

Isolation and Identification of Foot and Mouth Disease Virus from Clinically Infected Cattle in Ada Veterinary Clinic

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Abstract: Foot and mouth Disease (FMD) is a highly contagious and economically important disease of cloven-hoofed animals, predominantly for cattle, sheep and pigs. This study was designed to isolate and identify the serotypes of FMD virus from clinically suspected cattle in Ada veterinary clinic. Animals were clinically examined and tissue and serum samples were collected from each of FMD suspected cases. Out of 38 submitted samples, FMD virus was isolated from 36 (94.7%) of the samples on cell culture. Serotype identification was made using virus neutralization test (VNT) of the viral isolates and complement fixation test (CFT) of sera of positive cattle. The result from both tests Virus Neutralization Test (VNT) and Complement Fixation Test (CFT) showed that 24 (66.7%) of the animals were positive for serotype O while the remaining 12 (33.3%) were infected with South African territories type 2 (SAT2). Therefore, taking the most contagious nature of the disease, its economic impact and the non-existence of specific treatment after infection into account; practicing preventive strategies is the only means recommended to eradicate the disease from Ethiopia and to make the country to benefit from its huge livestock industry.

Key words: SAT2 • FMD Serotypes • DebreZeit • Virus Isolation • Foot-And-Mouth Disease

INTRODUCTION

Foot and mouth disease (FMD) is the most contagious viral disease of cloven-hoofed animals. The disease is characterized by fever and vesicular eruptions on the feet, buccal mucosa and, in females, the mammary gland [1, 2]. FMD is caused by a virus of the genus Aphthovirus, in the family picornaviridae. There are seven immunologically distinct serotypes; O, A, C, South African Territories (SAT) 1, SAT2, SAT3 and Asia1. FMD viruses are small RNA viruses that are enclosed with a non-enveloped protein shell (Capsid) [3]. The presence of seven serotypes and multiple subtypes and variants has added to the difficulty of laboratory diagnosis and control of FMD. The rise of new variants is inevitably caused by continued circulation of the virus in the field and quasi-species nature of RNA genome [5]. RNA viruses in general and FMD virus in particular have very high mutation rates, in the range of 10^{-3} to 10^{-5} per nucleotide site per genome replication, due to the lack of error correction mechanisms during RNA replication [6].

Of the domesticated species, cattle, pigs, sheep, goats and buffalo are susceptible to FMD while many species of cloven-hoofed wildlife, such as deer, antelope and wild pigs may become infected [7]. [8]. Transmission is generally affected by contact between infected and susceptible animals to the excretions and secretions of acutely infected animals [2].

FMD severely constrains international trade in animals and animal products[9]. FMD has considerable economic consequence and can be attributed to both direct and indirect costs. The direct effects of the disease are loss of milk production, loss of draught power, retardation of growth, abortion in pregnant animals, death in calves and lambs while the indirect losses are attributed to the disruption in trade of animals and derivative products [10]. The disease causes the greatest production losses in cattle and pigs and in particular in intensive dairy and pig production systems [11].

Today, many countries have either eliminated FMD by compulsory slaughter of infected animals or have reduced its incidence greatly by extensive vaccination

programs [8]. FMD is still a major global animal health problem, but its geographic distribution has been shrinking in recent years as control and elimination programs have been established in more and more countries [8]. Western and central Europe, North America, parts of South America, South Africa, Australia and some island regions of Asia are currently recognized as being free of FMD. However, the disease is still widespread in many countries of Africa, Asia and the Middle East [9]. The epidemiology of FMD in sub-Saharan Africa is probably more complicated than in any other region of the world. Not only have six of the seven serotypes occurred in Africa (Only Asia 1 has never been recorded), but also marked regional differences in the distribution and prevalence of serotypes and intratypic variants occur [12, 13].

FMD was first recorded in Ethiopia in 1957 when serotypes O and C were found [14, 15]. The recent study conducted by Ayelet *et al.* [16] on FMD samples collected between 1981 to 2007 throughout the country from different species of animals showed that serotype O, A, C, SAT1 and SAT 2 were recorded. Serotype O was the dominant serotype identified with the degree of 73.3% and widely distributed throughout the country, while the rate for serotype A was 19.5%, C 1.4%, SAT 2 was 4.1% and SAT 1 was 1.8 % with limited distribution.

Such a complex epidemiological situation of FMD in Ethiopia due to the circulation of multi-serotypes of the virus and involvement of four different host-species as well as additional factors such as the presence of high numbers of wildlife (Especially African buffalo), rooming free cross borders between the neighboring countries and lack of control of animal movements urges the need of having appropriate research, diagnostic and controlling programs of the disease. Vaccination against one serotype of FMD virus does not cross-protect against other serotypes and may also fail to protect fully or at all against other subtypes of the same serotype [2]. The objective of this study was therefore aimed: to isolate and identify FMD virus serotypes causing disease outbreaks around DebreZeit so as to design appropriate prevention measures.

Study Area, Clinical Case Description and Clinical Examination: The study was conducted in Ada veterinary clinic and National Veterinary Institute (NVI) over a period of November 2010 to May 2011. The clinic is located in DebreZeit town near Genesis farms while NVI is also located in DebreZeit in Kebele 15 next to AAUSVM. DebreZeit is located 45kms south east of the capital city,

Addis Ababa. It lies 9° N latitude and 4° E longitude at an altitude of 1850 meter above sea level central highlands of Ethiopia. The area has an annual rainfall of 866 mm of which 84% is in the long rainy season from June to September. The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 4°C, respectively, with mean relative humidity of 61.3% [17].

Cattle admitted in Ada veterinary clinic were registered and all case histories were collected and detailed physical clinical examinations were carried out according to standard procedures [2].

The cattle presented in the clinic were local breeds which were brought from different peasant associations (PAs) around DebreZeit and they are managed in intensive production systems. However, all the cattle were not vaccinated against FMD. The owners complained that the cattle showed rapid drop in feed and water consumption and they had lesions in their mouth and on the feet. Upon clinical examination of FMD suspected animals; they have showed a varying degree of clinical signs. Most of the animals were febrile with vesicular lesions on their feet, buccal mucosa and tongue with drooling of saliva. Generally, the disease was more severe in young than in adult animals and milking cows showed a drop in milk production. Animals showing such symptoms were tentatively diagnosed for Foot and mouth disease (FMD) and thirty eight animals showing clinical signs of FMD were selected for the study.

Collection and Transportation of Samples: Both epithelial tissue and serum samples were collected from each of the thirty eight FMD suspected cattle. Epithelial samples were collected from unruptured vesicles in the buccal mucosa and tongue of the cattle. The area over the vesicles was washed with phosphate-buffered saline (PBS) to remove gross contamination such as feed. Using sterile scissors and forceps, a piece of epithelial sample at least 2cm x 2cm (Postage stamp size) was collected without any attached subcutaneous fat or muscle. The samples were then placed in sterile screw capped test tubes containing transport medium composed of equal amounts of glycerol and 0.04 M phosphate buffer, pH 7.2–7.6, with added antibiotics (Penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU]). The samples were then labeled with the tag of the animals and dates of collection, kept in an ice box, transported and submitted to NVI virology laboratory. Sampling was done following procedures described in OIE [2].

In addition, blood was collected from the jugular vein of each of FMD suspected cattle with a plain vacutainer tube, labeled and it was allowed to clot. The serum was then harvested in a cryovial and labeled. The cryovials were packed with a plastic container and kept in an ice box for transportation to NVI immunology laboratory.

Sample Storage and Processing: Standard sample storage and processing for FMD test was employed (OIE, 2009). Epithelial samples were stored at -20°C until virus isolation. The samples were taken from the transport medium, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures and weighed. A suspension was prepared by grinding the samples in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium (pH = 7.2) and antibiotics under a class II safety cabinet. Further medium was added until a final volume of five times that of the epithelial sample has been added, giving a 20% suspension. This was clarified on a bench top centrifuge at 2000g for 10 minutes. Once clarified, such suspensions of field samples suspected to contain FMD virus were harvested and used to inoculate into cell cultures and the serum samples also stored at -20°C until they were tested for the presence of specific antibodies by the complement fixation test.

Analysis of Samples

Virus isolation on BHK-21 Cell Cultures: Established cell layer of baby hamster kidney (BHK-21) cells were inoculated with 0.2 ml of each of the clarified suspension of epithelial tissue samples. The cell cultures were examined for Cytopathic effects (CPE) for 48 hours within 6 hrs of interval. Cytopathic effect (CPE) was observed after 48 hours (Or even less) in positive cases. If no CPE was detected, the cells were frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours before the samples were declared to be negative. The infected BHK-21 monolayer cells were subjected to three freeze-thaw cycles to release the viral particles from the cells. The viral suspension was clarified from the cell debris by centrifugation at 800 × g for 10 min and stored at -70°C for virus neutralization test (VNT).

Virus Neutralization Test (VNT): The quantitative VN microtest for FMD antibody was performed with BHK-21 cells in flat-bottomed cell-culture grade microtitre plates. Stock virus was grown in cell monolayers and stored at -20°C after the addition of 50% glycerol. The sera were

inactivated at 56°C for 30 minutes before testing. The control standard serum was 21-day convalescent serum (Usually pig). Hank's balanced salt solution with yeast lactalbuminhydrolysate and antibiotics was used as a medium. VNT was performed according to procedures described in OIE [2]. Positive wells (where the virus has been neutralized and the cells remain intact) were seen to contain blue-stained cells sheets; the negative wells (Where virus has not been neutralised) were empty. Titres were expressed as the final dilution of antigen present in the serum/virus mixture at the 50% end-point. A titre of 1/45 or more of the final antigen dilution in the serum/virus mixture was regarded as positive. Titres of 1/16 to 1/32 were considered to be doubtful and further antigen samples were requested for testing. Animals were considered to be positive if the second sample has a titre of 1/16 or greater. A titre of 1/8 or less is considered to be negative.

Complement Fixation Test (CFT): Serum samples were tested by CFT at National Veterinary Institute (NVI), DebreZeit Ethiopia, according to the protocol described in OIE manual [2]. Sedimentation of hemolytic system (SRBC) was taken as a positive result while full hemolysis was observed in negative results.

Data Analysis: Data collected during the study period were stored in the Microsoft Excel spreadsheet program and analyzed using STATA 17 and descriptive statistics and frequency was used.

RESULTS AND DISCUSSION

Virus Isolation: Of the total 38 clinical samples examined, cytopathic effect (CPE) was observed in 36 (94.7%) samples in BHK-21 cell cultures for FMD virus, but the other 2 (6.3%) didn't show any CPE by blind passage 5 times (Table 1). The CPE was characterized by fast destruction of the cell monolayers and infected cells were round and formed singly. Complete destruction of the cell sheet was mostly seen within 48 hours of inoculation (Figures 2-5). The control cells remained intact after 48 hours of incubation.

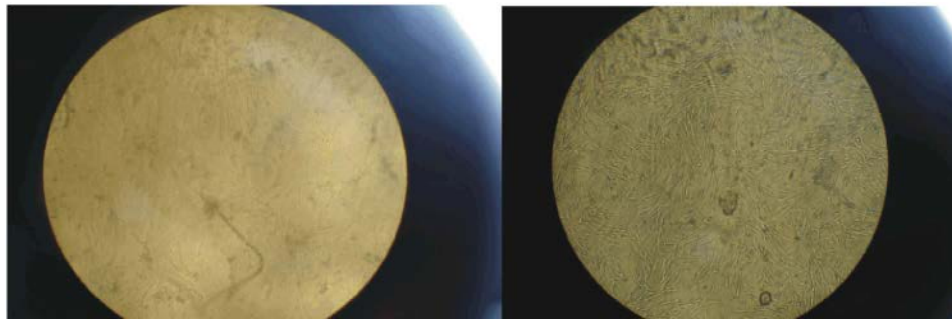
Virus neutralization (VNT) and Complement Fixation Test (CFT): FMD virus serotype identification was conducted by testing the first passages of those samples that showed CPE using virus neutralization test and sera of those animals were prepared for complement fixation test.

Table 1: Summary of cell culture results of suspected cattle in Ada veterinary clinic

Type of sample	Animal species	Number of inoculated flask/sample	Number (%) of samples showing CPE upto the 1 st passage	Number of samples that didn't show CPE after 5 th passage
Epithelial tissue	cattle	1	36 (94.7%)	2 (5.3%)

Table 2: Results of virus neutralization and complement fixation tests

Type of lab test	Type of samples	No of samples examined	Number (%) of Serotypes identified from the samples examined	
			SAT2	O
VNT	Isolated virus	36	12 (33.3%)	24 (66.7%)
CFT	Serum	36	12 (33.3%)	24 (66.7%)



A. at the time of inoculation

B. 48 hrs after inoculation

Fig. 1: BHK-21 cell uninoculated controls

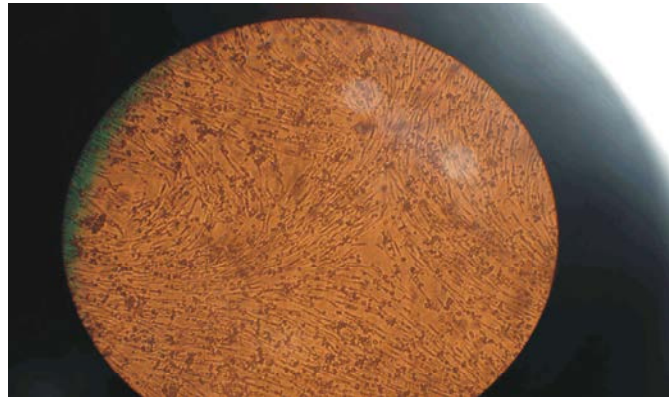


Fig. 2: Cytopathic effect observed after 12 hrs of incubation

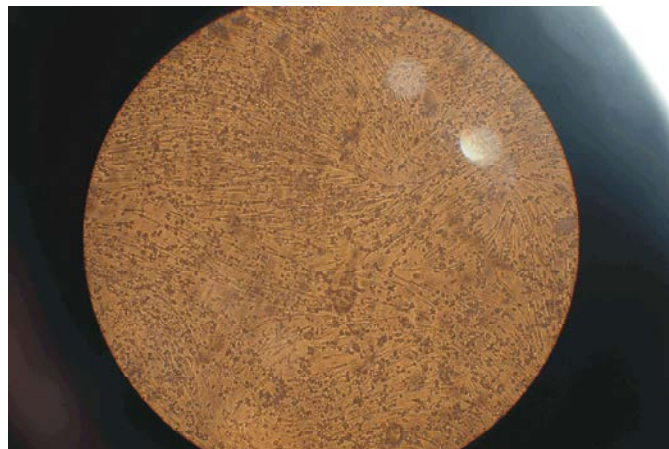


Fig. 3: Cytopathic effect observed after 18 hrs of incubation

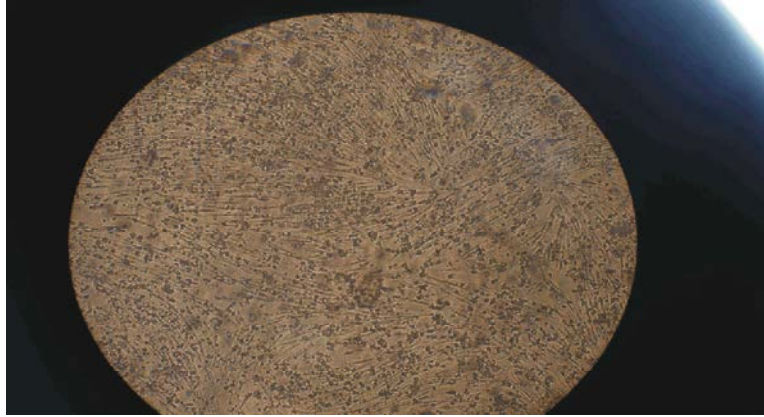


Fig. 4: Cytopathic effect observed after 24 hrs of incubation

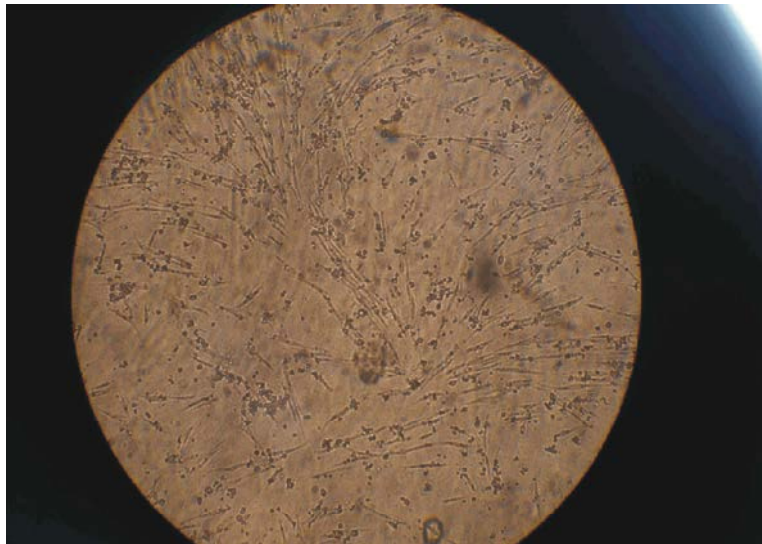
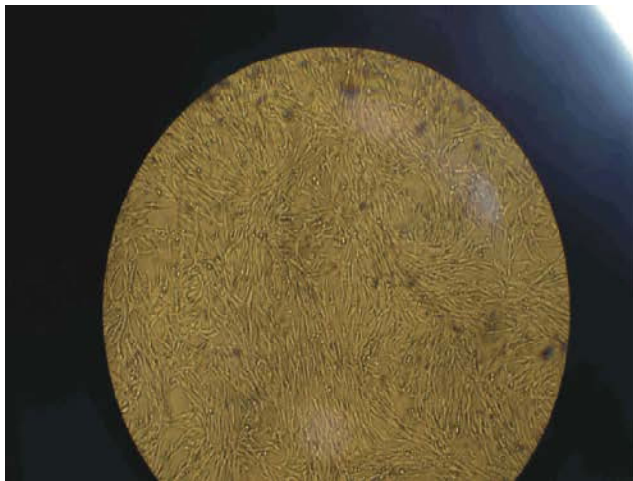
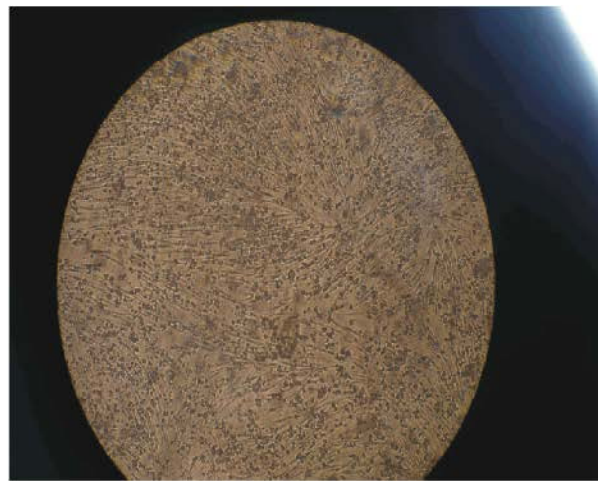


Fig. 5: Cytopathic effect observed after 48 hrs of incubation



A. Antibodies virus isolates neutralized by specific antigen



B. *Virus* isolates showing CPE on microplate that was not neutralized by antibodies

Fig. 6: Results of virus neutralization test

Out of 38 samples collected from clinically FMD suspected cattle, 36 (94.7%) were confirmed to be positive VNT and CFT. Samples from the remaining two cattle were found to be negative for FMD virus and circulating antibodies. Serotype identification of FMD virus of positive animals was performed by virus neutralization test (VNT) of the isolated virus and complement fixation test of their sera. Results from both tests have shown that 24 (66.6%) of the positive cattle were infected with serotype O while the remaining 12 (33.3%) were infected with South African Territories type 2 (SAT2) serotype of FMD virus (Table 2).

Based on the clinical examination and laboratory analysis performed, it can be concluded that 36 of the cases out of 38 under investigation were identified as FMD. 66.7% of the cases under investigation were found to be caused by serotype O while the remaining 33.3% were caught by serotype SAT2 of FMD virus. The result of the current study is fairly agreed with Ayelet *et al.* [11] reviewed that, serotype O was identified in 1979 by WRLFMD on samples collected from DebreZeit in 1977. However, no published data is available on the occurrence of SAT2 in and around DebreZeit. Therefore, this study implied that other serotypes of FMDV are emerging and this could lead to the difficulty of eradicating the disease from the area as well as the country at large.

Since the first report of FMD, Serotypes A and SAT2 were not identified until 1969 and 1989, respectively [15]. During 1988 to 1991 samples from 16 FMD outbreaks in Ethiopia were examined at the National Veterinary Institute (NVI), Ethiopia and at the Food and Agriculture Organization World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD), UK. Typing of the virus responsible was possible in 13 of these outbreaks representing 10 separate disease events; eight of these were caused by serotype O and two by serotype SAT2. In contrast to earlier studies serotypes A and C were not detected [18]. Antibodies to SAT 2 were also detected in 1971 from cattle in North Omo, Southwestern Ethiopia [14, 18]. From records of outbreak investigations conducted by NVI between 1982 to 2000, serotype O, A and SAT 2 FMD viruses were also identified [19]. The records of ministry of agriculture and rural development (MOARD) from 2000 to 2006 indicated that FMD outbreak occurred every year with the highest in 2001 with 88 outbreaks. FMDV is still active in many parts of Ethiopia; however, only limited efforts were made to characterize FMD virus isolates. During 1981 to 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. However, serotype C was not detected since 1983 [11].

CONCLUSION

The occurrence of 5 serotypes and multiple variants of FMD virus in the country, its economic impact and the highly contagious nature of the disease urges to implement appropriate eradication of FMD from Ethiopia so as to enable the country to benefit from its huge livestock potential. In this study, O and SAT2 serotypes of FMD virus were detected on samples collected from clinically presented and FMD suspected cattle in Ada veterinary clinic. This doesn't mean that other serotypes of the virus are not occurring in and around DebreZeit since the study was conducted on those animals which came into the clinic within a period of six months only. Furthermore, it was not possible to identify the subtype variants due to the absence of FMD-specific primers to perform molecular techniques.

Accordingly, the following recommendations are forwarded:

- Efforts should be made to make information available on the antigenic and genetic characteristics of isolates collected in all regions of the country.
- Ppolyvalent vaccines must be produced by the NVI including serotypes O, SAT2 and A which were found to be the most prevalent serotypes in recent years.
- A better coordination between regional and central laboratories must be made to improve the surveillance of FMD in the country.
- A more comprehensive system of vaccine matching should be made in order to select the subtypes of FMDV serotypes with the best ability of cross-protection as vaccinal strains.
- Further detailed Epidemiological and molecular study should be initiated to select the FMDV serotype circulating in the county.

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