2,528,903 and area of the Zone is approximately 19,825.22km^2. It receives biannual rain falls and the average temperature ranges from 10 to 25°C (CSA, 2015).

The third study area is woliso. Woliso is a district in south west Shewa zone of Oromia regional state, about 90-140km away from Addis Ababa. It is located at a latitude and longitude of 8^0 32′ 23.0″ N and 37^0 58′ 16.3″ E respectively. It has an area of 1,511.501 km^2 , and 37 rural kebeles and three urban centers including Woliso town. The district is bordered in South by the Regional State of Southern Peoples' Nations and Nationalities and Goro district, in the North by Dendi district of West Shoa and Dawo district, in North East by Becho district, in West by Amaya district, in North West by Wonchi district, in East by Saden Sodo of South West Shoa Zone (Ejeta *et al*., 2019).

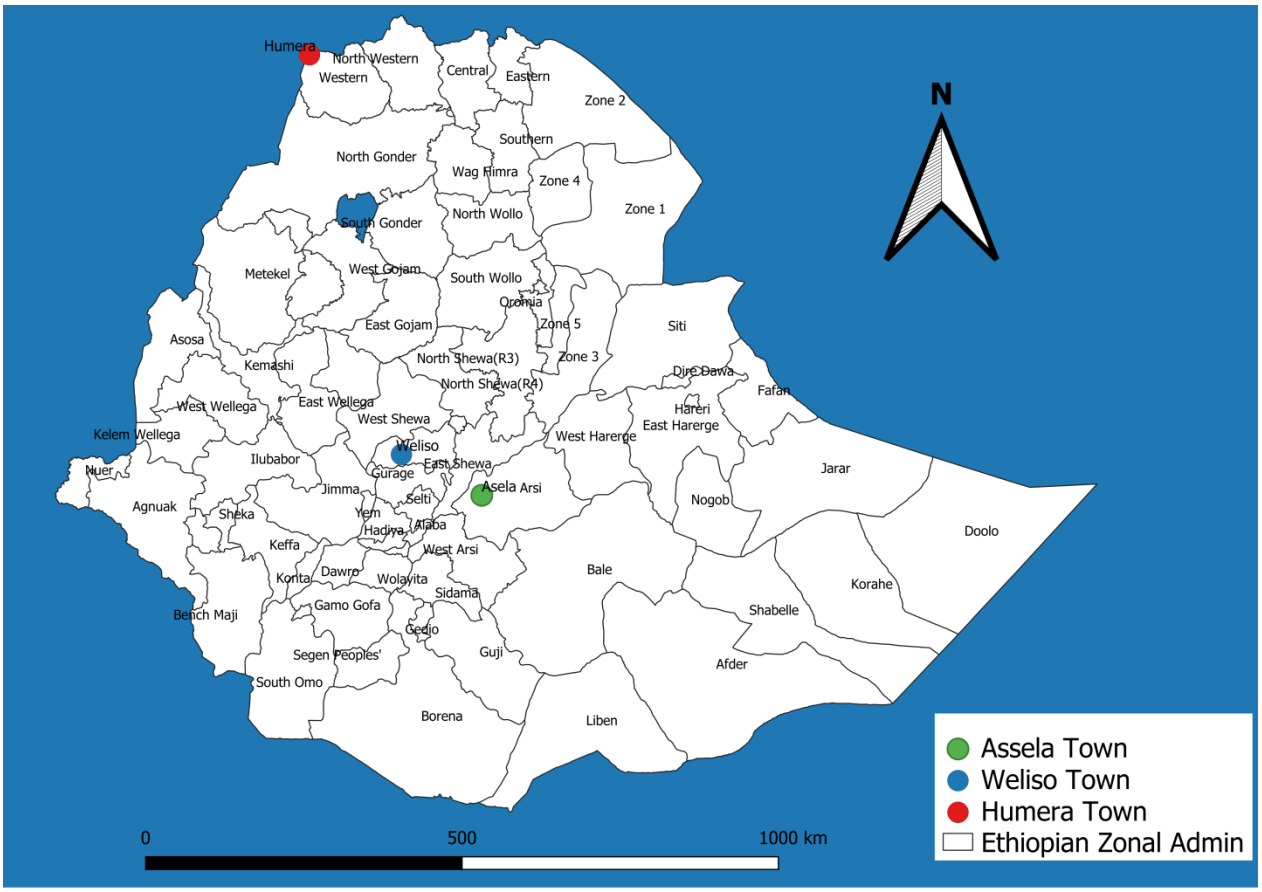

Figure 7: Map of the three study areas where FMD outbreak samples were collected.

3.2. Study animals

The study was conducted in cattle that had experienced outbreaks of FMD and showing clinical signs of the disease such as vesicles on the mouth, feet and teat. Cattle of both sexes with the extensive and semi intensive farms had been included in this research.

3.3. Study design

A cross sectional study design was used during the outbreak investigation, aimed at isolating and characterizing the serotypes of FMDV circulating in the study areas. Samples were collected by the regional veterinary diagnostic laboratories in the study areas and submitted and stored at the National Animal Health Diagnostic and Investigation Center (NAHDIC). For this study, tissue, swab and probing samples were used. Based on the data from the regional laboratories, animals

of all age, sex and breed with the history of infection and with healing lesions were used to collect samples.

3.4. Ethical clearance

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Ethical clearance for this study was obtained from Addis Ababa University College of Veterinary Medicine and Agriculture Minutes of Animal Research Ethics and Review committee. After the committee evaluated as part of the research project, it was approved.

3.5. Study methodology

3.5.1. Virus isolation

Before the virus isolation, the sample was first processed based on the procedure on OIE manual (OIE, 2014). The 10x sample suspension was made by grinding the sample in a sterile pestle and mortar using sterile sand and tissue culture medium containing antibiotic (penicillin, streptomycin and Amphotericin B solution). After grinding the suspension was centrifuged at 3500 xg for 10 minutes and then the suspension was filtered by Millipore filter of 0.22 µm sizes. Filtered tissue suspension (0.5 ml) suspected to contain the virus was inoculated on the confluent Baby hamster kidney cell grown on 25 cm^2 tissue culture flasks (appendix 1) and incubated at 37^0 C for one hour followed by addition of 10ml serum free Hank's media and incubated at 37^0 C with 5% CO_2 for 24-48 hr (appendix 2). It was observed daily for the appearance of CPE through inverted microscope. The cell was harvested when a CPE of >70% was observed, while samples was declared negative if CPE was not detected after three blind passages (OIE, 2012).

3.5.2. Identification of FMDV serotypes by antigen detection ELISA

Antigen detection sandwich ELISA (IZSLER, Bresica, Italy) was used to determine the serotypes of the FMDV. It is coated with and conjugated with monoclonal antibodies (MAbs) that can detect type O, A, C, Asia 1, SAT 1 and 2 serotypes. There is a pan-FMD test that can detect serotypes O, A, C, Asia 1 and some SAT serotypes which might have escaped to bind to the coated MAbs. The micro plates were supplied with one positive and one negative control with a catched Mabs to detect 10 samples per a well. The test was carried out using the manufacturers

guide lines in which samples were diluted in a diluent buffer and 50ul diluents per well were added into 8 wells from A-H and 50ul of diluents were added to the wells 11 and 12 (positive and negative controls, respectively). Then the plates were incubated for one hour at room temperature. After the incubation, all fluid in each wells were discarded and the plate were tap hard to remove all the residual fluid. 200ul of washing solution were added and incubated for 3 minutes at room temperature and then the well were emptied and washed two times. Finally, 50ul/well of conjugate A was added to rows A to F while the same volume of conjugate B was added to rows G and H and allowed to incubation for one hour. After the incubation, 50ul of substrate was added to all wells and left at room temperature for 20 minutes in the dark place. After 20 minutes, the reaction was stopped by adding 50ul/well of stop solution and the optical density of each well were read at wave length of 450nm using the micro plate reader (appendix 3).

Criteria for the validity of antigen detection ELISA

The positive inactivated controls are expected to give OD values of 1.0 unit or higher while the negative control for serotype O, A, C, Asia 1 and Pan-FMDV were expected to give OD values lower than 0.1 unit and the negative control for serotype SAT 1 and SAT 2 are expected to give OD value lower or equals to 0.2 unit.

Interpretation of antigen detection ELISA test

OD values of the samples were interpreted by subtracting the OD value of each negative control from the OD value measured for the test sample with the corresponding coated MAbs.

Table 1-Interpretation of antigen detection ELISA result.

Negative for FMDV	OD< 0.1
FMDV positive for type O	OD ≥ 0.1 with the type O MAb and with the pan FMDV MAb; some samples may cross react with the 1st MAb type A, but OD values with MAb O are higher.
FMDV positive for type A	OD ≥ 0.1 with at least one of the two type A MAba and with the pan-FMDV MAb.

FMDV positive for type Asia 1	OD ≥ 0.1 with the type Asia 1 MAb and with pan-FMDV MAb.
FMDV positive for type C	OD ≥ 0.1 with the type C MAb and with the pan-FMDV MAb.
FMDV positive for type SAT 1	OD ≥ 0.1 with the type SAT1 catching MAb; some samples could be positive also with the pan-FMDV MAb.
FMDV positive for type SAT2	OD ≥ 0.1 with the type SAT2 catching MAb; some samples could be positive also with the pan-FMDV MAb.
FMDV positive (untyped)	OD ≥ 0.1 with the pan-FMDV cathing MAb and < 0.1 with the type specific MAbs.

3.5.2. Molecular characterization

RNA extraction- total RNA was extracted from the cell culture isolate using RNAse mini kit (Qiagen, Germany) based on the manufacturer's protocol. For 350 ul of tissue sample an equal volume of lysis buffer RLT was added to 1.5 ml eppendrof and mixed by vortexing, then centrifuged at 13000xg using mini spine centrifuge for three minutes. After centrifugation, 350ul of 70% ethanol was added and vortexed as the previous one. The mixture was then transferred to RNeasy mini spin column placed on the 2ml collection tube under biosafety level II cabinet and spinned in a centrifuge for one minute at 12,500 rpm. The flow through was discarded from the collecting tube and repeated with remaining volume. 700 ul washing buffer RWI was added and centrifuged for 1 minute at 12.500 rpm to wash the RNA. After that, 500 ul RPE buffer was added and centrifuged 13400 rpm. Again the flow through was discarded and the RNAse mini spine column was centrifuged at 13400 rpm for 2 minutes. The column was transferred to a new clean and labeled eppendrof tube. Then, RNA free water was added and centrifuged at 13400 rpm for 1 minute. Finally, the RNA was elute with RNA free water in to eppendrof tube and stored at -20^{o} C until use.

One step RT-PCR- After extraction, master mix was done for the one step RT-PCR. Briefly, 8 µl RNase free water, 5 µl 5X PCR buffer, 1 µl Q Solution, 10 Mm dNTPs, 1 µl One step RT-PCR enzyme mix and universal primers (forward and reverse primers each with a volume of 2 µl were added to the total volume of 19 µl. Then 5 µl of the extracted RNA was added and the final volume was 24 µl.

Polymerase chain reaction cycles and primers used- At the molecular laboratory of NVI, samples were screened by universal primer set FMDV7- forward (FMDV7F) and FMDV7- reverse (FMDV7R) on the RT-PCR. The table below shows the primers and thermal profiles used for the amplification of the 5'UTR to screen the FMDV.

Table 2: List of universal primers used for the detection of FMDV and the thermal cycle protocol used.

Primer name	**Sequence**
Primer-FMDV7F	5'-GCCTGGTCTTTCCAGGTCT-3'
Primer-FMDV7R	5'-CCAGTCCCCTTCTCAGCTC-3'

Steps	**Temperature**	**Time**	**Cycle**
cDNA synthesis	50^{o} C	30 minutes	1 cycle
Intial denaturation	95^{o}C	5 minutes	1 cycle
1^{st} denaturation	94^{o}C	1 minute	
Annealing	54^{o}C	1 minute	30 cycles
Elongation	72^{o} C	1 minute	
Final elongation	72^{o} C	1 minute	1 cycle

Agarose gel electrophoresis of post RT-PCR amplicons- agarose gel is the most effective way of separating DNA fragments of varying molecular sizes. The principle of gel electrophoresis is that the negatively charged phosphate backbone of DNA will migrate to the positively charged anode when placed in an electric field. Due to the uniform mass/charge ratio of DNA, molecules are separated by size within an agarose gel in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight (Lee *et al*., 2012). Hence, it is used as a qualitative tool for estimation of molecular weight. The PCR products were analyzed on the prepared 1.5% Agarose gel by adding 4µl Gel red with loading dye then the PCR product were loaded in the volume of 10µl in each well and 10µl molecular marker(Ladder) was added started 100bp plus. Electrophoresis was run for one hour at 120v then the DNA band was visualized by UV illumination, using desktop according to the base pair (bp), and then the size was determined and documented (appendix 4).

Serotyping of FMDV by one step RT-PCR- after screening of FMDV by RT-PCR, positive samples were amplified using serotype specific primers for serotypes O, A, SAT2 and SAT 1 were indicated in table 5. The thermal cycling condition for each serotypes were also indicated in table 6. All the amplified PCR products were visualized on gel electrophoresis using 1.5% agarose gel.

Table 3: Location in the genome and sequences of primers for serotyping of each serotype

Primers	**Sequence (5'-3')**	**Location**	**bp**	**Serotype**
FMDVF	CTGCCACCGTCGAGAACTAC	VP3	591	O
FMDVR	CAGGCGCCACTATCTTCTTGT	1D		
IC562F	TACCAAATTACACACGGGAA	2B	866	A
EUR-2B52	GACATGTCCTCCTGCATCTGGTTGAT	1C		
FMDVSATF	CCACATACTACTTTTGTGACCTGGA	VP1	730bp	SAT2
FMD2B208R	ACAGCGGCCATGCACGCCAG	2B		
IC559F	CAAGACCGTGGACAACAAGA	VP3	506	SAT 1
2B208R	GCGTGGTCTTGTACCTGTCA	2B		

Table 4: Thermal cycling profile of each serotype (O, A, SAT 2, SAT1).

Thermal cycling for serotype O

Steps	**Temperature**	**Time**	**Cycle**
cDNA synthesis	50^{o} C	30 minutes	1 cycle
Intial denaturation	95^{o}C	5 minutes	1 cycle
1^{st} denaturation	95^{o}C	1 minute	
Annealing	58^{o}C	1 minute	35 cycles
Elongation	72^{o} C	1:30 minute	
Final elongation	72^{o} C	7 minute	1 cycle

Thermal cycling for serotype A

Steps	Temperature	Time	Cycle
cDNA synthesis	50^{o} C	30 minutes	1 cycle
Intial denaturation	95^{o}C	15 minutes	1 cycle
1st denaturation	95^{o}C	1 minute	
Annealing	55^{o}C	1 minute	35 cycles
Elongation	72^{o} C	2 minute	
Final elongation	72^{o} C	5 minute	1 cycle

Thermal cycling for serotype SAT2

Steps	Temperature	Time	Cycle
cDNA synthesis	50^{o} C	30 minutes	1 cycle
Intial denaturation	95^{o}C	5 minutes	1 cycle
1st denaturation	95^{o}C	1 minute	
Annealing	58^{o}C	1 minute	15 cycles
Elongation	72^{o} C	1:30 minute	
2nd denaturation	95^{o} C	1 minute	
Annealing	57^{o} C	1 minute	20 cycles
Extension	72^{o} C	1:30 minutes	
Final elongation	72^{o} C	7 minute	1 cycle

Thermal cycling for SAT1

Steps	Temperature	Time	Cycle
cDNA synthesis	50^{o} C	30 minutes	1 cycle
Intial denaturation	95^{o}C	5 minutes	1 cycle
1st denaturation	95^{o}C	1 minute	
Annealing	55^{o}C	1 minute	35 cycles
Elongation	72^{o} C	2 minute	
Final elongation	72^{o} C	5 minute	1 cycle

3.6. Data management

Results from virus isolation, antigen detection ELISA and RT-PCR results were recorded on Microsoft Excel spread sheet. Cell culture results, CPE development and molecular characterization results were recorded and tabulated. Virus isolation and molecular detection of FMD virus were elaborated using descriptive statistics analysis. Moreover, regarding the molecular characterization, the banding patterns of individual sample were scored based on the presence or absence of the bands with the appropriate base pairs.

4. RESULT

4.1. Virus isolation

Among the 23 samples collected from the three areas, 19 samples were subjected for virus isolation on BHK21 cell culture as shown in table 3. From 19 samples inoculated on BHK21 cell culture CPE was observed on 5 (26%) samples, while virus didn't grow on the rest of samples 14(74%) after third passage. CPE was observed on samples collected from woliso, but from Assela and Humera samples no CPE was seen even after the third passage.

Table 5- FMDV isolated from cattle in different areas.

Site of outbreak	**No. of sample inoculated**	**Type of sample**	**Date of collection**	**Virus isolated**
Humera	6	3- swab 3- probang	28/03/20	_
Assela	6	2- tissue 4- swab	22/05/20	_
Woliso	7	5 tissue 2 swab	02-04/05/20	5

But samples from Assela and Humera didn't show any CPE. The CPE observed in BHK-21 cell were fast destruction of mono-layer cell and the infected cell appeared as singly and round in shape (Figure 5). Additionally, the CPE was characterized by complete destruction of the cell and cell detachment which was mostly seen within 48 hrs of inoculation.

A B

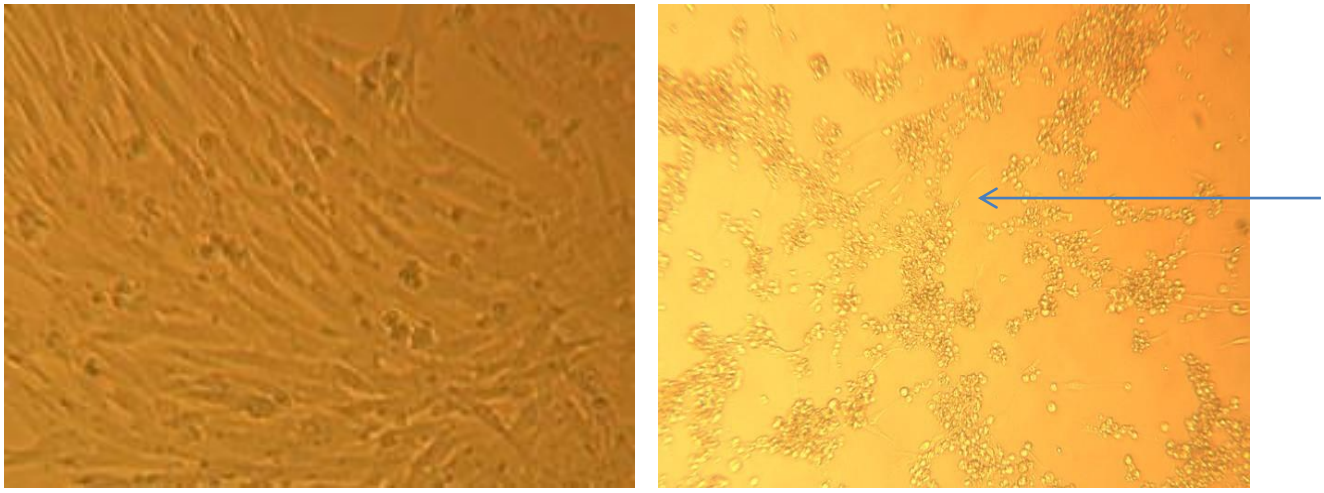

Figure 8: Picture showing FMDV on BHK cell. A: BHK-21 cell control (Virus was not inoculated), B: showed morphological change (CPE) after FMDV inoculation as indicated by the arrow. Arrow shows rounded cells and sloughing of the cell as a result of FMD virus infection.

4.2. Antigen detection ELISA

Out of the 23 original samples, three samples become positive on the antigen detection ELISA test. From the three, two samples were from Woliso and one sample from Assela. The woliso samples were typed as serotype O and SAT1 whereas the sample from Assela was serotype O as shown in the table 4 below.

Table 6- Serotypes of FMDV identified in the study areas.

Site of outbreak	**No. of sample collected**	**No of sample isolated**	**No. of serotype identified**	
			O	**SAT1**
Humera	7	-	-	-
Assela	6	1	1	-
Woliso	10	3	1	1
Total	**23**	**4**	**2**	**1**

4.3. Molecular characterization

Among the 19 cell culture samples tested on RT PCR only two (10.5%) of the samples from Woliso were found to be positive as shown in figure 8, where sample number 3 and 9 were found to be positive.

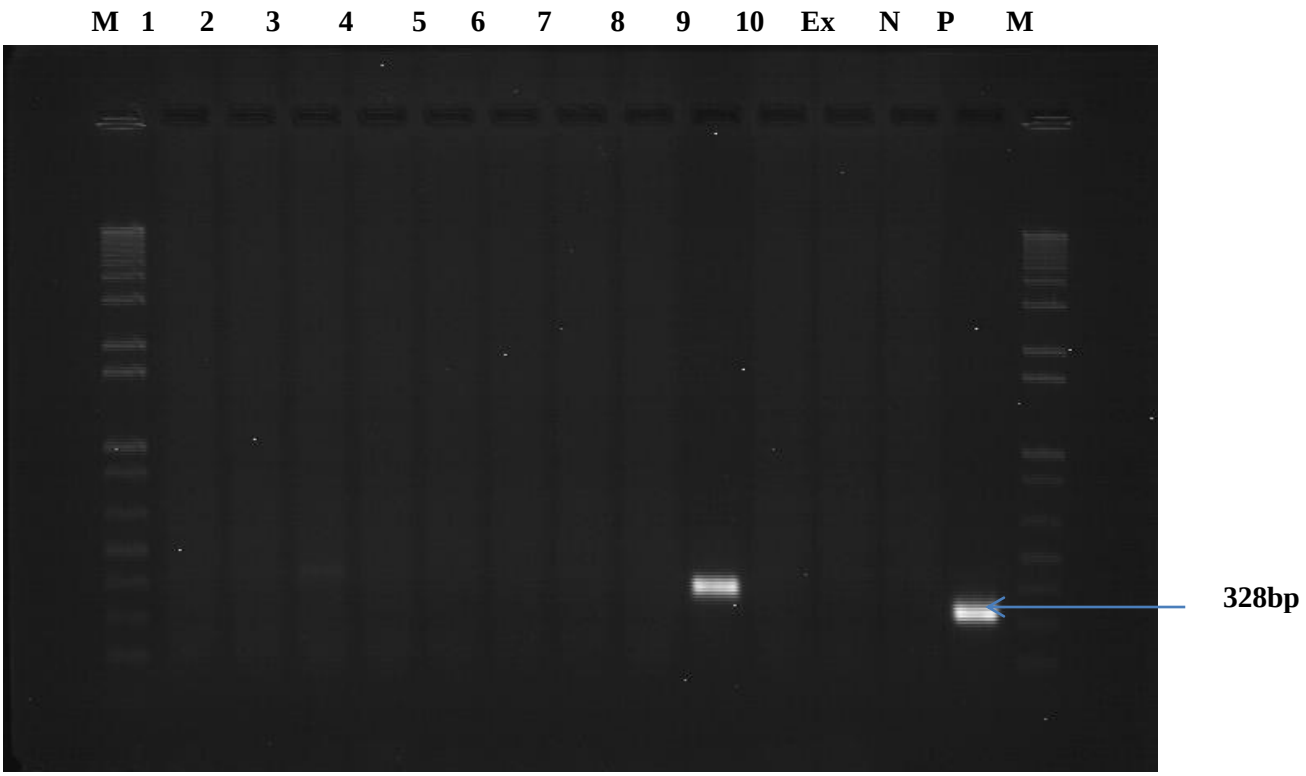

Figure 9: Detection of FMDV genome by RT–PCR using primer mixture of FMDV7F/ FMDV7R was used targeting the 5′ UTR coding region of the virus.

Note- M- molecular marker starts from 100bp

N- Negative control

P- Positive control

Ex- Extraction control

1-10- sample numbers

The two samples which were found positive on RT-PCR were tested for serotyping by using different serotyping primers. On serotyping, only one of the two shows a clear band serotype O primer which indicates that one of the sample was serotype O as shown in the figure 7. But the other sample didn't show any band by the four serotypes O, A, SAT 2 and SAT 1.

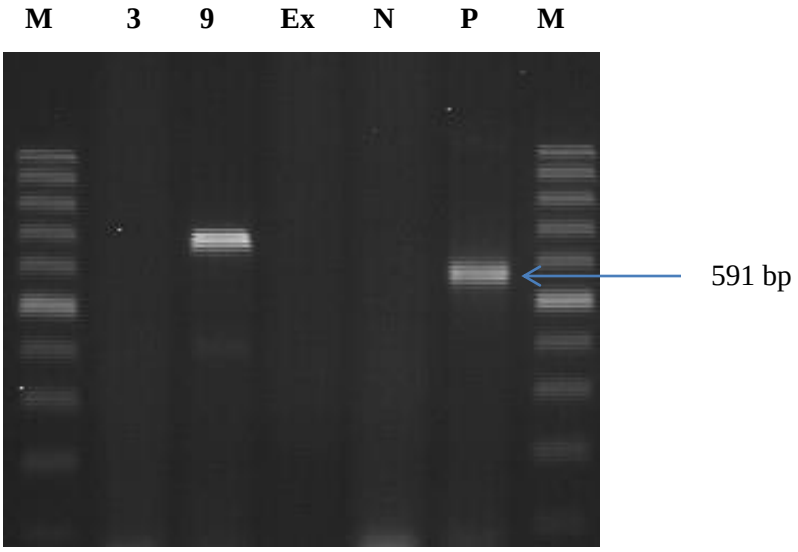

Figure 10: Detection of FMDV genome by RT– PCR. Primer mixture FMDVOF/FMDVOR was used for targeting the coding region of the virus.

Note - MM- molecular marker starts from 100bp

N- Negative control

P- Positive control

Ex- Extraction control

3 and 9- sample numbers

4.4. Sequencing of RT-PCR positive samples

The PCR product amplicons will be purified using the Wizard SV Gel and PCR clean up system kit (promega, Germany) in the molecular biology laboratory of NVI. The purified PCR product

mixed with sequencing primers will be submitted for the commercial sequencing company LCG Genomics (Berlin, Germany). Then, the sequences will be analyzed to determine the responsible serotype. Currently the purified PCR product shipment to the sequencing service provider is closed because of COVID-19 global crisis and will be submitted for sequencing after the restriction is lifted.

5. DISCUSSION

The highly contagious nature, wide host range, accompanied by the remarkable economic losses, make FMD of a primary animal health concern worldwide. Effective vaccines and strict control measures have enabled FMD eradication in most of the developed countries. However, the disease remains enzootic in many regions of the world, like Ethiopia, posing a serious problem for commercial trade with FMD free countries. Hence it is necessary to control FMD by reducing the dissemination of the causative virus to other non-infected regions and by using tailored made vaccines with antigenic similarity to the outbreak virus serotype. Therefore, characterization of the circulating FMDV serotype is essential for tracing source of the outbreak virus and for proper selection of effective vaccine.

The present study was aimed at isolation, molecular characterization and serotype identification of FMDV causing outbreaks in Humera, Assela and Woliso. In this research, from 19 suspected clinical samples subjected to BHK 21 cell line adaptation, 26 % (n=5) field samples showed FMDV induced CPE after 24-48 hrs of incubation. The CPE was characterized by rounding, detachment and sloughing of the mono-layer cell culture. This finding is in agreement with sulayeman *et al*. (2018), Metages (2018). But the rest of 14 samples didn't show any CPE even after the third passage. Most of the samples that didn't showed CPE were samples collected from Assela and Humera. This may be due to the animals where the sample had been taken may be at the late stage of recovery during sample collection (Metages, 2018). Also it could be due to the improper transportation of the samples from the collection site to the laboratory where the cold chain system might not be maintained that leads to the loss of FMDV. And may be the sample had been taken from animals that had been diseased by other vesicular diseases that have similar clinical signs like vesicular diseases.

In this study, all the 19 samples were detected using conventional PCR for the presence of FMDV genetic material in the sample, only two samples from Woliso were found to be positive. From the two samples, one was from epithelial origin while the other was swab sample. The epithelial sample had a clear band as compared to the swab sample which showed that the epithelial sample had more FMDV antigen. This is in agreement to OIE (2009), as this institution

described that epithelial tissues are the ideal samples for virus detection. This small number of positive conventional RT-PCR results might have been because of the virus destruction in the course of transfer from the field.

In Ethiopia, five of the seven serotypes had been diagnosed by clinically, serologically and molecular techniques during the period 1981 to 2018 (Yalew, 2019, Wubshet *et al*., 2019). In this study serotyping was done through antigen detection sandwich ELISA and serotype specific RT-PCR techniques. In antigen detection ELISA, two samples (10.5%) from Woliso and one sample from Assela have been found serotype SAT1 and O, respectively. But sample from Humera has been found to be negative for FMDV serotyping with OD value <0.1. This is agreement that all the samples from Humera didn't show any CPE after the third passage and were detected negative by the conventional PCR.

Antigen detection sandwich ELISA was developed for detection and typing of FMDV directly from field materials. But in this scenario, many samples failed to give positive results and such negative results have to be confirmed by inoculation into sensitive cell line which may take more than 4 days. As a result, a need for rapid confirmation of clinical diagnosis with more sensitivity resulted the development of serotype specific PCR. Also, a particularly high sensitivity was reported by RT-PCR ELISA (Longjam *et al*., 2011). Therefore, the two samples that were positive on the conventional PCR were serotyped by using serotype specific primers for serotype O, A, SAT2, and SAT1. The result was one sample was serotype O and the other sample was found negative by all serotype specific primers. The sample which was found to be serotype O by serotyping PCR was in agreement with the finding to the antigen detection ELISA result. The finding of the serotype O in this study is in agreement with Ayelet et al. (2009), Menda et al. (2014), Nigussie et al. (2011), Sulayman et al. (2018), Metages (2018).

The one sample that was positive by the universal primer and negative for other serotypes primer sets was serotyped as SAT1 by the antigen detection sandwich ELISA earlier than the conventional PCR. This might be attributed to different possibilities, one of which might be simple mutation. Point mutation at a critical site leads to failure of the primer to bind to the viral nucleic acid (Locher *et al*., 1995). Success in PCR depends mainly on the efficiency of the primer and template to bind together and amplify (Giridharan *et al*., 2005). Also High nucleotide

mutation rate is a common character of FMDV (Phologane *et al*., 2008) especially of the SAT serotypes that have the high substitution rates as compared to the other serotypes (Bastos *et al*., 2003, El-khabaz and Al-hosary, 2017). Hence, this sample was found SAT1 by antigen detection sandwich ELISA. The other sample which was from Assela, serotyped as O was found to be negative in conventional PCR. This may be due to the RNA degradation.

6. CONCLUSSION AND RECOMMENDATIONS

In this study, FMDV serotype O and SAT1 were identified. While the rest study areas found to be FMDV negative which could be attributed as a result of improper sample shipment from the study areas, samples may be collected at the stage of disease recovery or from other clinically the same vesicular diseases of cattle. The finding of serotype O indicates the dominating serotype circulating serotype in Ethiopia and still circulating in the country. But the one sample which had been detected as serotype SAT1 by the antigen detection ELISA and positive by the universal primers in the RT-PCR became negative by the serotyping RT PCR using serotype specific primers. However, the detection of SAT1 serotype is an alarm to include this serotype in the vaccine program of the country as it is circulating in outbreaks.

Based on the above conclusion, the following recommendations are forwarded.

- Research on continuous surveillance, serotyping and molecular characterization on FMDV should be conducted to check the introduction and circulation of new serotype and topotypes coupled emerging strains of the virus in the country.

- Government FMD control strategy through regular vaccination should be started and carried out in all susceptible animals and/or selected areas to control the disease and access export market.

- Further works such as nucleotide sequence is essential to explain the exact serotype of FMDV.

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8. APPENDICES

Appendix 1: Preparation of BHK -21 for virus inoculation

- First prepare a complete media containing 1x minimal essential media, 10% fetal calf serum, tryptose phosphate broth, 100x antibiotics and antimycotic solution.
- Pre-warm both Trypsin and phosphate saline solution (PBS).
- All the materials used for sub culturing are disinfected by 70% alcohol and placed in the biosafety cabinet.
- Take cell culture flask with confluent monolayer from the incubator to the biosafety cabinet after disinfecting by 70% alcohol
- Decant old media and wash by PBS to remove residual serum (which would inactivate the trypsin).
- Slowly add enough amount of trypsineand incubate for ten minutes.
- Add growth medium and pipette vigorously until monolayer become dispersed to a single cell.
- Add the final amount of the complete media and dispense to other 3 TC-25 flask and then incubate at humified incubator with 5% CO2 at 37^0c.
- Then follow the growth of the cell line and for any contamination using inverted light microscope.

Appendix 2: Virus isolation procedure on BHK-21 cell

- The tissue suspension virus samples and 2% DMEM were thawed.
- Put the cell, media and other materials that are used in inoculation into biosafety cabinet after disinfection.
- Discard the old media from the monolayer cell and wash the cell gently with 3ml of pre warmed PBS.
- Inoculate 1ml of virus sample to the cell in $25cm^2$ tissue culture flasks and rock the flask gently to distribute inoculum evenly over the monolayer cell.
- Incubate inoculated culture in incubator at $37\ ^0$c for 1hr to allow virus adsorb.

• Add 5ml of 2% DMEM to the inoculated cell and incubate at 37°C and 5% CO2 in a humidified incubator.

• Monitor the inoculated tissue flask daily for the development of CPE and any other contamination.

Appendix 3: Plate layout for antigen detection sandwich ELISA

Catching MAbs		Sp 1	Sp 2	Sp 3	Sp 4	Sp 5	Sp 6	Sp 7	Sp 8	Sp 9	Sp 10	Po. ctrl	Neg ctrl	
Type O A	A													Conjugate A (Pan-O-A-C-Asia 1+ some $SAT_{S)}$
Type A (1st MAb)	B													
Type A (2nd MAb)	C													
Type Asia 1	D													
Type C	E													
Pan-O-A-C-Asia 1	F													
Type SAT2	G													Conjugate B (SAT1-2)
Type SAT1	H													

Note- SP, Sample; MAb, Monoclonal antibody; Po.ctrl, Positive control; Ne.ctrl, Negative control

Appendix 4: Agarose Gel Electrophoresis Protocol

1. **Equipment**

To pour a gel you will need the following:

•Two 1L orange cap bottles.

• 250 mL flask

• Volumetric cylinders

• Spatula

• Gel casting tray

• Gel combs

For running the gel

• Tape

• Electrophoresis tank

• Power supply and cables

2. Pouring the gel

Agarose gels will always be made in 0.04M Tris-Acetate-EDTA, pH 8.3 (1X TAE) buffer.

1. Weigh out the appropriate amount of agarose (2% gel would be 2g agarose in 100 mL). Add the appropriate amount of 1X TAE. Make the mixture in a 250 mL flask, cover it with Wrap, and microwave for 1 minute until all the agarose has melted.
2. Tape both sides of the casting tray so that it will hold the liquid gel.
3. Add Ethidium Bromide (EtBr) to a final concentration of 0.05 μg/mL. So for a 100 mL gel add 5 μL of 10 mg/mL stock.
4. Pour the warm liquid agarose. Place the comb into the casting tray by placing the sides into the notches.
7. Wait until the gel polymerizes. It usually takes about an hour. The gel should look opaque and uniform.
8. Carefully remove the comb to expose the sample wells.

3. Running the gel

1. Place the gel into the tank.
2. Add 1 μL loading dye per 5 μL sample (because the dye is 6x).
3. Add samples:
    a) Loading 100-500 ng of DNA per lane is usually sufficient.
    b) Total sample volume should be from 10-35 μL (depends on the gel thickness of the gel and well size used).
    c) One of the samples should be a marker that contains DNA fragments of knownlengths that are in the range of your samples.
4. Connect the tank to the power supply and Set the voltage at ~150 V.

6. Run for about an hour or until the faster dye (BB) migrates most of the way through the gel. You can monitor the progress of the DNA directly (if the EtBr was added) by shining UV light on the gel as it is running. Just be careful with the UV lamp.

4. Staining a gel

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

5. Imaging the gel

To visualize the DNA bands, you must look at them under UV light.**WEAR GLOVES**. Be very careful with gels, as they can be very fragile.
1. Open the plastic cover.

2. Transfer gel from the casting tray by sliding onto the transilluminator. Close the plastic cover. This should protect you from UV, but you can use a face mask, a plastic shield, or other protection as well.

3. Turn on UV to 100%. You should see bands at this point.

4. TURN OFF UV. You want to minimize the UV exposure time as it may damage DNA. Turn off UV before you open the plastic cover.

Appendix 5: miscellaneous photos during research