



Farm based outbreak study and Genetic characterization of O serotype Foot and mouth diseases virus isolated from clinical cases in dairy farms of Welmera district, Central Oromia, Ethiopia

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ABSTRACT

Foot and mouth disease (FMD) is a trans-boundary viral disease that causes huge economic losses. A field based outbreak study was conducted in September 2016/2017 to identify and genetically characterize O serotype from clinical and asymptomatic cases at Welmera district Menagesha kolobo kebele of central Oromia. The investigation was purposively conducted in the respective farms where outbreak occurred. Accordingly, 50 dairy cattle were clinically examined and about 20 (40%) animals showed sign and clinical lesions of the disease. Accordingly, active epithelial tissues and probang samples were taken from acutely and severely infected animals for virus isolation, typing and genetic detection. These clinical samples were processed for antigen typing and O serotype was isolated from clinical and asymptomatic cases in the farm. The genetic material of this strain was tested by rRT-PCR and the viral genome was detected in the epithelial tissues and probang samples. In conclusion, the study showed that O serotype was circulating in the district. Therefore, foot and mouth disease control should be established based on the availability of potent and matched vaccines against the circulating field strains.

Keywords: Cattle; FMDV; Welmera; Serotypes; Outbreak

INTRODUCTION

Foot and mouth disease is an economically devastating and a highly contagious disease of cloven hoofed animals as a result of loss of production; cost of treatments and it is the main barrier to trade of animal and animal products at local and international markets (James and Rushton, 2002). The disease is characterized by low mortality in adult cattle and massive mortality in young calves due to acute Myocarditis (Kandeil *et al.*, 2013).

It is a virus of the genus Aphtho-virus in the family of Picornaviridae of which seven immunologically distinct serotypes (O, SAT2, SAT1, A, C, Asia 1 and SAT 3) are circulating in the wide field but multiple subtype strains have evolved within each serotypes. This variation causes failure of the prevention and control options as infection or immunization with one serotype doesn't confer protection against other serotypes and fails to protect other subtypes of the same strains (Paton *et al.*, 2005).

FMD outbreaks have been reported in Ethiopia and the situation indicated that, four serotypes (A, O, C and SAT 2) have been identified (Sahle, 2004). According to (Ayelet *et al.*, 2009) study, serotype O (73.30%) was the most prevalent followed by A (19.50%), SAT-2 (4.10%), SAT-1 (1.80%) and C (1.40%) during outbreaks. Besides this, Klein. 2009) indicated that serotype O is the most prevalent strain worldwide. The incident of new strains and uncontrolled movement are some of the risk factors of FMD outbreak in Ethiopia (Bewket *et al.*, 2012). However, a study on the specific serotype of virus that causes outbreak is limited. So, identification of the virus strain from outbreak is very important to apply emergency vaccines (Yang *et al.*, 2013). Outbreak based study is also important in the disease endemic areas to detect newly emerging variants despite the fact the existence of other serotypes or new virus subtypes might cause outbreak. Hence, generation of updated information on genetic characteristics of this strain could help to understand the circulating strains and institute disease prevention and control packages. Therefore, the objectives of this outbreak study were: to identify serotype O virus from clinical and asymptomatic Foot and mouth disease cases in the dairy farm and to genetically characterize the virus from farm outbreaks.

MATERIALS AND METHODS

Farm based outbreak study

Foot and mouth disease outbreak had been occurring in Welmera district Menagesha kolobo kebele in private dairy farm by mid-September 2016/2017. Welmera district is a part of Oromiya special zone Surrounding Finfinne and the district is 40 km away from the capital city, Addis Ababa. The area is situated at 90- 04'- 9o 13'

N latitude and 38o 29'-38o 39' E longitude. It is bordered on the south by the Sebeta Hawas, on the west by West shewa zone, on the North by Mulo district, on the Northeast by the Sululta. Menagesha Kolobo and Holeta are the towns located in the district. The average altitude of the area ranges from 2200-2500 meter above sea level and the area is characterized by short rainy pattern from February to April and a long rainy season from mid-June to September. The annual temperature and rainfall ranges from 18°C to 24°C and 1000 to 1100 mm, respectively (CSA, 2009)

FMD Infected cows reared by producers

Cattle that had manifested clinical signs of the disease and those with asymptomatic cases in the farm were included in the study. Cattle determinants such as age, sex, breed and body conditions were considered during sampling.

Sampling techniques and Ethical consideration

Clinically and acutely sick cattle were physically examined in the affected farm. Moreover, clinical parameters and clinical signs were recorded so as to know the status of infection in the farm. Further, clinically sick and acutely infected animals were purposively screened and ten cows were considered for clinical diagnosis and laboratory studies. Ethical Consideration was also used to alleviate suffering of animals from sampling during sample collection.

Sample collection from clinical and asymptomatic cases

Samples were collected from clinically sick cattle and from those which had healing lesion in the mouth, dental pad or on the feet and the asymptomatic cows in the farm (Kafeero *et al.*, 2016). Active epithelium were aseptically collected from gum and tongue of affected cows and put in a bottle with transport medium having glycerol and 0.04M phosphate buffer saline (PBS) with some antibiotics and antifungal drugs. Probang samples were also taken from foot and mouth disease infected and asymptomatic cattle (OIE, 2004).

Foot and Mouth disease virus isolation and characterization in cell culture

The samples were processed and cultured on BHK-21 cell monolayer with three subsequent passages. About 1 gram of tissue was washed three times using sterile phosphate buffered saline containing antibiotics and antifungal drugs on petridish and transferred to sterile mortar. The minced tissues were homogenized in sterile

sand with pestle and mortar. Nine ml of PBS was added to the homogenized tissues and five percent antibiotics were added to make it ten times as that of the epithelial tissue in order to produce ten percent suspension (OIE, 2012). Then, the suspension was transferred to test tube and clarified by centrifugation at 3500 rpm and the supernatant was inoculated to baby hamster kidney- 21 cells and incubated at 37^o c with 5% CO₂. The cells were monitored daily to see the formation of virus induced cythopathic effects (CPE). The virus is cytocidal and CPE is characterized by a fast destruction of the cell monolayer and infected cells were disrupted and detached from the flask. When CPE appreciated in the infected cases, supernatants of the clinical tissues had been typed by antigen detection ELISA as per the recommended procedure (Bhattacharya *et al.*, 1996). If no CPE was observed following three blind passages, the virus isolation was considered as negative (OIE, 2012).

Detection and subtyping of O serotype from field based outbreak cases

The foot and mouth disease virus serotyping was performed by antigen detection sandwich ELISA with appropriate combinations of antiFMDV monoclonal antibodies (MAbs), used as coated and conjugated antibodies. The test was done for typing of FMD viruses. The kit was made for detecting and typing of FMD viruses serotypes such as type O, A, SAT 1 and SAT 2. A pan FMD test, detecting any isolates of serotypes O, A, C, Asia 1 and some of the SAT serotypes were incorporated in the kit to complement the specific typing and to detect foot and mouth disease viruses which could have escaped binding to selected serotype-specific MAb. The micro plates were covered with catching MAbs. The test was done as per the manufacturer's procedure and six samples were tested on a microplate containing 96 wells, one positive control for each FMD types O,A, SAT1 and SAT2 and negative controls were included in each plate. These controls were already incorporated into the ELISA microplate trapped by the respective catching MAb. First samples were diluted half in diluent buffer and 50µl of each diluted sample was distributed in 72 wells of A-F rows and two replicates of each-specific catching MAb and for the pan-FMDV MAb. Then, 50µl of diluents per well were added in all wells of G and H rows (positive and negative control, respectively), then plates were incubated at 25o C for 1hour. After incubation, all fluids on the plates were discarded and the remaining residual fluids were removed. Then 200µl of washing solution were added and incubated for 3min at room temperature, subsequently wells were emptied and the washing repeated twice. Then the residual fluids were removed by tapping on clean absorbent paper and 50µl of conjugate A was added from columns 1 to 8 and the same volume of conjugate B was added from columns 9 to 12. Plates were covered and incubated at room

temperature for 1hour. After incubation, 50µl of substrate per well was added to all wells and plates were covered and left at room temperature for 20minutes in the dark. The reaction was stopped by adding 50µl of stop solution. Immediately after stopping, reading the optical density (OD) of each well was done at 450 nm wavelength using micro plate reader. Criteria for test validity: The positive controls were expected to give OD values of 1.0 unit or higher in the type-specific reactions and in the pan-FMDV reaction, the negative control usually gives OD values lower than 0.1 in wells H1 to H8 and slightly higher in wells H9 to H12.

Molecular based Detection and characterization of FMDV serotype O in the processed cell culture supernatants

The viral RNA was extracted from cell culture grown viruses and the viral genetic fragment in the clinical lesions was amplified by RT-PCR using primers that amplifies the VP1 of the virus (Knowles and Samuel, 2003). The total RNA was extracted from 140 micro-liter original cattle epithelial tissues and oral swab suspension using Qiagen RNA extraction kit following manufacturer's procedure as (Kafeero *et al.*, 2016). About 140 micro liters of active epithelial tissues and oral swab suspension was added to 560µl buffer AVL carrier RNA in the micro centrifuge and vortexed for 15 sec to mix and incubated at room temperature for 10 minutes. The tube was centrifuged to minimize drops from the inside of the lid. Then 560µl of ethanol (96%) was added to the processed sample and mixed by pulse vortexing for 15 seconds followed by centrifuging to remove drops from the inside lid. Then 630µl of the supernatants were applied to the QIAMP Mini column in a 2ml collection tube and centrifuged at 6000g (8000rpm) for a minute. The filtrate was removed and the column was put in a fresh 2ml collection tube. Then 500µl of buffer AW2 were added to the column and centrifuged at 20,000 ×g (14,000 rpm) for three minutes and the filtrate was removed. Then 65µl of Buffer AVE was added to the column at appropriate room temperature for one minute and centrifuged at 6000g (800rpm) for a minute. At the end the viral RNA genomic fragment was detected and characterized in the processed materials.

The strain genetic detection was done by rRT-PCR method and the amplicon from clinical samples and cell culture supernatants were tested by real time PCR method targeting universal 3D regions of FMD virus specific primers following forward primer sequence (5'-ACT GGG TTT TAC AAA CCT GTGA-3') and reverse primer 5' - GCG AGT CCT GCC ACG GA -3') to detect the viral genomic material in the clinical samples (Callahan *et al.*, 2002). By using a cut-off cycle threshold (Ct) value as shown by (Shaw *et al.*, 2007) the genome was detected in the clinical samples collected from field cases.

Data Analysis

The data generated from clinical study and laboratory investigations were recorded and coded using Microsoft Excel spreadsheet and analyzed using STATA version 13 for Windows (Stata Corp. College Station, TX, USA) and Statistical Analysis System (SAS version 9). The Odds ratio was used for determination of risk factor strength in relation to infection. In all the cases, 95% Confidence limit and P Values.

RESULTS

During the study period, the farm was acutely infected and about 50 dairy animals were clinically and physically diagnosed out of which 40% (N= 20) of them were infected with the disease. Severely infected cows developed vesicle on oral cavity, erosions, profuse salivation, fever and lameness (Table 1).

Table 1: Farm based clinical examination and morbidity cases

Outbreak area	Kebele based Outbreak	No. of examined	Morbidity cases
Welmera	Menagesha Kolobo	50	20
Total		50	20 (40%)

BHK21 cell based virus isolation

Clinical samples such as epithelial tissues and oropharyngeal fluids were collected from clinical and subclinical cases and inoculated on BHK-21 cell culture with three consecutive passages. The culture results

indicated that out of ten clinical samples cultured, 60% (N=6) were isolated and exhibited morphological alterations or FMDV cytopathic effect on BHK21 cell which was characterized by destruction of BHK-21 cells (Table 2).

Table 1: Virus isolated on BHK21 cells

District	Kebele	Species	Date of sampling	Clinical Samples	Cytopathic effect on BHK21 cell	
					With CPE	Without CPE
welmera	M.kolobo	B	9/11/016	BPS (3)	-	W-ETH/12/016
	M.kolobo	B	11/11/016	BPS(3)	W-ETH/12/016	W-ETH/12/016
	M.kolobo	B	11/11/016	BET(2)	CPE-detected WETH/11/016	-
	M.kolobo	B	14/11/016	BET(2)	CPE-detected WETH/11/016	-
Overall culture results			10		6(60%)	4(40%)

BET= Bovine epithelial tissues; Bos=Bovine oral swab; BPS-Bovine probang sample, B=Bovine; CPE= Cytopathic effect; OB=Outbreak. M-Menagesha, W- Welmera

The processed supernatants were inoculated onto BHK21 cell to appreciate CPE of the virus and the isolated virus that showed complete CPE was characterized by inducing destruction of BHK-21 cell and infected cells were found as round, swelling and formed

singly. There was sloughing and detachment of cells in severe cases within 72 hrs and death of the cell. The figure below illustrated the rounding and lysis of BHK21 cells inoculated with FMD virus and the control cells remained as uninfected (Figure 1).

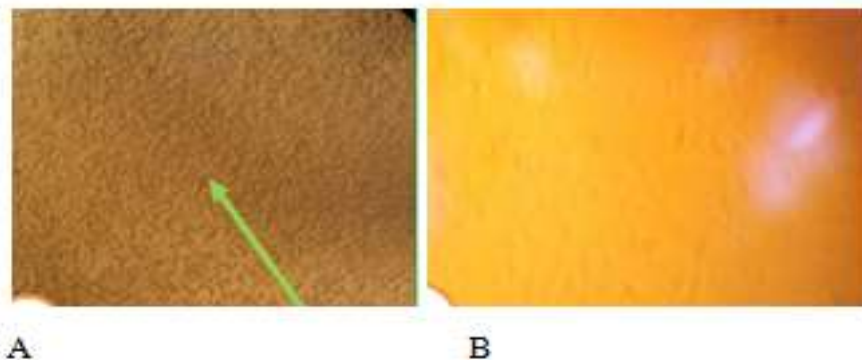


Figure 1: FMD virus infected (A) and control BHK21 cells (B)

7. Subtyping and rRT-PCR based characterization of FMD virus serotype O in clinical samples

Cell culture supernatants processed from clinical materials were subjected for subtyping by sandwich ELISA and serotype O was dominantly detected in the samples collected from outbreak farms. The strain had been detected and subtyped in the active epithelial tissues and probang samples. The presence of this viral

genetic material in the clinical samples was detected by rRt-PCR methods. The amplicon fragments extracted in the clinical materials including epithelium and probang samples were detected and characterized by rRT-PCR method (Amarel *et al.*, 1993). The targeting 3D regions of FMD O serotype virus genome was used to determine the presence of viral RNA in clinical samples (Callahan *et al.*, 2002) and there was high load of viral fragment in the clinical field samples (Table 3).

Table 2: Subtyping and genetic detection of FMD virus

Distinct	Outbreak at kebele	clinical samples	subtyped serotype	RT-PCR	rRT-PCR results
welmera	M-kolobo	BET		NVD	NFMDVGD
	M-kolobo	BP	-	NVD	FMDVGD
	M-kolobo	BP	-	NVD	
	M-kolobo	BET (2)	O	positive	FMDVGD
	M-kolobo	BET	-	NVD	FMDVGD
	Kolfe keranio	BP (2)	O	Positive	FMDVGD

NVD-no viral genome detected, NFMDVGD-No FMD viral genome detected, FMDVGD-FMD viral genome detected

DISCUSSION

Foot and mouth disease is a contagious viral disease of cloven hoofed animals which is characterized by formation of vesicles in the mouth, on the feet, teats and sudden death of young calves (Quinn *et al.*, 2005). The results of the current study showed that about 38.82% of infected cattle manifested clinical sign of the disease. This study was related with the previous findings of Belachew (2014) who reported that during outbreak of FMD in the country, 36.9% of animals manifested clinical sign of foot and mouth disease. In addition to this concept, some authors reported that about 28.2% of sick cows showed clinical signs indicative of foot and mouth disease (Nigussie *et al.*, 2011). This was supplemented by the studies of McLaws *et al.*, (2006) who stated that variations in clinical severity and manifestations were

associated with the virus strains, viral infection dose, species and breed susceptibility, management system and exposure to previous infection. The results of cell culture virus isolation showed that about 60% of inoculated BHK21 cells showed cytopathic effect and form rounding shapes. The previous studies reported by different authors also indicated that FMDV cytopathic effect was characterized by inducing fast destruction of BHK-21 cell monolayer. This was rationalized by (Shawky *et al.*, 2013) who stated virus isolated from clinical cases resulted in distortion of the cell and cytopathic effect within 24-48 hours after infection. The other clinical samples that didn't show cytopathic effect may be due to death of the virus during transportation and processing.

The subtyping analysis stated that about 40% of clinical samples contained the virus and O serotypes

were identified. This was substantiated with the report of Ayelet, (2009) who identified serotype O (73.3%), serotype A (19.5%), SAT-2 (4.1%), SAT-1 (1.8%) and C (1.4%) in the field outbreaks. The studies conducted by (Tesfaye, 2014) also indicated that serotype O (65.63%) was the dominant strain that circulated in Addis Ababa, Debre Berhan and Bishoftu areas. In line with this, studies conducted by Noureldin and Elfadil (2014) in Khartoum of Sudan indicated that serotype O (82.6%) and SAT-2 (40%) were the main circulating FMDVs in cattle. The current O serotype was identified from clinical samples such as epithelium, probang and oral swabs at Welmera district. This study was in line with the reports of Knowles (2010) who identified serotype O from clinical samples during FMD field outbreaks. There is a possibility of having more FMDV serotypes circulating in the area that could not be detected during the study period. The viral genetic material was detected in the clinical samples collected from field outbreaks. It was appreciated that there was higher concentration of viral load in the epithelial tissues and oro-pharyngeal fluids. This could be justified by the evidence that epithelial tissues were taken from clinical cases while probang samples were taken from symptomatic and asymptomatic cases. The preferred site for virus detection and multiplication is epithelial tissues and the oral mucosa for producing vesicles (OIE, 2012). This indicated that there was high load of viral RNA in the epithelial tissues than in the probang samples. Moreover, clinical samples that didn't induce the pathologic effect could have been due to virus degradation during transfer from the field and difficulties in maintaining the cold chain during transportation (Mwiine *et al.*, 2009)

CONCLUSION AND RECOMMENDATIONS

Foot and mouth disease is a rampant problem in the current study area. This was further confirmed through clinical investigation, serological screening, virological detection and molecular based diagnosis. Hence, the results of the current study indicated about 38.6% of infected cattle showed clinical signs during FMD outbreak. As a result of this, young calves mostly suffered from this disease in the farm. The dominant serotype circulating in the area and responsible for the recent outbreak was FMDV serotype O. In conclusion, the presence of enormous susceptible animals, age variability, breed susceptibility, poor prophylactic immunization, uncontrolled cross border movement, and high contact of animals at common points were identified as major risk factors that could result in the occurrence of foot and mouth disease. Therefore, field outbreak investigation will be required to detect the emergence of new strains in circulation and identification of the potential risk factors as well as vaccine matching test should be conducted to apply potent vaccines against the serotypes per areas.

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