SEROTYPING AND MOLECULAR CHARACTERIZATION OF FOOT AND MOUTH DISEASE OF CATTLE IN CENTRAL ETHIOPIA

MSc. THESIS

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Final approval and acceptance of the Thesis is contingent upon the submission of its final copy to the council of Graduate Studies (CGS) through the candidate's Department or School of Graduate Committee (DGC or SGC).

DEDICATION

This Thesis is dedicated for loving memory of my mother Buzu Tolessa, who passed away in May 2012 and for my beloved daughter Bikiltu Getachew, who missed her mother by emergency death in May 2016. For both mothers, may God rest their souls in peace!

STATEMENT OF THE AUTHOR

By my signature bellow, I declare and confirm that this thesis is my own work. I have followed all ethical and technical principles of scholarship in preparation, sample collection, laboratory investigation, data analysis and completion of this thesis. In addition, I affirm that I have cited and referenced all sources of information, knowledge and materials used in this document. Every serious effort has been made to avoid any plagiarism in the preparation and development of this thesis. This thesis has been submitted to Haramaya University in partial fulfillment of the requirements for the Degree of Master of Science in the program of Biotechnology and deposited at the Library of the University to be made available to borrowers under the rules and regulations of the Library. I solemnly declare that I have not submitted this thesis to any other institution anywhere for the award of any academic degree, diploma or certificate.

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BIOGRAPHICAL SKETCH

Getachew Mulatu (author) was born on November 11, 1988 in Ilfeta, West Shewa Zone, Oromia Regional State from his father Mr. Mulatu Dilba and his mother Mrs. Buzu Tolessa. He attended his primary school at Gute Ilfeta (Ilfeta District) and Tule (Ambo District) Elementary School from 1998 - 2005, secondary and preparatory school at Ambo Secondary and Preparatory School from 2006-2009. After completion of his preparatory school, he joined Ambo University in September 2010 and graduated with Bachelor of Science in Veterinary Laboratory Technology in July 27, 2012. In January 13, 2013, he got employment opportunity at Ambo University as graduate assistant. He was successfully doing his responsibility until he joined Haramaya University, College of Natural and Computational Sciences, Department of Biology to pursue his MSc. Program in the field of Biotechnology starting from September 2015.

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ACRONYMS AND ABBREVIATION

BHK	Baby Hamster Kidney
cDNA	Complementary Deoxyribonucleic Acid
CFT	Complement Fixation Test
CPE	Cytophatic Effect
CSA	Central Statical Agency
C_T	Threshold Cycle Value
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
ICTV	International Committee on Taxonomy of Viruses
MAB	Monoclonal Antibody
NAHDIC	National Animal Health Diagnostic and Investigation Center
NSP	Non- Structure Protein
OD	Optical Density
OP	Oro-pharyngeal Fluid
OIE	Office International Des Epizooties
PAB	Polyclonal Antibody
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RT- PCR	Reverse Transverse Polymerase Chain Reaction
SAT	South Africa Territory
SP	Structure Protein
+SS RNA	Positive Sense Single Stranded Ribonucleic Acid
VNT	Virus Neutralization Test
VP	Virus Protein
VTM	Viral Transporting Media
WRL	World Reference Laboratory
WRLFMD	World Referenced Laboratory Foot and Mouth Disease

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ABSTRACT

Foot and mouth disease (FMD) is one of the most economically important, highly contagious and trans-boundary animal diseases in developing countries including Ethiopia causing huge amount of economical loses in different geographical areas of the country. However, the information about genetic characteristics and strains of FMD virus circulating in Ethiopia is scarce. A cross sectional study was conducted between September 2016 and April 2017 to undertake serotyping and molecular characterization of FMD virus from outbreak cases in central Ethiopia. A total of 105 samples (35 oral swabs, 6 epithelial tissues, 64 oro-pharyngeal fluids) were collected purposively from cattle outbreaks cases in central Ethiopia including Oromia Special Finfine Surrounding zone (Mulo woreda), North Shewa zone (Aleltu, Kimbibit and Wochale woredas), East Shewa zone (Adea woreda) and Addis Ababa (Kolfe Koraneyo sub city) and transported to NAHDIC Sebeta for laboratory investigation. All samples collected, stored, prepared in accordance with standard methods and subjected to RNA extraction and real-time RT-PCR. Positive samples for FMDV were further subjected to viral isolation (cell culture) and identification of isolated virus by antigen detection ELISA. Obtained data were entered into Ms-excel and interpreted by SPSS program. Out of the total 105 samples tested by real-time RT-PCR, 34 (32.38%) samples were found to be positive for FMD virus. Eight positive samples representing each outbreak areas were further cultured or inoculated into 25 cm^2 flask containing monolayer BHK-21 cell lines, the result showed the cytopathic effect (CPE) which is characterized by the rounding and flattening of the cells, breaking down of the intracellular bridges and finally cell death. Among the eight isolates, six samples were characterized by antigen detecting ELISA. Serotypes O (4) and A (3) were identified. In one of the isolates both serotype were identified. This study revealed that serotypes O and A of FMD virus were causing outbreaks in cattle in Ethiopia inflicting huge economic losses. Therefore, proper control and intervention should be designed and implemented to minimize the burden of the disease.

Keywords: *Cattle, Cythopatic Effect, Foot and Mouth Disease, rRT-PCR, Serotype O, A*

1. INTRODUCTION

Ethiopia has the largest livestock population in Africa and 5th in the world and estimated as 56.71 million cattle, 29.33 million sheep, 29.11 million goat, 2.03 million horses, 7.43 million donkeys, 0.4 million mules, 1.16 million camels and 56.87 million poultry (CSA, 2015). According to Behnke and Metaferia (2011) livestock contribution to the national economy is estimated at 19% of the total GDP, 45% of the agricultural GDP and about 20% of the country's export earnings.

Animal diseases are widespread in all parts of the country causing death of animals, reducing the production and productivity of livestock. Among total livestock population, 4.09 million cattle die every year, of which 3.45 million die from diseases (CSA, 2011). Foot and mouth disease (FMD) is one of the seven trans-boundary animal diseases that restrict Ethiopia from international trade. In addition, the most contagious disease of cloven hooves mammals have a great potential for causing severe economic loss in susceptible cloven hoofed animals such as domestic species, cattle, pigs, sheep, goats and water buffalo (Larska *et al*., 2008). It is one of the most important diseases that hamper productivity and production in Ethiopia (Ayelet *et al*., 2012).

Foot and mouth disease virus (FMDV) belongs to the genus *Aphthovirus*, family *Picornaviridae* (OIE, 2004). It has seven serotypes, namely: O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1 and by one time or another, one type or the other was widely reported in most parts of the world (Quinn *et al.,* 2005). Serotype O and A reported in France by Valee and Caree in 1922, they named these serotypes after their place of origin; O for the department of Oise in France and A for Allemagne (Germany). Waldmann and Trautwein 1926 reported serotype C. Serotypes SAT1, SAT2, and SAT3 of FMDV were observed in 1948 a sample collected from the FMD outbreak in South Africa. Asia 1 was reported from Pakistan in 1954 (Brooksby, 1958; Radostits *et al*., 1994).

The disease spreads rapidly by movement of infected animals or mechanically on fomites such as clothing, shoes, vehicles and veterinary instruments. The reasons for the rapidity of spread to fully susceptible populations, the production of high titer in respiratory secretions, the large volumes of droplets, aerosols of virus shed by infected animals, the rapid replication cycle with

very high virus yields and the short incubation period as stated by Sahle *et al*., (2004).

Foot and mouth disease is a global disease that throughout the years has affected most of the countries, commonly in Asia, Africa, the Middle East and parts of South America (Sahle *et al*., 2004). The annual global economic impact of FMD in endemic regions ranges 6.5-21 billion USD resulting from visible production losses and vaccination costs, while outbreaks in FMD free countries and zones cause additional losses exceeding 1.5 billion USD per year (Knight-Jones and Rushton, 2016). FMD is frequently listed as the most economically important disease of livestock in many developed and developing countries (Grace *et al*., 2015).

Foot and mouth disease is an endemic disease in Ethiopia affecting mainly cattle in the majority of cases, but also causing problems in small ruminants at infrequent intervals. Historically the disease was first reported in 1957 (Gulima, 2011; Ayelet *et al*., 2012), although the disease had been undoubtedly in the country for longer time prior to this report. Most of livestock keepers were familiar with the disease and some were using traditional methods of immunization against it, i.e. 'mouthing' (Gulima, 2011). In Ethiopia, reports indicate that FMD serotypes O, A, and C were responsible for FMD outbreaks during the period of 1957-1979 (Martel, 1974) and 1981-2007 additional serotypes of the virus including SAT 1 and SAT 2 were reported (Gelagay *et al.,* 2009).

The annual costs of FMD were assessed based on production losses, export losses and control costs. According to the report of Wudu *et al*. (2016), the total annual costs of FMD under official control program were estimated at 1354 (864-2042) million Ethiopian birr (ETB). The major cost (94%) was due to production losses and the rest were export losses and control costs. Besides causing direct losses to livestock economy, it also causes indirect losses in terms of severe trade restrictions, impact which may be higher than direct losses. FMD is the most important livestock disease in terms of economic impact on export earnings. Wagari (2016) has documented about 71026.8 USD losses and Alemayehu *et al*. (2014) in 2011 the total annual economic loss due to bulls rejection from international market was estimated to be 3,322,269 USD.

The occurrence of FMD outbreaks in Ethiopia is increasing from time to time and cattle were under risk of infection, however, there is no government strategy in FMD control and prevention. Lack of vaccination strategies (quality, coverage and timing), presence of free animal movement

without certification, the high rate of contact among animals at commercial markets, in communal grazing areas and watering points, poor surveillance and diagnostic facilities were among the reasons forwarded for the increasing incidence of the disease. The study areas were known with the abundance of cattle and for trading purpose, movement of cattle from border toward the central Ethiopia is high. This leads to an increase in the incidence of the disease in the area. So the impact of FMD could be solved by developing an adequate control and prevention, generating more knowledge about etiology, epidemiology, transmission, pathogenesis, diagnosis and others to community (cattle holder). Effective control strategies of this disease needs sensitive, specific and rapid diagnostic tools like molecular detection (rRT-PCR), virus isolation and antigen detection by ELISA is needed. However, in Ethiopia the information and knowledge of molecular characterization and serotyping is scarce. Considering the above problems, the current study was planned to investigate the serotyping and molecular characterization of the FMDV isolate from suspected cattle, which is useful for vaccine strains selection, tracing the source of outbreaks, implementation of good control programmers and the eradication of the disease in the country.

The objectives of the study were as follows:-

General Objective was

- To undertake Serotyping and molecular characterization of FMD virus from outbreak cases in central Ethiopia.

Specific Objectives were

1. To detect and isolate the FMD virus from outbreak cases.
2. To identify the FMD virus serotypes circulating in the study area.

2. LITERATURE REVIEW

2.1 Overviews Foot and Mouth Disease

Foot and mouth disease (FMD) is a highly contagious viral disease that primarily affects cloven-hooves livestock and wildlife. Although adult animals generally recover, the morbidity rate is very high in naive populations, significant pain and distress occurs in some species. Sequel may include decreased milk yield, permanent hoof damage and chronic mastitis. High mortality rates can sometimes occur in young animals or in some wildlife populations. Foot and mouth disease was once found worldwide; however, it has been eradicated from some regions including all of North America and Western Europe. Where it is endemic, this disease is a major constraint to the international livestock trade. Unless strict precautions are followed, FMD can be readily re-introduced into disease-free regions via animals or animal products. Once introduced, the virus can spread rapidly, particularly if livestock densities are high or detection is delayed. Outbreaks can severely disrupt livestock production, result in embargoes by trade partners and require significant resources to control. Direct and indirect economic losses estimated several billion USD. Since the 1990s, a number of outbreaks have occurred in FMD-free countries (OIE, 2014; 2016).

2.2 Etiology

The name of foot and mouth disease virus differ from place to place; fiebre aftosa, fievre aphteuse, and maulund-klauenseuche. According to the International Committee on Taxonomy of Viruses, this disease has the following characterization (ICTV, 2011): Family: *Picornaviridae*, Genera: *Aphthovirus*, Baltimore Classification: Group IV (+) ssRNA.

2.2.1 Morphology

Foot and mouth disease as indicated in (Figure 1) non-enveloped, 25-30 nm, icosahedra symmetry and single stranded positive sense RNA virus with the whole virus particles having sedimentation coefficient of 146S and genome of ~8.5 Kb size. The viral genome is translated as a single polyprotein, which is posttranslational cleaved by viral proteases into four structural proteins (VP1, VP2, VP3, and VP4) and several nonstructural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D). The P1 region of genome encodes the four structural proteins VP1, VP2, VP3, and

VP4 encoded by 1D, 1B, 1C and 1A genomic regions, respectively. Sixty copies of each structural protein (VPl-4) assemble to form the capsid. VP4 is internal whereas others are exposed on virion surface and VP1 is the most immunogenic protein described by Neeta *et al*. (2011).

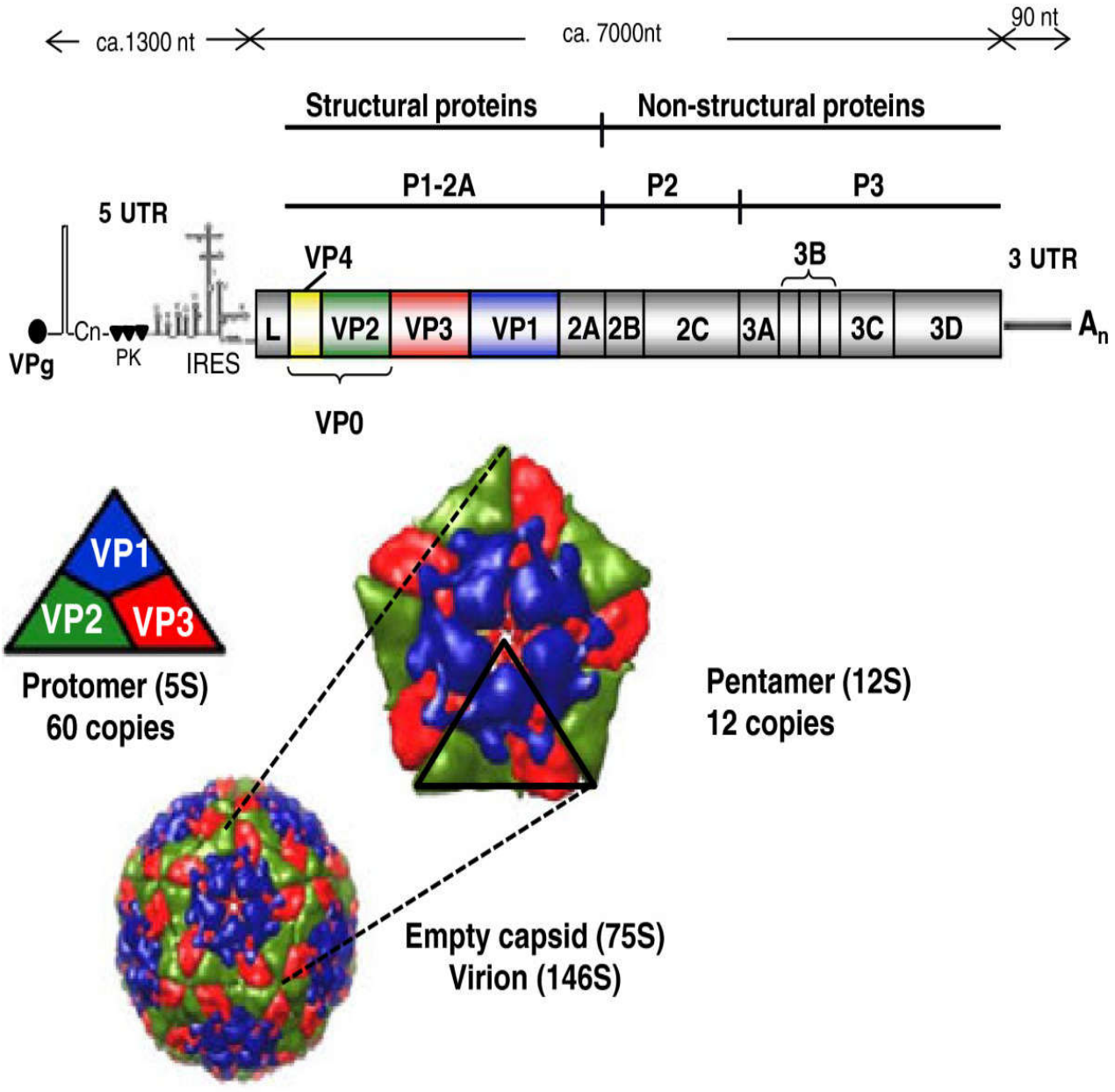

Figure 1:- Genome organization of FMDV and the structure of virus (source: Belsham, 2005; Goodwin *et al*., 2009).

2.2.2 Foot and Mouth Disease Serotypes and Strains

There are seven serotypes of FMD virus: O, A, C, Asia-1, and South African Territories (SAT) 1, SAT2, and SAT3. Type O is the most prevalent serotype worldwide, followed by Asia-1. Within these serotypes, 65 different strains have been officially identified as explained by Rweyemamu *et al*. (2008). Generally, FMDV is highly susceptible to change, resulting from errors in RNA replication, recombination and host selection. There is no cross protection between the distinct serotypes. Protection within serotypes varies based on the antigenic similarity of the strains. Subsequently, any vaccine must be carefully matched with the field strain to be effective (OIE, 2009).

2.2.3 Susceptible Species

FMDV infects cloven hooves mammals (order *Artiodactyla)*, as well as a few species in other orders. Livestock susceptible to FMD virus include cattle, pigs, sheep, and goats. In addition, deer, bison and elk are also susceptible to the virus. Llamas and alpacas have been infected experimentally, but do not seem to be highly susceptible to natural infection. Wild pigs, antelope, African buffalo, Bactrian camels, and giraffe are also all susceptible species. Other animals like rats, mice, guinea pigs, and armadillos have all been experimentally infected. Strains may have a predilection for one animal species over another (CFSPH, 2007).

2.2.4 Carrier State

A carrier is defined as an animal in which FMDV or viral RNA can be detected for more than 28 days post infection. However, with FMD, carrier animals may or may not be able to transmit infection. African buffalo are the only carrier animals that have been demonstrated to be able to definitive transmit SAT FMDV to other buffalo and potentially cattle (Alexandersen *et al*., 2002). Serotypes A, O, Asia-1, and C do not seem to be transmitted from carriers to naive animals (Kitching, 2002). In addition to African buffalo, cattle, sheep, goats, and water buffalo can also become carriers; however, there is no sufficient evidence to suggest a carrier state exists in pigs. In comparison to cattle and buffalo, sheep and goats are carriers for a shorter period typically between 1-5 months. On the other hand, most cattle carry FMDV for six months or less, there is evidence of cattle carrying FMDV for more than 3 years (Alexandersen *et al*., 2002).

2.2.5 Transmission and Shedding of FMD Virus

FMD is highly contagious since typically introduced via contact with infected animals, their secretions, excretions, fomites or products contaminated with FMDV. FMD can also be introduced into a naive animal population by feeding contaminated meat, milk or garbage. Conveyances may be responsible for transmitting the disease between infected and uninfected premises (USDA APHIS, 2011). Cattle typically become infected through aerosolized virus. Pigs usually become infected by eating virus-contaminated food or through direct contact with the vesicular lesions of other animals. However, animal-to-animal contact is a common mode of transmission. FMDV is shed in all secretions and excretions, including saliva, milk, semen, feces, urine, and ruptured vesicular fluid. Pigs produce nearly 30 to 100 times as much FMDV in respiratory secretions as either cattle or sheep (USDA APHIS, 2011).

2.2.6 Incubation Period

The incubation period for FMD is typically 2–14 days (OIE, 2013). If there is a significant amount of FMDV in a naive population, the incubation period may be as short as 24 hours. How fast clinical signs appear depends on the dose of the virus, species of the animal, as well as the route of infection. Animals may shed FMDV before the appearance of clinical signs (OIE, 2016).

2.3 Epidemiology

2.3.1 Distribution

FMDV has an essentially global distribution, with the exception of North America, Western Europe and Australia. The FMD status of any particular country or region can be defined as endemic, epidemic (sporadic) or free. National borders (e.g. Australia, Indonesia) can define FMD-free regions, by supranational borders (Europe, North America) or by disease-free zones within non-free areas, which are maintained by movement control (e.g. Zimbabwe). Sporadic regions are characterized by repeated incursions of FMD viruses into regions where disease does not usually occur. The disease either is eliminated through control program or disappears naturally without intervention until the next introduction months or years later (Samuel and Knowles, 2001). It is endemic in Africa, Asia and South America, Middle and Far East and parts of Europe (Table 1)

Table 1. Serotypes commonly isolated from certain geographical regions continent or subcontinent Virus serotypes.

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

Source: Asseged, (2005)

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

2.3.2 Molecular Epidemiology

Phylogenetic analysis of the virus protein 1 (VP1) region of FMD virus has been used extensively to investigate the molecular epidemiology of the disease worldwide. These techniques have assisted in studies of the genetic relationships between different FMD virus isolates, geographical distribution of lineages and genotypes. It is also used for the establishment of genetically and geographically linked topotypes and tracing the source of virus during outbreaks (Knowles and Samuel, 2003). Sequence differences of 30 to 55% of the VP1 gene were obtained between seven serotypes of FMD while different subgroups (genotypes, topotypes) were defined by differences of 15 to 20% (Knowles and Samuel, 2003). Since 1987, the analysis of the genetic distance and phylogenetic resolution of the sequence of VP1 encoding gene have provided crucial epidemiological information covering different degree of genetic

relationships between field isolates (Sahle *et al*., 2004). The evolutionary changes of virus are determined by comparing genomic material from more than one virus with each other. DNA sequencing and phylogenetic trees are widely used to illustrate the genetic relationship between strains of FMD viruses (Sahle *et al.,* 2004).

2.4 Pathogenesis

The main route of infection in ruminants is through the inhalation of droplets, but ingestion of infected feed, inoculation with contaminated vaccines, insemination with contaminated semen and contact with contaminating clothing, veterinary instruments, and so on can all produce infection. In animals infected via the respiratory tract, initial viral replication occurs in the pre-pharyngeal area and the lungs followed by viremic spread to other tissues and organs before the onset of clinical disease. FMD virus is then distributed throughout the body, to reach best sites of multiplication sites such as the epithelium (*aphthae*) of Oro-pharynx, oral cavity, Feet, udder and heart. Virus probably replicate in the mammary gland and pituitary gland of susceptible cow. Viral excretion commences about 24 hours prior to the onset of clinical disease and continues for several days FMD virus. The acute phase of the disease lasts about one week and viremia usually declines gradually coinciding with the appearance of strong humeral responses (Murphy *et al.,* 1999). Recovered cattle produce neutralizing antibodies and can resist re-infection by the same subtype of virus for up to one year. It was suggested that heat intolerance was a sequel to FMD and was caused by damage to the endocrine system by Radostits *et al. (*1994).

The morbidity and mortality in FMD depends upon the breed and age of the animal where mortality in adult animals is very low (2%) in comparison to 20% in young stock (Radostits *et al.* 1994). The calves show prominent signs of myocarditis, whereas piglets manifest gastroenteritis (Knowles and Samuel, 2003). In sheep and goats, symptoms are frequently less severe and may make the detection of the disease difficult. Asymptomatic, persistent infection can also be established in ruminants, during which infectious virus can be isolated from the oesophagus and throat fluids of the animals from a few weeks up to several years of the initial infection. There is epidemiological evidence to support the hypothesis that carrier animals may be the origin of outbreaks of acute disease when brought into contact with susceptible animals. This mode of transmission has been experimentally reproduced for serotype SAT isolates (Knowles and Samuel, 2003).

2.5 Clinical Sings

The incubation period of FMD is 2-14 days (OIE, 2004). In cattle, the initial signs are fever of 103 -105ºF (39.4 - 40.6ºC), dullness, anorexia, and fall in milk production. There is abundant salivation, the saliva hanging in long, ropy strings, a characteristic smacking of the lips and drop in milk yield (Quinn *et al.,* 2005). The predilection sites for vesicles are areas where there is friction such as on the tongue, dental pad, gums, soft palate, nostrils, muzzle, inter digital space, coronary band and teats (Sahle *et al*., 2004).

Cattle with FMD, especially the highly productive breeds, they usually become febrile and develop lesions on the tongue, dental pad, gums, soft palate, nostrils and/or muzzle. The vesicles on the tongue often coalesce, rupture quickly, highly painful and the animal becomes reluctant to eat. Profuse salivation and nasal discharge are common in this species; the nasal discharge is mucoid at first, but becomes muco-purulent. Affected animals become lethargic, may lose condition rapidly and may have gradual or sudden, severe decreases in milk production. In some cases, milk may not be produced again until the next lactation or milk yield may be lower indefinitely. Hoof lesions, with accompanying signs of pain, occur in the area of the coronary band and interdigital space. Young calves may die of heart failure without developing vesicles. In areas where cattle are intensively vaccinated, the entry of FMD into the herd can sometimes cause swelling of tongue and severe clinical signs that resemble an allergic disease (OIE, 2014).

In addition to other complications such as mastitis or hoof malformations, some cattle that recover from FMD are reported to develop heat-intolerance syndrome (HIS, ‘hairy panters’). This poorly understood syndrome is characterized by abnormal hair growth (with failure of normal seasonal shedding), pronounced panting with elevated body temperature and pulse rate during hot weather and failure to thrive. Some affected animals are reported to have low body weight and reproductive disturbances (OIE, 2014; 2016).

2.6 Immune Response

The protection of a susceptible host against FMD virus correlates with the neutralizing antibodies level. Infection with one-serotype produces complete protection against homologous virus, but little or no protection against heterologous viruses. Serotype specific immunity is based on the

presence of neutralizing antibodies to one of the viral capsid proten, VP1, develops 7 to 21 days after exposure to the virus (Knowles and Samuel, 2003). The immunoglobulin M (IgM) is most prevalent in the early convalescent serum and is less specific to the different serotypes than immunoglobulin G (IgG). IgG is produced in the later stage during the FMD infection and the reaction between the serotype and the homologous antibodies is highly specific. It has been reported that healing of lesions and clinical recovery in infected animals would not occur until a few days after the IgG1 antibodies have developed. The localized antibody response, specific to anti-FMD IgM and IgA antibodies in the pharyngeal fluid of cattle develops 7 days after exposure to the virus, while IgG activity reaches pick in serum only 14-21 days after infection (Quinn *et al*., 2005).

The age of individuals has also been shown to influence the antibody response against FMD virus. Calves (age one week to six months) but deprived of maternal antibodies responded as well as, or better than 18 months old cattle to initial vaccination against FMD. Although serum antibody levels play an important role in host protection against FMD virus infection, the cellular responses mediated by T-helper and T-cytotoxic cells also play a role in the immune response to FMD virus infection (Radostits *et al*., 1994).

2.7 Diagnostic Techniques

2.7.1 Identification / Isolation of Virus

A range of sample types including epithelium, OP samples and serum, will be examined by virus isolation or RT-PCR, in contrast; ELISA, CF and the lateral flow device are suited to the examination of epithelial suspensions, vesicular fluids or cell culture supernatants, but are insufficiently sensitive for the direct examination of OP samples or serum.

The epithelium sample should be taken from the PBS/glycerol, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000rpm for 10 minutes. Then, such suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures. Sensitive cell culture

systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells. Established cell lines, such as BHK-21 (baby hamster kidney) and IBRS-2 cells, may also be used but are generally less sensitive than primary cells for detecting low amounts of infectivity. The use of IBRS-2 cells aids the differentiation of swine vesicular disease virus (SVDV) from FMDV (as SVDV will only grow in cells of porcine origin) and is often essential for the isolation of porcinophilic strains, such as O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours (OIE, 2009). In the case of OP fluids, pretreatment with an equal volume of chlorofluorocarbons may improve the rate of virus detection by releasing virus from immune complexes.

2.7.2 Immunological Methods: - Enzyme-Linked Immunosorbent Assay (ELISA)

The preferred procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Goris and DeClercq, 2005). Different rows in multi well plates are coated with rabbit antisera to each of the seven serotypes of FMDV in this indirect sandwich test. These are the ‘capture’ sera. Test sample suspensions are added to each of the rows and appropriate controls are included. Guinea-pig antisera to each of the serotypes of FMDV are added, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents.

A color reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMDV can also be identified. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once; a CPE has developed (OIE, 2009).

2.7.3 Nucleic Acid Recognition Methods

RT-PCR can be used to amplify genome fragments of FMDV in diagnostic materials including epithelium, milk, serum and OP samples. RT combined with real-time PCR has sensitivity

comparable to that of virus isolation and automated procedures enhance sample throughput. Serotyping primers have also been developed (OIE, 2009).

The use of polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origin and it has become an essential tool in the research laboratory. The potential of this format to provide sensitive, specific, and swift detection and quantification of viral RNAs has made it an indispensible tool for state of the art diagnostics of important human and animal viral pathogens. Real-time PCR has engendered wider acceptance of PCR due to its improved rapidity and sensitivity overcoming poor precision, low sensitivity, low resolution, absence of automation, only size-based discrimination, absence of expression of results in numbers, poor quantitative performance (Ethidium bromide for staining is not very quantitative), and post-PCR processing, rendering the conventional PCR not very suitable for accurate diagnosis (Callahan, 2002).

There are five main chemistries used for the detection of PCR product during real-time PCR. These are the DNA-binding fluorophores, the 5 endonuclease, adjacent linear and hairpin oligo-probes, and the self-fluorescing amplicons (Reid *et al*., 2014). This approach is a highly sensitive technique enabling simultaneous amplification and quantification of specific nucleic acid sequences. In addition to enhanced sensitivity, the benefits of real-time PCR assays over conventional endpoint detection methods include their large dynamic range, a reduced risk of cross-contamination, an ability to be scaled up for high through put applications and the potential for accurate target quantification.

According to OIE Reference Laboratory at Pirbright procedure, the RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) Detection of the PCR products by agarose gel electrophoresis. The real time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmed for the RT and PCR steps (Reid *et al*., 2003). PCR amplification of the RT product is performed by a different procedure. A one-step method for combining the RT and PCR steps has also been

described by Shaw *et al. (*2007). Detection of the PCR products in agarose gels is not required following real-time amplification.

Real-time PCR assays recommended by the World Organization for animal health (OIE) for detection of FMDV incorporate universal primers and fluorescent labeled probes that recognized conserved region within the 5 UTR (Reid *et al*., 2014) or conserved gene regions within the RNA-dependent RNA polymerase gene (3Dpol). TaqMan technology has combined the 5-nuclease activity of the Taq DNA polymerase and foster resonance energy transfer to detect and quantify amplification product. Using this technology real-time PCR has been developed to detect the nucleic acid (Oleksiewicz *et al*., 2001). This is most sensitive and rapid method to detect the nucleic acid. The viral RNA can be consistently detected over a seven-log range, the lowest of which corresponded to as few as 10-100 RNA per volume tested. The test can be performed in 2 hours or less on a portable instrument and sample can be held at ambient temperatures. Real-time chemistry allows for the detection of PCR amplification during the early phases of the reaction and real-time PCR monitors the progress of a PCR reaction in the real-time. At the same time, a relatively small amount of PCR products (DNA, CDNA, or RNA) can be quantified (Shaw *et al.,* 2007).

2.7.4 Serological Tests

Serological tests for FMD are performed in support of four main purposes namely; 1) to certify individual animals prior to import or export (i.e. for trade), 2) to confirm suspected cases of FMD, 3) to substantiate absence of infection and 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programmed of vaccination (OIE, 2009). Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and nonstructural proteins (NSPs).

The SP tests are serotype specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralization test (VNT), the solid phase competition ELISA and the liquid phase blocking ELISA (Paiba *et al*., 2004). Those tests are serotype specific and highly sensitive, providing that the virus or antigen used in the test is closely matched to the strain

circulating in the field. The VNT requires cell culture facilities, the use of live virus and takes 2-3 days to provide results. The ELISAs are blocking or competition based assays that use serotype specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. Low titer false-positive reactions can be expected in a small proportion of the sera ELISA. An approach combining screening by ELISA and confirming the positives by the VNT minimizes the occurrence of false-positive results.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore, the tests can be used to confirm suspected cases of FMD and to detect viral activity or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may not be detected in some anti NSP tests, causing false-negative results (Brocchi *et al*., 2006). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of *in-vitro* expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered the most reliable indicators of infection. In animals seropositive for antibody to 3AB or 3ABC antibody to one or more of the other NSPs can aid in the final interpretation of the test (Bergmann *et al*., 2000). However, lack of vaccine purity may affect diagnostic specificity as the presence of NSPs in some vaccine preparations may result in misclassification in animals that have been repeatedly vaccinated. International standard sera for testing of cattle have been developed and are available from the OIE Reference Laboratories in Brazil and UK (Campos *et al*., 2008).

2.7.4.1 Virus Neutralization Test

The quantitative VN micro test for FMD antibody is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue culture grade microtiter plates. Stock virus is grown in cell monolayer and stored at -20°C after the addition of 50% glycerol. Virus has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. The control standard serum is 21-day convalescent or post vaccination serum. A suitable medium is Eagle's complete medium or LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with hepes buffer and antibiotics (OIE, 2009).

2.7.4.2 Solid-Phase Competition Enzyme Linked Immuno-Sorbent Assay

The method described by Paiba *et al*. (2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea pig or rabbit antisera, suitable MAbs can be used coated to the ELISA plates as capture antibody or peroxidase conjugated as detecting antibody. A commercial kit is available for serotype O with a different format but similar performance characteristics by Chenard *et al*. (2003).

Rabbit antiserum to the 146S antigen of one of the seven types of FMDV is used as the trapping antibody at a predetermined optimal concentration in carbonate or bicarbonate buffer pH 9.6. Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which after addition of an equal volume of diluents, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% NBS and 5% normal rabbit serum and phenol red indicator is used as a diluents (blocking buffer). Guinea-pig antisera, prepared by inoculating guinea pigs with 146S antigen of one of the seven serotypes and pre-blocking with NBS is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer PBS containing 0.05% Tween 20 and 5% dried, nonfat skimmed milk (PBSTM). Rabbit (sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and pre-blocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer. Test sera are diluted in PBST blocking buffer. The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA by Goris and De Clercq (2005). Methods have been described for the development of secondary and working standard sera and for charting assay performance (OIE, 2009).

2.7.4.3 Liquid-Phase Blocking Enzyme Linked Immuno-Sorbent Assay

Antigens are prepared from selected strains of FMDV grown on monolayer of BHK-21 cells. The unpurified supernatants are used and pretitrated in a twofold dilution series but without serum. The final dilution chosen is that which after addition of an equal volume of diluents, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as diluents

(PBST). The other reagents used in the test are the same as those in the solid-phase blocking ELISA (OIE, 2009).

2.7.4.4 Nonstructural Protein (NSP) Antibody Tests

Antibody to expressed recombinant FMDV NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations (Brocchi *et al*., 2006).

Briefly, the test was carried out stepwise as per the manufacturer's manual. First the reagents were kept at room temperature and homogenized by vortexing. The test was carried out in 96 well micro plates. Then 50µl of dilution buffer 18 were added in to each well. Thirty µl of positive control were added in to wells A1 and B1 and the same volume of negative control were also added to wells C1 and D1 while, the rest wells were filled by 30µl of test sera. Then incubated at 37^{o}C for 2 hours, after incubation the wells were washed 5 times with adding 300 µl of wash solution. After washing 100µl of the conjugate 1 times were added in to each wells and incubated for 30 min at 21^{o}C. After incubation the wells were washed 5 times with 300 µl of wash solution, then 100 µl of the substrate solution (TMB) was added in to each wells and incubated at 21^{o}C for 15 minutes in dark. To stop color reaction 100 µl of stop solution was dispensed into each well. Finally, the optical density (OD) readings were recorded using a spectrophotometer at wavelength of 450 nm. The test result was validated if: The mean value of negative control O.D. (OD_{NC}) was > 0.7. OD_{NC} > 0.7. The mean value of the positive control O.D. (OD_{PC}) is < 30 % of the OD_{NC} OD_{PC}/OD_{NC} <0.3. Interpretation for each serum sample, the competition percentage was calculated (S/N %): S/N % = OD sample/ODNC X 100Sample presented S/N %: =<50% were considered positive, >=50% were considered negative (OIE, 2014).

2.8 Economic Importance

FMD threatens the livelihoods of small-scale farmers, large sophisticated farming practices, the national and the international economies of the countries (OIE, 2004). The direct effects of the disease are loss of milk production, loss of draught power, retardation of growth, abortion in pregnant animals, death in calves and lambs, while indirect losses can be attributed to the

disruption in trade of animals and derivative products of animals (Bayissa *et al*., 2011). Since, FMD causes substantial economic loss to farmers and the nation from embargoes of livestock and livestock product trade (Megersa *et al.,* 2009) and become the major constrain hampering the export of livestock and livestock products to the Middle East and Africa country FAO (2005).

2.9 Prevention and Control

Routine vaccination is used where the disease is endemic; in contrast, a number of disease free countries have never vaccinated their livestock but have preferred the use of strict movement controls and slaughter of infected and in contact animals when outbreaks occur (OIE, 2004). Immunity to one serotype provides protection only against the homologous viruses. In some cases, inactivated bi-, tri-, or polyvalent vaccine, which contains the representative strains of the serotypes that are in circulation in the region, must be used. Therefore, active disease surveillance must be effective which needs a strong field service as well as proper laboratory facilities with efficient methods of detection and characterization of the virus (OIE, 2004).

Import regulations help prevent FMDV from being introduced from endemic regions in infected animals or contaminated foodstuffs fed to animals. Waste food (swill) fed to swine is a particular concern. Heat-treatment can kill FMDV in swill and reduces the risk of an outbreak; however, some countries have completely banned swill feeding, due to difficulty in ensuring that adequate heat treatment protocols are followed. Protocols for the inactivation of FMDV in various animal products such as milk products, meat, hides and wool have been published by the OIE (2014).

Measures taken to control an FMD outbreak include quarantines and movement restrictions, euthanasia of affected and exposed animals and cleaning and disinfection of affected premises, equipment and vehicles. Additional actions may include euthanasia of animals at risk of being infected and/or vaccination. Infected carcasses must be disposed of safely by incineration, rendering, burial or other techniques. Rodents and other vectors may be killed to prevent them from mechanically disseminating the virus. People who have been exposed to FMDV may be asked to avoid contact with susceptible animals for a period, in addition to decontaminating clothing and other fomites. Good bio-security measures should be practiced on uninfected farms to prevent entry of the virus (OIE, 2012).

Vaccination may be used to reduce the spread of FMDV or protect specific animals during some outbreaks. The decision to use vaccination is complex and varies with the scientific, economic, political and societal factors specific to the outbreak. Vaccines are also used in endemic regions to protect animals from illness. FMDV vaccines only protect animals from the serotypes contained in the vaccine. For adequate protection, the vaccine strains must also be well matched with the field strain. Wildlife transmission may need to be considered in some locations. However, wildlife fencing may not be practical in some areas, and there are some disadvantages to its use. Another issue is the protection of highly susceptible wildlife species from FMDV (OIE, 2014).

In Ethiopia context the control of FMD is practiced by involvement of quarantine, restriction of animal movement, isolation of infected animals, vaccination programs, proper disposal of infected carcass and other methods, which are feasible to Ethiopian economy. There is no countrywide vaccination program aimed to control FMD and a ring vaccination is carried out around an infected area. Considering the wide prevalence of serotypes O, A and SAT 2 the National Veterinary Institute (NVI) is producing an inactivated trivalent vaccine (Tesfaye, 2014).

2.10 Status of Foot and Mouth Disease in Ethiopia

FMD in cattle in Ethiopia was first recorded by FAO and WRL, which indicated that FMD serotypes O, A and C were responsible for FMD outbreaks during the period of 1957 to 1979 (Martel, 1974). The antibodies of SAT2 also were detected in 1971, in sera collected from cattle in the region now known as North Omo, southwestern Ethiopia (Roeder, *et al*., 1994). Extensive movement of livestock, the high rate of contact among animals at commercial markets, in communal grazing areas and at watering points, were among the reasons forwarded for the increasing incidence of the disease (Mersie *et al.,* 1992).

During the period of 1988 - 1991, serotyping of FMDV was conducted at National Veterinary Institute (NVI) Ethiopia and WRL for FMD United kingdom (UK) and serotypes O and SAT2 were identified. This is the first record of the presence of SAT2 FMDV in Ethiopia from sample collected from Borana, Southern Ethiopia (Roeder *et al.,* 1994). From record of outbreak investigation in cattle by NVI, between 1982 and 2000, three serotypes: O, A and SAT2 FMDV were identified (Gelaye *et al*., 2001). The serological survey on FMD indicates that Serotypes A,

SAT1 and SAT2 were isolated from buffalo at Omo National Park; O, A, SAT1 and SAT2 were isolated from cattle and O and A were isolated from Small ruminants in Ethiopia (Sahle *et al*., 2004). SAT1 antibody was detected for the first time in both buffalo and cattle (Leforban, 2005). So FMD is a notifiable disease in Ethiopia and the Federal Veterinary service of Ministry of Agriculture send monthly and annually office reports to OIE (Table 2).

Table 2. History of the FMD virus types isolated in Ethiopia.

Period	**Number of samples**	**Types**
1957-1973	98	O,A, C
1973 – 1988	Unknown	O, A
1988 – 1994	16	O, SAT2
1994 – 2000	67	O, A, SAT2
2000 – 2005	7	O, A, SAT2
2005 – 2009	Unknown	O, A, C, SAT1, SAT2

Source Salhe *et al*., 2004; Leforban, 2005; Gelagay *et al*., 2009

Geographically, the outbreaks were widely distributed within central Ethiopia, including the Addis Ababa administrative region and Oromia; Ahmara and Tigray in the north; Dire Dawa in the northeast; Beneshangul-Gumuz bordering Sudan in the west and Southern Nations Nationalities and Peoples Region bordering Kenya and Sudan in the south. In eastern Ethiopia, poor veterinary services and inaccessibility to the area could have resulted in the lack of samples submitted (Gelagay *et al*., 2009).

In terms of species, cattle were found to be infected with all circulating serotypes of FMDV, whereas swine had only type O. SAT 2 was recorded in 2007, after an apparent gap of 16 years, from a bovine sample collected from Bambas, Beneshangul-Gumuz and western Ethiopia bordering Sudan. The first recorded occurrence of FMDV type SAT 1 in Ethiopia was identified from a bovine sample collected in November 2007 from the Mizan Teferi area bordering Kenya (Megersa *et al*., 2009). Seroprevalence of FMD at herd and individual levels assessed in different regions of Ethiopia was a herd prevalence of 57.6% and individual prevalence of 11.9% **(**Gulima, 2011). Samples tested for FMD certification on 3, 2284 animals for export showed an overall positivity of 14.82%. Serotyping of 120 seropositive samples took from Borana pastoral herds indicated that serotypes O (99.2%), A (95.8%), SAT 2 (80%) and C (67.5%) (Rufael *et al.,* 2008). All the virus serotypes and topotypes were similar, i.e. O serotypes, EA-3 except the Makelle outbreak topotype, which was identical with Sudan topotype (EA-3 Sudan).

According to report of Bewket *et al.,* 2012 on 58 FMD outbreaks of 2011 in Addis Ababa, Amhara, Gambella, Oromia, SNNP and Tigray Regional States were 17577 cases comprising 13249 cattle, 4063 goats, and 265 sheep with mortality of 746 heads of animal (373 cattle, 309 goats and 64 sheep). This same report also revealed the involvement of swine in Adaa district, Eastern Shewa Zone of Oromia, in which 11 of 19 samples examined turned to be positive for FMD virus. During 2015, 83 samples have been collected from outbreaks in Ethiopia and characterized at NAHDIC by Ag-ELISA (supported by additional testing and sequencing at OIE/FAO Reference Laboratories). FMDV serotypes O (O/EA-3) and SAT 2 (SAT 2/VII/Alx-12) have been detected (Donald and Mark, 2015). Jemberu *et al*. (2014) identified as serotypes O, A, SAT2 and SAT 1 were the causal serotypes of the outbreaks during the year 2007–2012. In the past seven years (2009–2015) on average 93 numbers of FMD, outbreaks were reported to Ministry of Livestock and Fisher annually. The outbreaks occurred every year, but most were reported in 2011 and 2012 each 124 and 205 outbreaks, respectively (MoLF, 2016).

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

3. MATERIAL AND METHODS

3.1. General Description of the Study Area

The study was carried out at six purposively selected study areas, in Oromia Special Finfine Surrounding zone (Mulo woreda), North Shewa zone (Aleltu, Kimbibit and Wochale woredas), East Shewa zone (Adea woreda) and Addis Ababa (Kolfe Koraneyo subcity) all are located in central Ethiopia. The study was conducted between September 2016 and April 2017. The location of the study areas were latitude: 9°0'0" North, longitude: 44°0'0"- 46°0'0" East and average of elevation 2324 m above mean sea level, which indicate that this located in tropical climate zone though the climate conditions is marked to rainy (March- September) and dry (October- February) season and influenced by altitude variation topography High land, Mid-Highland and Lowland. Mean annual temperature also varies 10-15c^{o} minimum and 20-25 c^{o} maximum, also annual rainfall 1089-1699mm (Figure 2).

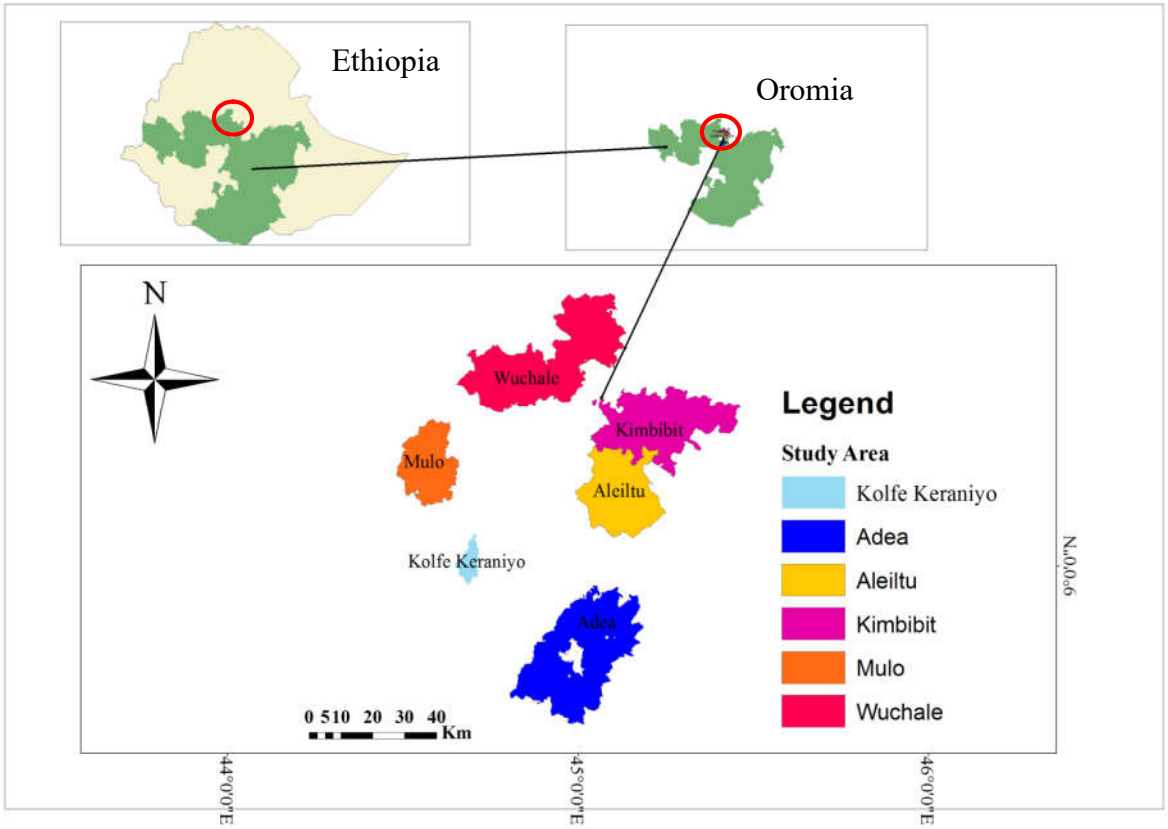

Figure 2: Map of showing the study area

The study areas were rich in livestock resource having huge number of animal population of various species, breeds (local and cross), among livestock cattle was more popular because of their product and by products. The clay-loam soil type more favors the crop farming mainly *teff, wheat, barley, maize*, etc. Natural vegetation, grass and cereal residue are common feeding sources for livestock. Anthrax, blackleg, foot and mouth disease, hemorrhagic septicemia, lumpy skin disease etc, are the endemic diseases of cattle in the study area (CSA, 2015).

3.2 Study Population, Sample Size Determination and Sampling Methods

The study population constituted of indigenous zebu and cross breed cattle managed under smallholder mixed crop-livestock farming system. Most of animals are kept under traditional extensive husbandry system with communal grazing and watering points. Six suspected FMD outbreaks areas were investigated. From each suspected FMD outbreak, cattle with typical clinical signs including vesicular lesions in the mouth, lips, tongue, feet, teat, salivation and lameness (manifestation of clinical signs of the disease) were selected for the sample collection.

The sample size was determined based on the purpose of the study. Since the main objective of the study was to determine the serotypes of FMD virus circulating in the study areas and to characterize molecularly, large sample size was not required. Only some representative sample size for each sample type, which could be manageable in terms of cost for processing and investigation, were considered.

Accordingly, 6 epithelial tissues (tongue), 35 oral swabs and 64 oro-pharyngeal fluids (probang) samples were collected from 105 suspected cattle. After sampling, the specimens were labeled, put in icebox, transported to the National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratory at Sebeta and stored at -80°C until processing. Besides, sampled cattle characteristics including address and owner's name, age, sex, breed, body condition score, production and management system were carefully recorded. Age determination was taken by observing dental eruption of sampled cattle and asking the owner of cattle (Appendix Table 1, 5 and 6).

3.3 Study Design

The design of the study was laboratory based cross-sectional investigation of FMD, which involves sampling, serotyping and molecular characterization. The laboratory works were carried out at National Animal Health Diagnostic and Investigation Centre (NAHDIC), Sebeta, Ethiopia.

3.4 Sample and Data Collections

About 1g of tongue epithelial tissue was collected from non-ruptured or freshly ruptured vesicles by using sterile forceps and scissor then placed in 10% volume of tissues in universal bottle with viral transport medium (VTM). VTM contains 0.5% bovine serum albumin (BSA), benzyl penicillin (2 x 10^6 IU), streptomycin (200 mg), polymyxin B (2 x 10^6 IU), gentamicin (250 mg), nystatin (0.5 x 10^6 IU), ofloxacin hydrochloride (60 mg) and sulfamethoxazole (0.2 g) per 1 liter in distilled water, equal amount of phosphate-buffered saline (PBS) with pH 7.2-7.6 which contains (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g) and distilled water to make 1 liter solution. Oral swab was collected from fresh lesions in mouth by rayon swab with plastic stick sterile disposable into 0.5 ml of VTM sterile plastic screw-cap vials (Cryovials). Probang samples also collected by probang cup from oro-pharyngeal (op) or upper trachea into 2-3 ml of VTM in universal bottle from persistently (late exposure) infected. All samples collected were labeled with identification number, date of collection, disease suspected and other appropriate information listed under sampling method. Then samples were kept at +4 C^o in ice packs box and transported to the NAHDIC, Sebeta, Ethiopia; again coded by laboratory code at reception room then transfer to molecular biology diagnostic, cell culture and serology lab. The samples were stored at -80 C^o until further processing.

3.5 Laboratory Tests

3.5.1 Sample Preparation

A suspension epithelial tissue sample was prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue and adding VTM until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. All epithelial tissue suspension, oral swab and probang (oro-pharyngeal fluid) samples were clarified on a

bench centrifuge at 2000 rpm for 10 minutes then the supernatant were collected into new cryovial and labeled (OIE, 2014).

3.5.2 RNA Extraction and Amplification rRT-PCR

RNA of FMDV was extracted following QIAamp viral Mini Spin Columns (Qaigen, Germany) in accordance with manufacturer's recommendation. Each 140 µl epithelium tissue sample suspensions, oral swabs and probangs samples were separately added with 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml micro centrifuge tube, mixed by pulse-vortexing for 15s then incubated at 15-25 C^o for lysing purpose. Binding by using 560 µl of ethanol (96-100%), mixed by pulse-vortexing for 15sec then briefly centrifuged the tube to remove drops from inside the lid. A 630 µl of the solution transferred into QIAamp Mini spin (silica) column, then centrifuged at 8000 rpm for 1min through placing the QIAamp Mini column into a clean 2 ml collection tube and discarded the tube containing the filtrated, the left solution again transferred similarly. Washed by 500 µl of Buffer AW1, again by 500 µl of Buffer AW2, then dry centrifuged and discarded the tube containing the filtrated. Finally, the RNA was eluted after adding 60 µl of Buffer AVE and incubated at room temperature for 3-5 min then centrifuged at 8000 rpm for 1 min (QIAGEN, 2014).

Extracted RNA was kept at +4°C for immediate use or stored at -80^{o}C until used for real-time reverse transcription polymerase chain reaction (rRT-PCR). Reverse transcription of FMDV RNA and PCR amplification of reversed transcribed RNA was conducted using automated rRT-PCR as prescribed by Moniwa *et at*. (2007); OIE (2012). This method amplified a conserved segment of the FMDV RNA polymerase gene (3D). The following primers were used; the forward primer (Callahan 3DF) was 5′-ACTGGGTTTTACAAACCTGTGA-3′ and the reverse primer (Callahan 3DR) was 5′-GCGATGCCTGCCACGGA-3′ alongside with the probe 5′-FAM-TCCTTTGCACGCCGTGGGAC-TAMRA-3′ i.e. labeled reporter dye with 6-carboxyfluorescein at the 5' end and the quencher tetra-methyl-rhodamine at the 3' end (Reid *et al*., 2003).

The reactions component of master mix for one step RT-PCR were performed using, 2x reaction mix (12.5µl), nuclease-free water (1.5µl), forward primer (2µl), reverse primer (2µl), Taqman probe (1.5µl) and superscript (0.5µl) to make total 20µl per sample for each reaction of wells of

PCR plate including positive and negative control master mix then thoroughly mixed by pulse-vortexing. RNA sample template (extracted RNA) (5µl) added to each reaction and the total volume of PCR plate was 25µl (Appendix Table 2). The PCR plate was sealed with adhesive film then inserted into the thermal cycler machine slots (adjusted) according to QIAmgen one-step RT-PCR kit protocols. The one step rPCR amplification started with reverse transcription (RT) cDNA synthesis at 50°C for 30 min; followed by activation or denaturation of reverse transcriptase at 95°C for 10 min; followed by annealing and extension of 95°C for 15sec. and 60°C for 1min respectively described by Oleksiewicz *et al*. (2001). Totally 50 cycles and 2:39 hrs were taken to finish amplifications or final extensions.

The Automated Applied Biosystems Thermal Cycler Machine was connected to a computer loaded with 7500 Fast system SDS software displaced the cycling profile, then FMDV was detected through threshold cycle (C_T) values based on baseline and graphs. Threshold cycle C_T values <30.0 were considered as positive. Amplification with C_T value between 30.0 and 35.0 and/low curve to the threshold sample was considered suspicious or doubtful (retest) while, C_T value >35.0 was considered negative. Acceptable C_T range for positive was 12-30.0 (Callahan *et al*., 2002; Reid *et al*., 2014).

3.5.3 Virus Isolation and Cell Culturing

Among 34 positive samples that were detected by real-time PCR, eight (Kolfe Koraneyo: 1 oral swab and 1 oro-pharyngeal fluid, Mulo: 1 oral swab, Adea: 1 oral swab, Wuchale: 1 epithelium tissue and 1 oro-pharyngeal fluid, Kimbibit: 1 oro-pharyngeal fluid and Aleltu: 1 oro-pharengeal fluid) were selected based on their representative of study area. The supernatant from the prepared samples were collected and filtered by millipore filter of 0.4 µm again by 0.22 µm pore size. About 1ml of filtered sample suspension was inoculated on baby hamster kidney (BHK-21) monolayer cells grown by using complete media and washed by 2 ml PBS on 25 cm^2 sample culture flask and incubated at 37°C for 1hr for adsorption of the virus to cells and then 2 ml of maintenance media or growth media (2% minimum effective media) was added for each flask and incubated at 37°C and 5% CO_2 in a humidified incubator for 24-72 hrs. The cells were examined twice daily under inverted microscope until it shows characteristic cytopathic effect (CPE) by FMDV. FMD virus produced cytopathic effects (CPE) in the BHK-21 cell at different intervals post infection (24-472 hrs) which was characterized by the rounding and flattening of

the cells, breaking down of intracellular bridges and finally cell death, which are characteristic findings of FMDV infected cells. Initially the growth of virus in BHK-21 cell line lower, which increased after, subsequent three time's passage of infection into BHK-21 cell line (Quddes *et al.,* 2016). The Monolayer that was shown CPE harvested when 85-100% of CPE were observed after consecutive three passages then declared as positive. If CPE was not detected within 48 -72 hrs of inoculation, the cells had been freezed and thawed, inoculated into fresh cell cultures and to re-examine for CPE for another 48-72 hrs up to three passages. Then sample was declared as negative if no CPE was observed after third blind passages (OIE, 2012; 2013).

3.5.4 Serotyping of FMD Virus

Six Samples (3 oral swabs, 1 epithelium tissue and 2 probangs) were taken from cell cultures that had CPE positive were tested by indirect sandwich ELISA, according to the protocol recommended by the Institution of Animal Health Pirbright, UK, FMDV antigen detection ELISA serotyping of O, A, SAT1 and SAT2. Micro plates were supplied pre-coated with catching MABs and with both positive and negative controls already incorporated onto plates from manufacturer. Test samples were dispensed 50 µl/well into micro plate in row (1-12), 50 µl/well of diluents buffers also added to positive and negative control wells and incubated for 1 hrs at 18-25C^{o} with the coated MABs. The corresponding type-specific MABs and Pan-FMDV MAB detected the presence of FMD virus antigen. After washing three times within 3min interval by adding 200 µl/well washing solution (PBS-Tween) and incubated at room temperature to remove unbounded material, then two detector conjugates (50 µl/well) horse radish peroxide-conjugated (HRPO) were added in corresponding wells: a pan-FMDV MAB (O,A,C, Asia1) into 1-8 column wells and pan-SAT MABs pool (SAT1, SAT2) into 9-12 column wells, then covered and incubated for 1hrs at room temperature and washed again. A 50 µl/well of substrate (chromogen) solution added to all wells then covered and left at room temperature for 20 min in the dark. Finally, the reaction was stopped (blocked) by adding 50 µl/well of H_2SO_4 0.6N then mixed well for immediate reading of optical density (OD) of each well at 450 nm wavelength using a micro plate reader, BIOTEK Photometer (OIE, 2014, 2016; Appendix Table 3).

The positive controls were expected to give OD values of 1.0 units or higher in the type-specific reactions and in the pan-FMDV reaction, the negative controls usually gives OD values lower

than 1.0 in wells 1-8H and slightly higher in wells 9-12H. The mean background reactions were calculated for each plate by adding the optical density (OD) values of negative control wells of each row (serotype) and dividing by number of well coated with the same serotype. These OD values were due to the reagents and not to a specific reaction between antigen and antisera. Each mean background OD was subtracted for each serotype from the actual OD for that serotype to obtain a corrected OD value. If corrected OD was >= 0.1 positive to coated MAB (serotype), unless negative (Namatovu, 2015); (OIE, 2013, 2014); (Sentayhu *et al*., 2014). Corrected OD = OD value of sample (each well) – OD value of corresponding negative control.

3.6 Data Analysis and Management

Data obtained from laboratory tests; real-time RT-PCR detection, virus isolation by cell culture and serotyping FMDV by antigen detection ELISA and Characteristics of sampled cattle collected during sample collections were entered into Microsoft Excel before being analyzed using Statistical Package for Social Sciences (SPSS) software, version 20 to use statistical descriptive to summarize the results. The map of study area was generated using ArcGISv9.0.

4. RESULTS AND DISCUSSIONS

4.1 Characteristics of Study Cattle and Examined Sample Types

In the study of serotyping and molecular characterization of FMD virus all epidemiological information were recorded and numbered with the character of cattle sampled during study period. The basic characteristics of the study cattle (N= 105) are summarized and presented in Table 3.

Regarding the age the study cattle 38 (36.2%), 38 (36.2%) and 29 (27.6%) were young (<3 year), adult (3<6 year) and old (>6 year), respectively (Appendix Table 5). Out of total 105 sampled cattle, 59 (56.2%) were cross (indigenous zebu with Holstein Friesian breed) and 46 (43.8%) were local (indigenous zebu) breed. Concerning the sex of study cattle, out of total 105 sample cattle, 42 (40%) were males and 63 (60%) were females (Table 3).

Cattle production system in Ethiopia mostly practiced extensive production system managed under smallholder mixed with other susceptible FMD domestic animals like small ruminant, pigs; however, in the study place three types of production systems were exercised. Among 105 sample cattle, 24 (22.9%), 49 (46.7%) and 32 (30.5%) were kept under intensive, semi-intensive and extensive production systems, respectively (Table 3). The production systems have major role in transmission of FMD and other diseases, since those productions ought to be kept in communal grazing area, watering points, movement of cattle place to place would be increase the incidence of the disease (Megersa *et al*., 2009).

Besides, the body condition scoring of sampled cattle, which is useful management tools for distinguishing differences in health status of cattle in the herd, was recorded. Out of the total 105 sample cattle, the body condition scores of 19 (18.1%), 29 (27.6%), 44 (41.9%) and 13 (12.4%) were categorized as emaciated, thin, moderate and good, respectively (Table 3 and Appendix Table 6).

Table 3. Characteristics of study cattle (N= 105) for molecular detection and serotyping of FMD virus in central Ethiopia during the study of September 2016 – April 2017.

Study Place	Age			Breed		Sex		Production system			Body condition score				Total
	Young	Adult	Old	Cross	Local	Male	Female	I	S	E	E_m	T	M_o	G	
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
A	4 (3.8)	3 (2.8)	6 (5.7)	--	13 (12.4)	11 (10.5)	2 (1.9)	--	--	13 (12.4)	2 (1.9)	2 (1.9)	9 (8.6)	--	13 (12.4)
B	3 (2.8)	7 (6.7)	1 (0.9)	9 (8.6)	2 (1.9)	7 (6.7)	4 (3.8)	--	--	11 (10.5)	3 (2.8)	2 (1.9)	4 (3.8)	2 (1.9)	11 (10.5)
C	13 (12.4)	7 (6.7)	4 (3.8)	13 (12.4)	11 (10.5)	6 (5.7)	18 (17.1)	--	24 (22.9)	--	7 (6.7)	4 (3.8)	11 (10.5)	2 (1.9)	24 (22.9)
D	2 (1.9)	3 (2.8)	7 (6.7)	12 (11.4)	--	--	12 (11.4)	12 (11.4)	--	--	--	--	11 (10.5)	1 (0.9)	12 (11.4)
E	4 (3.8)	7 (6.7)	1 (0.9)	3 (2.8)	9 (8.6)	3 (2.8)	9 (8.6)	3 (2.8)	9 (8.6)	--	--	7 (6.7)	3 (2.8)	2 (1.9)	12 (11.4)
F	12 (11.4)	16 (15.2)	5 (4.8)	22 (21)	11 (10.5)	15 (14.3)	18 (17.1)	9 (8.6)	16 (15.2)	8 (7.6)	7 (6.7)	14 (13.3)	6 (5.7)	6 (5.7)	33 (31.4)
T_t	38 (36.2)	38 (36.2)	29 (27.6)	59 (56.2)	46 (43.8)	42 (40)	63 (60)	24 (22.9)	49 (46.7)	32 (30.5)	19 (18.1)	29 (27.6)	44 (41.9)	13 (12.4)	105 (100)

(-): Unavailable, Study area (A= Adea, B= Aleltu, C= Kimbibit, D= Kolfe koraneyo, E= Mulo, F= Wochale, T_t= summation of all study area), Production system (I= intensive, S = sem-intensive, E= extensive), N= total number of cases, (%)=percentage of total number, Body condition score (E_m= Emerciate, T= Thin, M_o= Moderate, G= Good), Age (young = <3, Adult= 3<6, Old= >6) year.

4.2 Detected FMD Virus

In this study, out of total 105 samples tested by real-time RT-PCR, 34 (32.38%) samples (13(38.2%) oral swabs, 4(11.8%) epithelial tissues and 17(50%) oro-pharyngeal fluids) were positive (Table 5, Figure 2 and Appendix Table 3). The result of RNA extraction, cDNA synthesis and amplification protocol conducted showed that FMD virus 3D gene in all sample types ranged from C_T = 15.19 - 30.24 and total mean and stander deviation 20.99 and 4.83, respectively recorded (Table 5). Among woredas, Kimbibit was the highest positive scorer 11 (32.4%) while Aleltu was the lowest positive scorer 1 (2.9%).

Other samples might be due to improper sample collection, storage, transportation from field of study to NAHDIC and dilution of sample with viral transport media (VTM), leads to being negative, since FMD virus is highly susceptible to harsh conditions including excessive temperature, extremes of pH, disinfectants and desiccation. Therefore, advantageous to protect samples during the interval between collection and testing (especially for viral isolation). In addition, suspicious or doubtful result was not detected by rRT-PCR. All positive FMD viruses detected by real-time RT-PCR with low C_T values indicate high concentration of viral RNA (Table 5 and Appendix Table 3).

The molecular diagnosis of FMD by real-time RT-PCR method used in this study is concurred with the report of Callahan *et al*. (2002), who characterized field isolates resulted in positive reactions with low C_T values. Cycle threshold (C_T) values are not only used to determine the presence of FMD virus RNA but they are also used to quantify the level of viral RNA in a sample. The C_T values obtained with the assays reported here were consistent with that of the pan-FMDV 3D gene (Reid *et al*. 2014). Moreover, Kasanga *et al*. (2014) reports revealed that from a total of 179 oro-pharyngeal fluid samples were tested for the 3D region of the FMD virus genome by rRT-PCR, 31 samples (17.3%) tested positive for FMD virus, which was more comparable with the present result.

Indeed, previous study demonstrated that FMD virus 3D gene detection method performed similarly to another real-time RT-PCR protocol for amplification of the 5' un-translated region (5'UTR) of the FMD virus genome (King *et al*., 2006). Also Waheed *et al*. (2011) extracted from epithelial tissue samples (before cell culture) were also tested by one-step real-time RT-PCR

targeting the 5'UTR and 3D regions of the FMDV genome using an automated robot to extract total nucleic acids during their study molecular characterization of FMDV 2008-2011 in Pakistan. Similar work was done by Callahan *et al.* (2002) and Shaw *et al.* (2007) on experimental animal and approved that it is possible to extract RNA of FMD virus from 5'UTR and 3D region of gene. However, in this study RNA of FMD virus was extracted only from 3D region of gene.

During real-time RT-PCR detection, the amount of amplified cDNA is measured after each cycle of amplification via dyes that generate fluorescent signals, the magnitude of which is proportional to the amount of the amplicon generated. The amplification curves, generated by plotting the fluorescence against the number of cycles, represent the accumulation of product over the duration of the reaction. The fluorescence signals increase exponentially with the number of thermal cycles after 13-cycler number (Figure 3). This analogue observation reported (King *et al*., 2006).

Normalized reporter (Rn) (reporter and quencher dye at both end of probe) is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye i.e. a level of ΔRn used for the determination of the threshold cycle (C_T) during amplification by real-time RT-PCR assays (figure 3). The level was set above the baseline, but sufficiently low to be within the exponential growth region of the amplification curve reported by Callahan *et al*. (2002); Reid *et al.* (2003). The value of the rRT-PCR can be rapidly facilitate the molecular analysis of field isolates FMD virus and provide important epidemiological information (basic characters of cattle) regarding the source of outbreaks. The rRT-PCR could be determined the serotype of FMD virus; However, due to lack of primers and probe unable to identify serotypes. For this limitation, virus isolation (VI) and antigen detection ELISA (Ag-ELISA) was done for identification of FMD virus serotypes.

The number and types of samples collected for investigation or detection of FMD virus by cattle character are summarized and presented in Table 4. Oro-pharyngeal fluid were more appropriate site of FMD virus during late exposure, but epithelial tissue better at active cases and oral swab collected during healing stage of scar (OIE, 2014). Epithelial tissues samples were collected only in Wochale, since this type of sample can only found in active cases freshly ruptured or non-ruptured lesion within 2-3 days. Oral swab samples were not collected in Aleltu while oro-

pharyngeal fluid samples were collected in all study places. A total 16 of oro-pharyngeal fluid and 16 oral swab samples were collected highest in Kimbibit and Wochale, respectively **(**Table 4). Out of total 6 collected epithelial tissues 4 (66.7%) were positive detected by rRT-PCR. This indicates that epithelial tissue is more appropriate site of FMD virus than oral swab and oro-pharyngeal fluid (Table 5 and Appendix Table 3). Oro-pharyngeal fluids out of total 64 samples collected 17(26.6%) and oral swabs out of total 35 samples collected 13(37.1%), respectively positive detected by rRT-PCR during study periods (Table 4, 5 and Appendix Table 3).

Table 4. Number of FMD virus detected from each study areas by rRT-PCR method and comparisons between sample types during study.

Study Place	**Sample Type**	**№ of Examined**	**№ of Pos (%) by rRT-PCR**	**Mean±SD C_T Value**
Adea	Oral Swab	2	2(100)	15.33 ± 0.24
	Oro-pharyngeal fluid	11	1(9.1)	15.19 ± 0.00
	Subtotal	13	3(23.1)	15.28 ± 0.19
Aleltu	Oro-pharyngeal fluid	11	1(9.1)	28.42 ± 0.00
	Subtotal	11	1(9.1)	28.42 ± 0.00
kimbibit	Oral Swab	8	4(50)	20.68 ± 3.79
	Oro-pharyngeal fluid	16	7(43.8)	24.15 ± 5.05
	subtotal	24	11(47.8)	22.89 ± 4.76
Kolfe Koraneyo	Oral Swab	5	2(40)	17.24 ± 1.97
	Oro-pharyngeal fluid	7	1(14.29)	30.24 ±0.00
	Subtotal	12	3(25)	21.61 ± 7.69
Mulo	Oral Swab	4	1(25)	21.69 ± 0.00
	Oro-pharyngeal fluid	8	5(62.5)	22.67 ± 2.36
	Subtotal	12	6(50)	22.52 ± 2.15
Wochale	Oral Swab	16	4(25)	20.71 ± 5.33
	Epithelial Tissue	6	4(66.7)	16.49 ± 2.38
	Oro-pharyngeal fluid	11	2(18.2)	19.37 ± 2.16
	Subtotal	33	10(30.3)	18.75 ± 3.98
Total		105	**34(32.38)**	20.99 ± 4.83

Mean ± Stander Deviation, C_T = threshold cycle, rRT-PCR = real-time reverse transcriptase polymerase chain reaction, Pos (%) = percentage of positive rRT-PCR in corresponding sample type.

The basic characteristic of sampled cattle were useful for analysis of risk factor, accordingly, out of total tested by rRT-PCR 38 young, 38 adult and 29 old, 9 (23.8%),16 (42.1%) and 9 (31.1%) were positive FMDV in age category, respectively (Table 5). Adult cattle were more affected, which renders the productivity. This is in lines with Rufael *et al*. (2008) in Borena pastoral area and Megersa *et al*. (2009) in Gamo gofa and Sidama zones, which revealed that a significant variation on sero-positivity of foot and mouth disease among young, adult and old cattle. Higher seroprevalence of FMD was observed in young and adult cattle than in old cattle. In addition, according to Thrusfield (1995), young cattle with less prior exposure did not show a measurable response. The low exposure in-immature age groups were because of keeping young animals around the homestead and around camps separately from the adult animals. On the other hand, Gelaye *et al.* (2009) who have done their research in Bench Maji zone of southern Ethiopia documented no significant association between seropositivity of FMD between groups different age of cattle.

Concerning sex of sampled cattle, out of total detected by rRT-PCR 63 females and 42 males, 20 (31.8%) and 14 (33.3%) were FMDV positive, respectively (Table 5). This finding was consistent with the previous findings reported from different parts of Ethiopia (Galaye *et al*., 2009; Megersa *et al*., 2009), where sex appeared not to have a significant effect on sero-positivity for FMD. On the contrary, Hailu *et al*. (2010) in their report on the incidence of FMDV among dairy cattle in Northwest part of Ethiopia documented a higher rate of incidence in female cattle than that of male cattle.

Regarding production systems, 32 extensive, 49 semi-intensive and 24 intensive were tested by rRT-PCR and 8 (25%), 19 (38.8%) and 7 (29.2%) were positive FMDV respectively (Table 5). The studies of cattle in extensive production systems in different regions of Ethiopia have reported a direct association with FMD, which an indication of the nature of disease transmission (Bayissa *et al*., 2011).

The breed of sampled cattle, out of total 59 cross and 46 local breeds examined by rRT-PCR 18 (30.5%) and 16 (34.8%) were positive to FMDV (Table 5). There was no more variation between local and cross. This is parallel in line with Misganu *et al*. (2013) which report the seroprevalnce of FMD between local and cross breeds were found to be statically non-significance difference; However, the higher prevalence in local breed than that of cross breed might be attributed to

uncontrolled movement and extensive management unlike that of relatively control movement in cross breed. Moreover, the purposive samples taken might also contribute to the differences in all characters of sampled cattle.

Table 5. Number of samples examined by rRT-PCR and character of cattle examined for the detection of FMD virus in central Ethiopia during study September 2016 - April 2017.

Characters of cattle		**№ of Examined**	**№ of pos(%) by rRT-PCR**	**Mean±SD C_T Value**
Age	Young	38	9 (23.8)	20.86 ± 4.78
	Adult	38	16 (42.1)	20.31 ± 4.51
	Old	29	9 (31.1)	20.09 ± 4.2
Sex	Female	63	20 (31.8)	20.48 ± 4.5
	Male	42	14 (33.3)	23.88 ± 4.23
Breed	Cross	59	18 (30.5)	20.75 ± 4.79
	Local	46	16 (34.8)	20.24 ± 4.33
Production	Extensive	32	8 (25)	20.85 ± 4.78
system	Semi-intensive	49	19 (38.8)	21.16 ± 4.7
	Intensive	24	7 (29.2)	24.52 ± 4.05
Body	Emaciation	19	6 (31.6)	19.32 ± 4.54
condition	Thin	29	12 (41.4)	20.34 ± 4.5
	Moderate	44	15 (34.1)	20.9 ± 4.78
	Good	13	1 (7.7)	27.95 ± 000
Sample type	Epithelium tissue	6	4 (66.7)	16.49 ± 2.21
	Oral swab	35	13 (37.2)	19.41 ± 3.93
	Oro-pharyngeal fluid	64	17 (26.6)	22.87 ± 4.79

Mean ± Stander Deviation, C_T = threshold cycle, rRT-PCR = real-time reverse transcriptase polymerase chain reaction, Pos (%) = percentage of positive rRT-PCR in corresponding cattle characters and sample type.

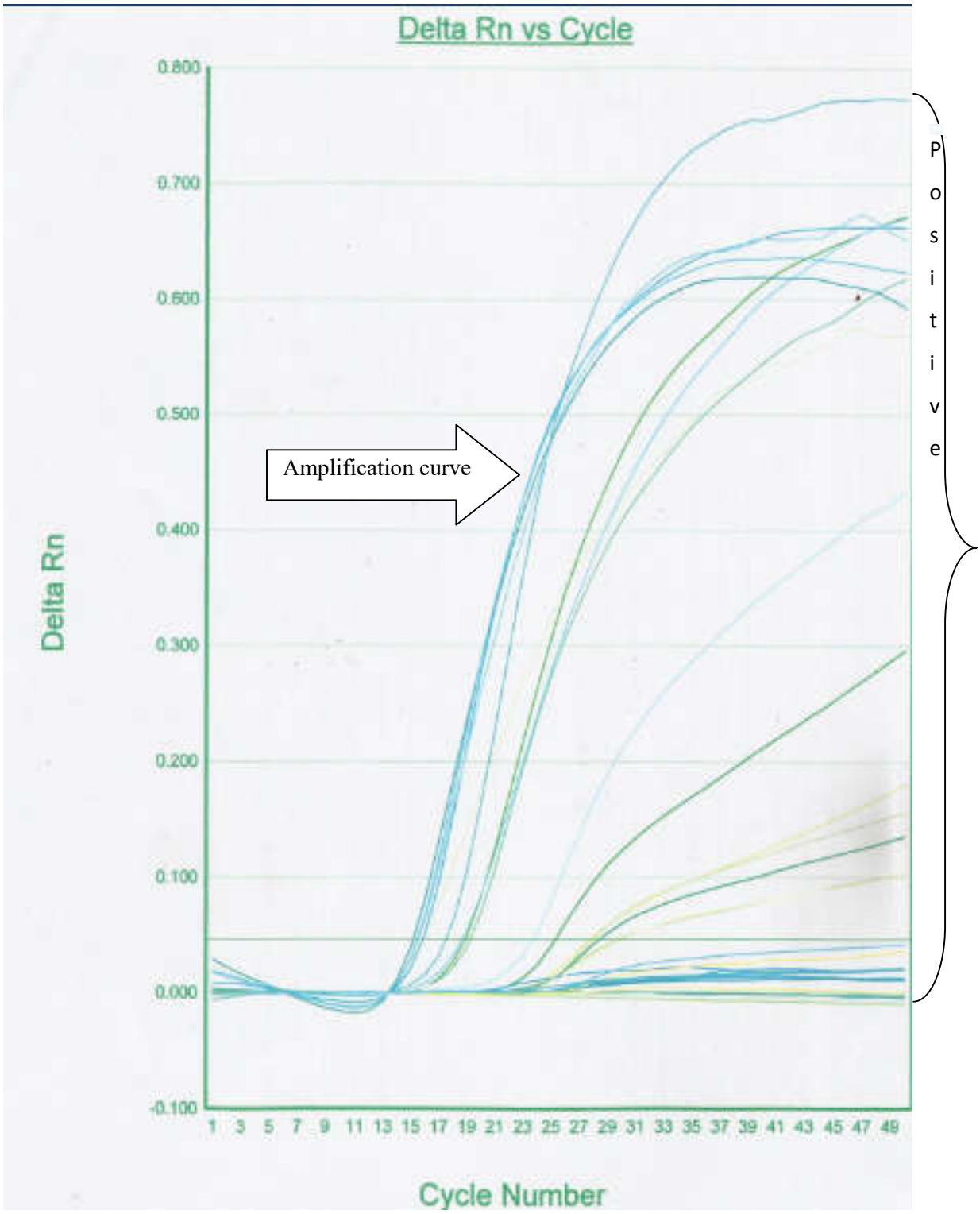

Figure 3: The results of real-time RT-PCR Delta normalized report (Rn) versus cycle number indicated the extraction of FMDV RNA and cDNA synthesis.

4.3 Cytopathic Effect of FMD Virus

Cytopathic effects on monolayer BHK-21 cell cultures, FMD virus were observed after 24-72 hrs in all passages, which indicates the presence of the virus in the collected samples (Figure 4). The CPE is characterized by a fast destruction of the monolayer BHK-21 cell lines, infected cells were found singly, the cells lines were manifested by the rounding and flattening of the cells, multinucleated giant cells formation, breaking down of the intracellular bridges and finally BHK-21 cell deaths which are characteristic findings of FMD virus infected cells (Figure 4). Mostly cytopathic effect (CPE) in the form of clumping, detachment and destruction of the cell lines sheets were seen within 48-72 hrs of the second passages of inoculation. Moreover, the morphology of uninfected control BHK-21 cell line was compared with the FMD infected BHK-21 cells were shown in Figure 4.

The finding of cell culture following infection with FMD virus was correlated with the findings of Shahiduzzaman *et al*. (2016) who reports the CPE effect produced by the inoculation of FMD virus in BHK-21 cell lines were manifested by the rounding and flattening of the cells, breaking down of the intracellular bridges and cell deaths were characteristic findings of FMD virus infected cells. Hossen *et al*. (2014) and Alam *et al*. (2015) reported similar findings. Moreover, Haileleul *et al*. (2013) from the total of 33 bovine epithelial tissues cultured samples,19 (57.57%) samples were showed CPE on BHK-21 monolayer cells culture for FMD virus in which CPE was characterized by a fast destruction of the monolayer cell, infected cells were round, formed singly and complete destruction of the cells were mostly seen within 48 hrs of inoculation. In addition to above reports, Sentayhu *et al*. (2014) during 2011- 2012 of their study in five regions of Ethiopia including Oromia, Amhara, and Tigray, they isolated FMD virus 43(72.88%) from the total of 59 epithelial tissues that showed CPE on BHK-21 monolayer cell culture and 16 of samples were negative CPE due to death of virus during transportation to NVI. However, the samples in present study were selected from positive at rRT-PCR, 8(100%) showed CPE.

The current result is in agreement with Alaa *et al*. (2014) who used BHK-21 cell lines as a susceptible cell lines for isolation of FMD virus that resulting in 12 samples were positive from total 35 epithelial tissues and vascular fluid, explained the characteristic of CPE as rounding and lysis of cell sheets; BHK-21 was the most reliable diagnostic method of FMD virus isolation. In addition, FMDV isolation on BHK-21 cell lines were done in Ethiopia by many researchers like

Kiros *et al*. (2013) in and around Mekele, National Veterinary Institution (NVI) Bishoftu, Ethiopia, for vaccine production of FMD virus in large scale.

Viral isolation or grown on monolayer BHK-21 cell lines and showing CPE implies the presence of virus in the sample, but this method cannot differentiate the type of virus in the sample, since other virus (without FMD virus) can grow on monolayer BHK-21 cell culture. So it must be detected by antigen detection ELISA or other detection techniques.

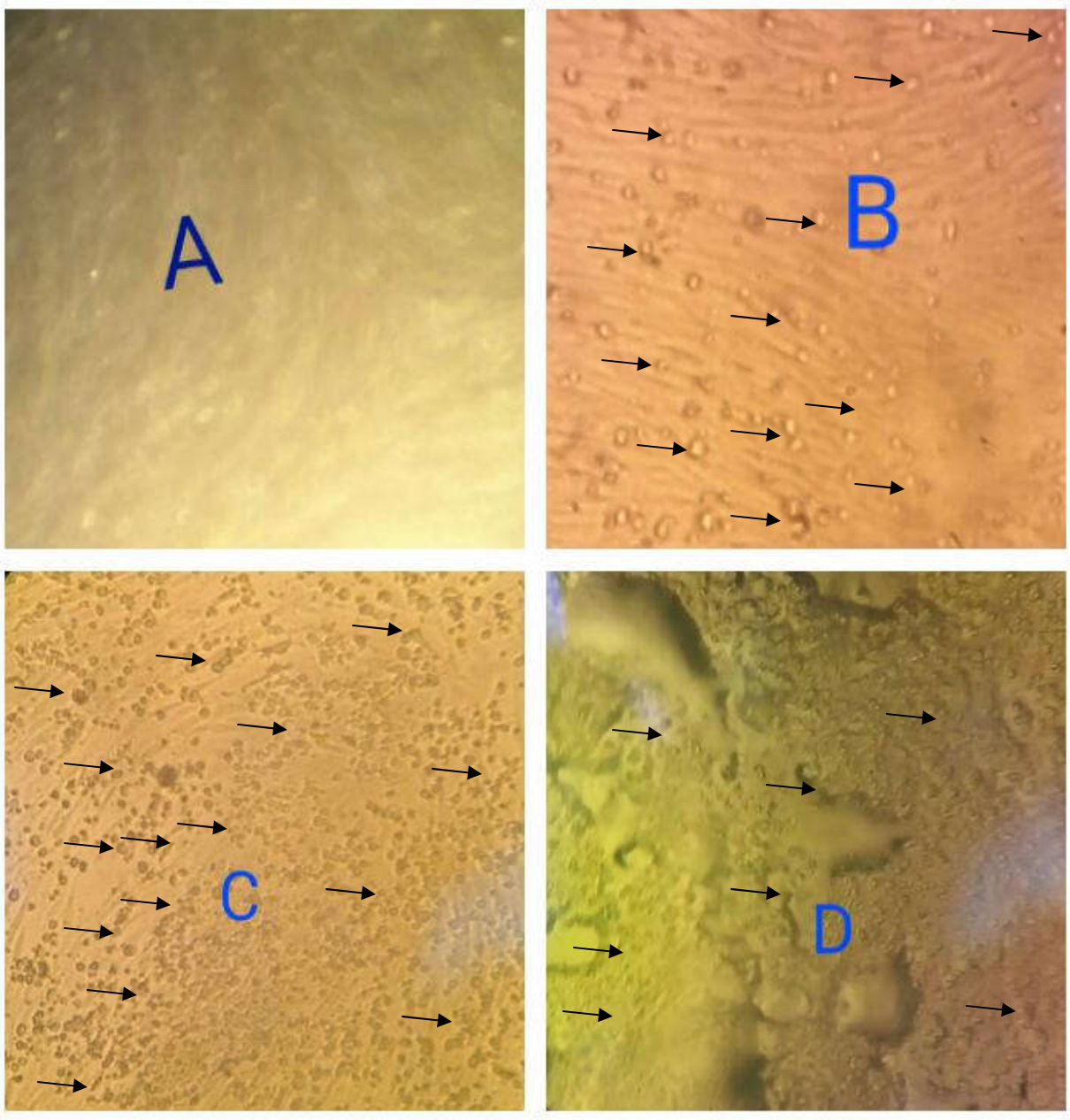

Figure 4: - Comparison of CPE

A. Free culture of BHK-21 cell line,

B. FMDV infected BHK-21 cell (Passage 1st: initiation of infection and cell rounding started),

C. FMDV infected BHK-21 cell (Passage 2nd: almost 85-100% cell infected and

D. FMDV infected BHK-21 cell (passage 3rd: almost all BHK-21 cells were destroyed and formation flattening) all photos were taken after 48 hrs from each passages by observing under inverted microscope 40X**.**

→ = Indicate the cythopatic effect of the virus at all passages.

4.4 Detected FMD Virus Serotypes

Six samples were tested by antigen detection ELISA to identify the FMDV serotypes circulating in the study area (Table 6 and Appendix Table 4). The result showed that serotype "O" and "A", were circulating in the outbreaks areas of central Ethiopia during the study period. In one of the sample collected from Wochale district, serotype O and A were identified. FMD virus serotype O 4(57.14%) was more dominant than serotype A 3(42.86%) from findings. All the results of FMD virus serotypes were summarize and presented in Table 6.

This finding is not in agreement with the FAO Reference Laboratories report during 2015, where FMD virus serotypes O (O/EA-3) and SAT 2 (SAT 2/VII/Alx-12) have been detected but serotype A was not detected by Donald and Mark (2015) in Ethiopia. The study of Sahle *et al.* (2004) reported O, A, SAT1 and SAT2 from cattle in Ethiopia, while Rufael *et al.* (2008) serotyping of 120 seropositive samples obtained from Borana pastoral herds (Southern Ethiopia) indicated that serotypes O (99.2%), A (95.8%), SAT 2 (80%) and C (67.5%). Moreover, Gelagay *et al*.(2009) during their study 1981-2007, serotypes have reported O, A, C, SAT 1 and SAT 2 from bovine, swine, ovine, and caprine samples collected from the outbreaks in central Ethiopia, out of this serotype O was the dominant serotype (73.3%), followed by types A (19.5%), SAT 2 (4.1%), SAT 1 (1.8%) and C (1.3%). In previous reported by Gelaye *et al*. (2005) and Ayelet *et al*. (2009), Serotype O was isolated from the samples collected from Kolfe sub-city (Addis Ababa). The present findings are concurred with Haileleul *et al*. (2013) who identified from 19 epithelia tissue samples that showed CPE, three samples were serotype A (Africa topotype, G-VII strain), while 16 tissue cultured samples were found serotype O (East Africa-3 topotype) during their study 2008-2009 in Oromia, Amhara and Addis Ababa city.

In another study by Kiros *et al*. (2013), serotype O was isolated in Tigray region and Sentayhu *et al*. (2014) reported that serotype O was circulated throughout Ethiopia where outbreaks occurred. On the other hand, national disease outbreak investigation records from NVI and NAHDIC showed that FMD outbreaks that occurred in the 2007- 2012 were caused by serotypes O, A, SAT 1 and SAT 2 in Addis Ababa, North Shewa zone, East Shewa zone and other many parts of Oromia region. According to report of Jemberu *et al.* (2014), the most dominant serotype O accounted for 70% of 173 samples that tested positive (N=379), followed by SAT 2 with 20.8% identified as serotypes O, A, SAT2 and SAT 1 were the causal serotypes of FMD outbreaks

during their study 2007- 2012 in Ethiopia. The current study identified serotype O and A of FMD virus. Serotype O was common and dominant when compared with other serotype followed by serotype A. Serotype O was highly prevalent and a dominant serotype causing an outbreak in Ethiopia, as well as, genetically diverse topotype that can affect many host range or cloven hooves mammals both domestic and wild animals, distribution was all over the world compare to other serotypes. Yet, there was no report about co-circulation serotype of FMD virus in Ethiopia.

Table 6**.** Summary of FMD virus serotype by antigen detected ELISA from study area.

No	**Woreda (Study Place)**	**Sample type**	**No of sample**	**C_T value rRT-PCR**	**CPE**	**OD value**	**Serotype**
1	Kolfe koraneyo	Oral Swab	01	18.64	+++	0.16	A
2	Mulo	Oral Swab	01	21.69	++	0.15	O
3	Adea	Oral Swab	01	15.5	+++	0.49	O
4	Wochale	Epithelial Tissue	01	15.13	++	0.54,0.19	O, A
5	Kimbibit	Oro-pharyngeal	01	15.13	++	0.199	O
6	Aleltu	Oro-pharyngeal	01	28.423	+++	0.101	A

OD= optical density, C_T= threshold cycle, rRT-PCR = real-time reverse transcriptase polymerase chain reaction, CPE= cytopathic effect, (++) = harvested at second passage, (+++) = harvested at third passage in cell culture.

5. SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Summary and Conclusion

Foot and mouth disease (FMD) is a debilitating and highly contagious, trans-boundary disease of cloven-hoofed animals especially cattle. It has seven serotypes, namely: O, A, C, SAT1, SAT2, SAT3 and Asia 1. It was an important veterinary pathogen, which can cause huge economical loss in the global with wide spread epidemics. FMD in Ethiopia is notifiable disease and considered as a slow down or impede by creating an obstruction to the livestock productivity and production. Rapid detection and identification of FMDV serotypes is important and essential for both health and control of FMD. However, the information of molecular characterization and strain of FMD virus in Ethiopia is scarce. Considering the above facts, the current study was focused on detection, isolation and identifying FMD virus serotypes circulating in central Ethiopia purposively from FMD suspected cattle.

A total of 105 (35 oral swabs, 6 epithelial tissues and 64 oro-pharyngeal fluids) samples were collected from 105 cattle in Adea, Aleltu, Kimbibit, Mulo, Wochale and Kolfe Koraneyo. All the study places are found in central Ethiopia, with basic characters of cattle. All the samples were collected from study place and transported in icebox to National Animal Health Diagnostic and Investigation Centre (NAHDIC) Sebeta, for laboratory investigation. Laboratory tests like sample preparation, RNA extraction, amplification, viral isolation (cell culture) and antigen detection by sandwich ELISA were done NAHDIC at Sebeta. Out of total 105 sampled 34(32.38%) were positive FMD virus as detected by rRT-PCR. Negatives results might be due to improper sample collection, storage, transportation, dilution with VTM and other factors.

Eight samples were selected among positive results by rRT-PCR based on representative of outbreaks occurred in the study areas and inoculated into prepared monolayer BHK-21 cell lines. All showed CPE which characterized by the rounding and flattening of the cells, multinucleated giant cells formation, breaking down of the intracellular bridges and cell death.

Out of harvested in cell culture six of them were selected for serotyping by antigen detection ELISA or sandwich ELISA. Serotype O 4(57.14%) and A 3(42.86%) were identified and from one sample both serotype were circulating.

The findings of the current study confirmed that, FMD virus serotypes O and A are circulating in the central Ethiopia during study between September 2016 to April 2017. FMD in central Ethiopia is endemic due to factors such as, the presence of high number of susceptible domestic animals (small ruminant, pigs), free movement of livestock and their products, lack of monitoring and surveillance, lack of immediate report of outbreaks, lack of vaccination of FMD and others have contribution of FMD to pose recurrent outbreaks and devastated the economy of the country. The detection of FMD virus in persistently infected or carriers among exposed cattle from the study areas were the great importance that needs suitable, sensitive, specific and rapid techniques. Early diagnosis of FMD during outbreaks using the combination of real-time RT-PCR diagnostic technique followed by culturing on monolayer BHK-21 cell line and antigen detection are essential diagnostic method for detection, isolation, identification of serotype and molecular characterization of FMD virus. However, the lack of infrastructure in study areas posed high risk of FMD, which may be a limiting factor for using rRT-PCR as a routine diagnostic tool.

5.2 Recommendations

Based on the above findings and conclusion, the following points were recommended:

- Modern diagnostic techniques should be practiced and trained personnel.
- Animals should be vaccinated with appropriate strains of vaccines.
- Serotyping and molecular characterization to ensure that field strains must be matched with the vaccine strains to be effective.
- Quarantine and restriction of livestock movement from FMD suspected outbreak areas.
- Surveillance and monitoring during import and export of livestock and their products.
- Generally, proper control and intervention should be designed and implemented to minimize the burden of the disease.
- A further study should be conducted at a wider range of serotyping, topotyping and molecular characterization of FMD virus and socio-economic impacts of FMD in Ethiopia with neighboring countries for further prevention, control and eradication of the disease.

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7. APPENDIX

Appendix: - Tables

Appendix Table 1. Sample (data) collection format of FMD cases in cattle

	Owner's Name	Address		History of Cattle							Sample Collected (√)				Clinical Sign (√)				Parameters		
		Woreda	Kebele	ID	Breed	Sex	Age	Body con.	Reproduction	Prod. System	Tissue	Blood	Swab	Probang	Salivation	Lesion	Lameness	severity	Tem.Rate	Pulse	Resp.
1	Tola	A	G	0	L	F	3	1	Preg	ex	√	-	√	-	√	√	√	S	.	.	.
2	Bontu	W	T	2	C	M	6	3	-	In	-	-	-	√	_	√	√	M	.	.	.
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105	Biyansa	K	X	-	C	F	7	2	Lact	S	-	-	√	√	√	√	√	M	-	-	-

Body Condition Score (1= Emaciate, 2= Thin, 3= Moderate, 4= Good), Production System(Ex= Extensive, S= Semi-Intensive, In= Intensive)

Appendix Table 2. Composion of master mix reactions

Reaction component	Volume added to each reaction PCR plate
2X reaction mix	12.5 µl
Nuclease-free water	1.5 µl
Forward primer	2 µl
Reverse primer	2 µl
Taqman probe	1.5 µl
Superscript III RT/ platinum taq mix	0.5 µl
Extracted sample/ positive/negative control	5 µl
Total	25 µl

µl = microlitre

Appendix Table 3. The C_T values of each positive FMD virus detected by rRT-PCR during study.

Woreda	Breed	Sex	Age	Pro. System	Sample Collected	Body condition	RT-PCR C_T-Value
	Local	F	3	Extensive	Oral Swab	Thin	21.77
	Local	M	6	Extensive	Oral Swab	Emaciate	27.7592
Wochale	Cross	M	5	Extensive	Oral Swab	Emaciate	15.794
	Cross	F	5	Semi-Extensive	Oral Swab	moderate	17.4975
KolfeKoraneyo	Cross	F	5	Intensive	Oral Swab	moderate	15.8505
	Cross	F	7	Intensive	Oral Swab	Thin	18.6353
Mulo	Local	F	4	Semi-Extensive	Oral Swab	Thin	21.69
	Local	M	7	Extensive	Oral Swab	moderate	15.4993
Adea	Local	M	10	Extensive	Oral Swab	moderate	15.1636
	Local	F	7	Semi-Extensive	Oral Swab	Thin	19.5145
	Cross	F	1	Semi-Extensive	Oral Swab	Emaciate	25.2764
Kimbibit	Local	M	8	Semi-Extensive	Oral Swab	moderate	16.24
	Local	F	1	Semi-Extensive	Oral Swab	moderate	21.68
	Cross	F	6	Intensive	EP. Tissue	Thin	20.06
	Cross	M	3	Intensive	Ep. Tissue	Emaciate	15.13
Wochale	Cross	F	5	Intensive	Ep. Tissue	Thin	15.3591
	Cross	M	5	Extensive	Ep. Tissue	Emaciate	15.41
Kolfe Koraneyo	Cross	F	5	Intensive	Oro-pha	moderate	30.3393
Wochale	Cross	M	2 .5	Semi-Extensive	Oro-pha	Emaciate	20.8924
	Cross	M	5	Semi-Extensive	Oro-pha	Thin	17.8385
	Local	F	6	Semi-Extensive	Oro-pha	moderate	24.9447
	Local	F	2	Semi-Extensive	Oro-pha	Thin	19.9777
	Local	F	4	Semi-Extensive	Oro-pha	Thin	21.2059
Mulo	Local	M	4	Semi-Extensive	Oro-pha	Thin	21.9415
	Cross	F	5	Intensive	Oro-pha	moderate	25.3603
Adea	Local	M	10	Extensive	Oro-pha	moderate	15.1869
	Cross	F	1	Semi-Extensive	Oro-pha	Thin	29.9559
	Local	F	7	Semi-Extensive	Oro-pha	Thin	23.6653
	Cross	F	4	Semi-Extensive	Oro-pha	moderate	19.6486
	Local	F	7	Semi-Extensive	Oro-pha	moderate	18.8556
	Local	M	8	Semi-Extensive	Oro-pha	moderate	19.2123
	Cross	F	4	Semi-Extensive	Oro-pha	Good	27.9541
Kimbibit	Cross	M	3	Semi-Extensive	Oro-pha	moderate	29.7789
Aleltu	Cross	M	3	Extensive	Oro-pha	moderate	28.423

Oro-pha = oro-pharyngeal fluid, Sex (F= female, M= male)
Interpretation: C_T (threshold cycle) <30 = positive, C_T = 30<35 doubtful (retest) C_T =>35 negative. Best positive C_T value rages 12-30.

Appendix Table 4. Plate layout for FMDV detection and serotype ELISA

Catching MABs		**Type O**		**Type A**				**Pan O,A,C, As1**		**Type SAT 1**		**Type SAT 2**	
		MAb 3B11		MAb4D12		MAb 5F6		MAb 1F10		Pool 1MAbs		Pool 1MAbs	
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Sample 1	**A**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Sample 2	**B**												
Sample 3	**C**												
Sample 4	**D**												
Sample 5	**E**												
Sample 6	**F**												
POS cnt	**G**												
NEG cnt	**H**												

Corrected OD = OD value of sample (each well) – OD value of corresponding negative control.
OD= optical density, pos= positive, neg= negative, MAB= monoclonal antibody.
If corrected OD was >= 0.1 positive to coated MAB (serotype), unless negative.

Appendix Table 5. Age estimation of study cattle based on dental eruption.

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

Source: (Merck veterinary manual, 1998)

Appendix Table 6. Body condition score of study cattle

No	BCS	Description Body Condition
1	Emaciate	Starving and weak; no palpable fat detectable over back, hips or ribs; tail head and individual ribs prominently visible; all skeletal structures are visible and sharp to the touch; animals are usually disease stricken. Under normal production systems cattle in this condition score are rare
2	Thin	Borderline; individual ribs noticeable but overall fat cover is lacking; increased musculature through shoulders and hindquarters; hips and backbone slightly rounded versus sharp appearance
3	Moderate	Moderate; increased fat cover over ribs, generally only 12th and 13th ribs are individually distinguishable; tail head full, but not rounded.
4	Good	Cattle appear fleshy and carry fat over the back, tail head, and brisket; ribs are not visible; area of external rectum contains moderate fat deposits.

BCS= Body Condition Score