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Isolation, Molecular Detection and Genotyping of Foot-and-Mouth Disease Virus from Naturally Infected Cattle in Central Ethiopia

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Abstract: Foot-and-mouth disease is an endemic and economically important, contagious viral infection of cloven-hoofed animals that poses a huge economic impact. This study was an outbreak investigation study to detect and isolate the circulating foot-and-mouth disease serotype in central Ethiopia between December 2020 and March 2021. For this 35-sample outbreak (26 epithelium and 9 swabs), samples were purposively collected. Virus isolation was performed on baby hamster kidney cells line (BHK-21), followed by nucleic acid detection of the agent in the samples using real-time and conventional polymerase chain reactions. Serotyping was done by antigen detection sandwich enzyme-linked immunosorbent assay (ELISA). In the present study, out of 35 samples, only 24 (68.75%) showed a cytopathic effect on a monolayer of the BHK-21 cell line. The nucleic acid of the virus was detected in 24 samples targeting a specific gene called 3D Pol. With regard to the serotype distribution, four serotypes were identified (O, A, SAT2, and SAT1). The dominant serotype circulating in the study area was serotype O (87.5%), followed by serotype A (25%), SAT2 (12.5%), and SAT1 (12.5%). Based on this finding, we recommend that regular outbreak investigation encompassing all the regions and districts be done in order to identify the circulating serotype and that further sequence based analysis of the topotypes be done. This will help with the prevention and control of the diseases through vaccination.

Keywords: Foot-and-Mouth Disease, Virus, Ethiopia

1. Introduction

Foot and mouth disease is contagious for cloven-hoofed animals (cattle, buffalo, sheep, pigs, and goats) [2]. It is characterized by fever, loss of appetite, salivation, vesicular eruptions in the mouth, on the feet, and teats, highly contagious transboundary disease of both domestic and wild cloven-hoofed animals [17] and possesses significant economic losses [3, 28]. It is caused by an RNA virus called Foot and Mouth Disease (FMDV), genus of Aphthovirus, family of Picornaviridae. This virus possesses seven serotypes: A, O, C, South African Territories (SAT) 1, 2, 3, and Asia 1 [16]. Currently, serotypes O, A, SAT-1, and SAT-2 are endemic in Ethiopia, whereas serotype C was last diagnosed in 1983 [1, 14].

The genome of a virus is divided into three parts: the 5' untranslated region (UTR), a single open reading frame (ORF), and the 3' untranslated region (UTR) [4, 20]. The genome of the virus is enclosed with four structural proteins and eight nonstructural proteins (L, 2A, 2B, 2C, 3A, 3B1-3, 3C, and 3D) [4, 10].

Vaccination is increasingly being recognized as a potential tool to supplement 'stamping out' for controlling

foot-and-mouth disease (FMD) outbreaks in non-endemic countries [26]. However, control is difficult due to their genetic variation, and cross-protection is impossible between serotypes and subtypes [13, 6]. FMD vaccines contain inactivated, purified antigen from which nonstructural viral proteins are removed, usually by chromatographic purification, and are formulated with different proprietary adjuvant formulations [8].

In Ethiopia, several outbreaks have been occurring with different serotypes of FMDV. This is due to a lack of strategy to control the disease. On top of this, a lack of vaccination strategies (quality, coverage, and timing) and the presence of free animal movement without certification are thus the main factors that could increase the spread of FMD along the cattle market chain [1]. With this in mind, this study was aimed to isolate, detect, and serotype the foot-and-mouth disease virus from an active outbreak in central Ethiopia.

2. Materials and Methods

2.1. Study Area and Population for Outbreak Investigation

The study was conducted in Sebeta, Bishoftu, Sululta, Bole, Dima Guranda, and Tafki from December 2020 to March 2021 (Figure 1) (Table 1). The cattle of both intensive and extensive farms were included, and specific district peasant associations and farms were purposefully selected following the report of the FMD outbreak. The study population consists of cattle that show clinical signs of FMD in the outbreaks in the study areas.

Table 1. *Full Description of Study Area.*

District	Population size (Sheep)	Population size (Goat)	Population size (Cattle)	Reference
Sebeta	32006	116136	188619	SALO, 2020
Bishoftu	53921,	47303	181256	BALO, 2020
A. A, Bole	11100	4100	26300	[33]
Sululta	115791	21521	284442	SALO, 2020

BALO: Bishoftu agricultural livestock office, SALO: Sebeta agricultural livestock office, $SALO_2$: Sululta agricultural livestock office, MAR: Mean Annually Rain. Dima Guranda and Tafki town was included under Sebeta with population size, evenly Dima Guranda have no own description it included at Sebeta at all.

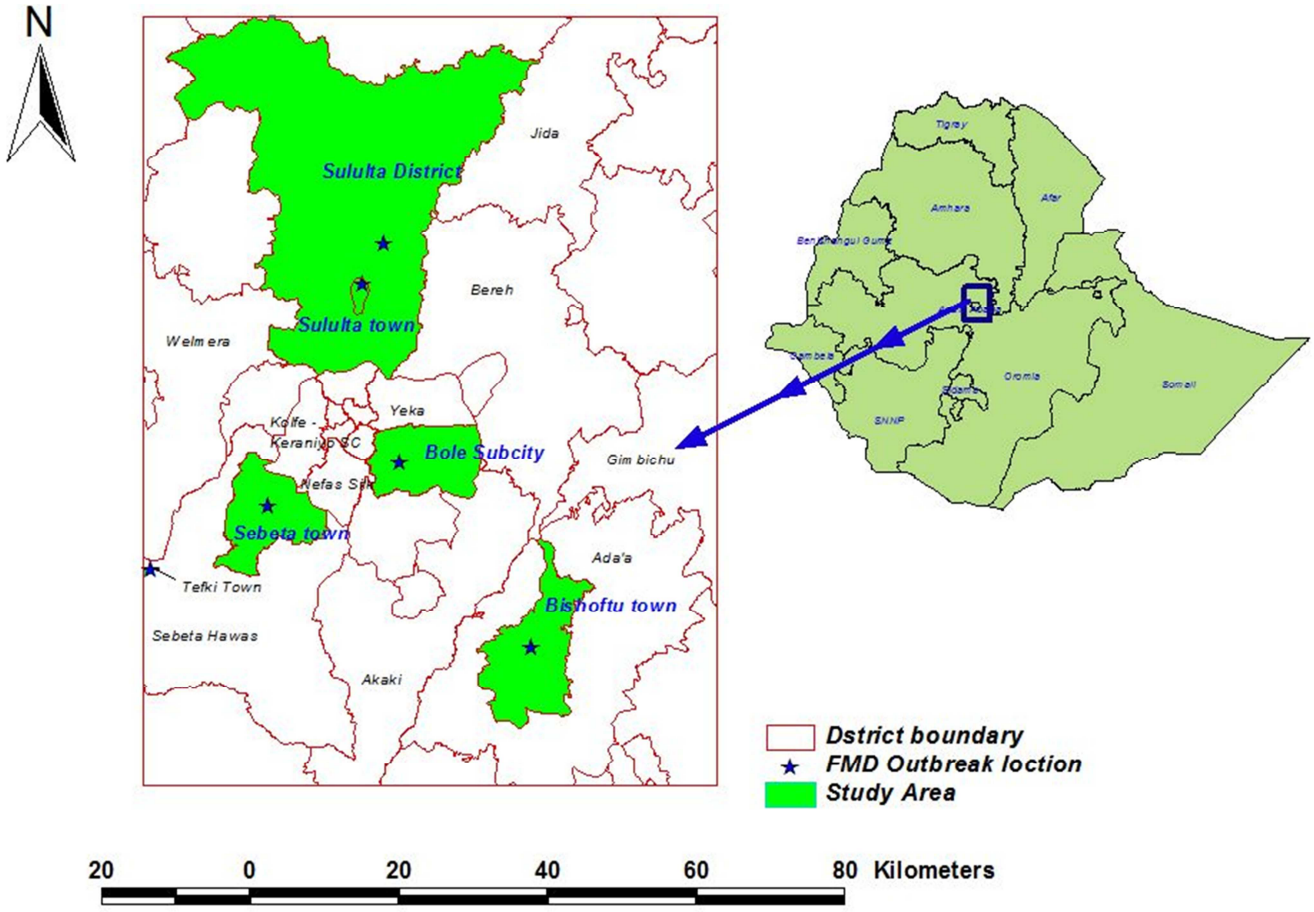

Figure 1. *Map Showing the Districts Reporting FMD Outbreaks in 2020 in Central Ethiopia.*

2.2. Study Design and Sampling

A cross-sectional study design was used between December 2020 and March 2021 for the outbreak investigation of FMD in central Ethiopia, based on a report from key informants. When an active outbreak was reported, a field investigation was conducted at the specific site of the outbreak. Sick animals are clinically examined for any lesions on the mouth, feet, teats, and udder. Specimens were collected from clinically sick animals with vesicular lesions suspected of FMDV infection. To collect the sample, a clinical sign examination was done, the perfect sample was determined, and the sample was collected after the animal was restrained. For this purposefully small study, 120 animals were examined from five study areas, but only 35 were sampled because of the presence of clinical signs. The epithelium tissue was collected from the vesicle, which is freshly ruptured on the tongue and buccal mucosa. Oral swab specimens were also obtained by swabbing under the tongue and an area of contact between the lower gum and the inner surface of the lower lip [9]. In addition, oral swab samples were also collected from fresh lesions in the mouth by sterile cotton-tipped wood applicators. The collected sample was placed in a falcon tube that contains viral transport medium (VTM), which contains glycerol, antibiotics and antimycotics (trademarks VA 20109 USA, and 100x), and phosphate buffer solution [22]. For analysis in the laboratory, collected samples were transported at 4°C by using an icebox with ice packs from the collection site to AHI, Sebeta, Ethiopia. All collected samples followed the sample submission process of AHI and were submitted to the viral isolation laboratory, to isolate, share with the molecular lab, and stored at -80°C until processed.

2.3. Sample Processing

All collected samples were processed at the Animal Health Institute (AHI), formerly the National Animal Health Diagnostic and Investigation Center (NADHIC), at the viral serology lab, the cell culture laboratory, the molecular biology laboratory, and the viral isolation laboratory. Tissue, and swab samples for FMDV were processed manually by sterile pestle and mortar with sterilized sand in the form of grinding with 10% volume of tissue culture medium containing penicillin, streptomycin, and amphotericin solution (trademarks: VA 20109 USA, and 100x). To achieve supernatant suspensions of the processed samples, the sample was centrifuged on a bench centrifuge at 2000 rpm for 10 minutes and filtered through Millipore filter paper of 0.22 µm pore size [25].

2.4. Virus Isolation

A stored cell line known as Baby Hamster Kidney-21 (BHK-21) was taken out of liquid nitrogen, grown on a tissue culture flask by complete Glasgow Minimum Essential Medium (which have twice the concentration of amino acids and vitamins compared to the original Basal Medium Eagle,), and kept at 370°C for 24 hours until 70% cell confluence. On twenty-four-well tissue culture plates, a confluent growth cell line was sub cultured. Onto the cell monolayer, 0.08 ml of the virus suspension from all processed samples was injected. As a negative control, phosphate buffer solutions were added to one well. Then, this was incubated for viral adsorption for 1 hour at 37°C.

Finally, 0.42 ml of GMEM maintenance media (2% fetal calf serum) was added to the infected cell, which was then kept at 37°C and 5% CO_2 in a humidified incubator. While the control cell line was the one that wasn't infected. For 24-48 hours, the plates were checked daily for abrupt contamination and cytopathic effect (CPE) [22]. Then, CPE was assessed based on the detachment of the cell from the flask, disruption of the monolayer cell, and rounding of the cell. Those samples that did not exhibit CPE were kept and subjected to multiple freeze-thaw cycles in virus-adapted cell lines until the third passage [23] said that if no CPE was seen after 48 hours, the sample was deemed to be virus-free. The virus isolate and negative sample are then sent to a molecular biology lab for real-time PCR and RT-PCR virus genome detection as well as viral serology for serotype determination.

2.5. RNA Extraction

The sample was first lysed under highly denaturing conditions to inactivate RNases and to ensure the isolation of intact viral RNA during RNA virus sample extraction in accordance with QIAamp Viral RNA spin methods. The sample was then placed onto the QIAamp Mini spin column after the buffering conditions were modified to provide the best possible binding of the RNA to the QIAamp membrane. Contaminants were effectively removed in two phases using two distinct wash buffers after the RNA attaches to the membrane. According to the manufacturer's instructions for the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) (Lot number, 160036446) and as stated by [19]. FMDV detection in real-time RT-PCR.

Real-time RRT-PCR was used to evaluate the extracted RNA samples, specifically the 3D regions of the RNA viral detection using the FMD virus genome. The 3D probe (5'-6-FAM-TCC TTT GCA CGC CGT GGG AC TAMRA-3') created by [9] was utilized along with the forward primer (5'-ACT GGG TTT TAC AAA CCT GTGA-3'), reverse primer (5'-GCG AGT CCT GCC ACG GA-3', and 3D probe. 39 samples, including two positive and two negative controls, were used in a reaction total volume of 17 l to perform the one-step real-time RT-PCR for FMD.

Extracted template [31] sample on the master mix plate was added after the prepared solution was dispersed on a plate and mixed by a spinner before being sealed with adhesive film. Then, in accordance with the instructions for the Qiagen one-step RT-PCR kit, insert the device into the thermal cycler machine's slots.

It required 50 cycles to complete all amplification processes [19], after which the results were read by an applied biosystem 7500 real-time PCR thermocycler [24], and positivity was calculated using the Ct value.

2.6. Virus Detection with Traditional PCR

Extraction of template RNA from the test or control

sample, reverse transcription of the extracted RNA, PCR amplification of the RT product, and detection of the PCR products by agarose gel electrophoresis are the three sequential steps that make up a convectional PCR assay [23]. For a total of twenty-eight samples, including two positive and two negative controls, the master mix solutions were created. The reverse primer (5'-CCA GTC CCC TTC TCA GATC-3') and forward primer (5'-GCC TGG TCT TTC CAG GTCT-3') were created by the research [27] and predicted the size of the PCR.

2.5 µl l of RNA template mixed with control was added to 22.5 l of the prepared master mix for real-time PCR in order to detect the genome of the positive virus. In an Applied Biosystem 2720 thermal cycler PCR machine, RNA was amplified using a Flex cycler for initial reverse transcription at 50°C for 30 minutes, PCR activation at 95°C for 15 minutes (denatures cDNA template), followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minute in Applied Biosystem 720 thermal cycles PCR machine.

2.7. Gel Electrophoresis

The 1.5% agarose gel that had been prepared and stained with 18 l of SYBER safe was used to evaluate the PCR results. Then it was added to a gel tray with combs to create loading holes, and it was left there for 20 minutes until it set. Each well was then loaded with a volume of 10 l of loading dye mixed with PCR product before a 6 l molecular marker (Ladder) with a minimum of 150 bp was added. The target bp positive result is shown by the existence of a 328bp band corresponding to FMDV partial sequence in the Vp1 region of the genome. Electrophoresis was done for one hour at 100v and 110mA, after which the DNA fragments were seen by Gel documentation machine (Bio-rad).

2.8. Serotype identification virus by antigen detection Sandwich ELISA

By using sandwich ELISA (Lot number F19070/20, IZSLER: Brescia, Italy) and specific combinations of anti-FMDV monoclonal antibodies (MAbs), utilized as coated and conjugated antibodies, the serotype of FMDV was determined. Six different FMD virus serotypes, including types O, A, C, Asia1, SAT1, and SAT2, were intended to be detected and typed with the kit. A pan-FMD test was also added in the kit to supplement the specific typing and to find FMD viruses that might have eluded binding to certain serotype-specific MAbs. This test can identify isolates of serotypes O, A, C, Asia1, and some of the SAT serotypes. With one positive and one negative control for each serotype, the micro plates (Batch number: PlAg3/2020) were supplied with captured MAbs to detect 10 samples at once. The ELISA microplate captured by the corresponding cached MAb already contained the controls. According to the manufacturer's instructions, the test was conducted. The confirmation Sandwich ELISA antigen detection criteria: According to the manufacturer's manual, a positive virus was defined as having an OD of "A" >0.1, "O" >0.1, "SAT-1 >0.1," and "SAT-2 >0.1."

2.9. Data Analysis and Management

The characteristics of the sampled cattle were collected during sample collection and the data from laboratory tests, including molecular detection, virus isolation by cell culture, stereotyping FMDV by antigen detection ELISA, and stereotyping FMDV by antigen detection ELISA, were entered into Microsoft Excel before being analyzed using Statistical Package for Social Sciences (SPSS) software, version 20, to summarize the results.

3. Results

3.1. Field Clinical Examination of the Animals

Most samples taken from epithelial tissues (foot, gum, and tongue) of all examined cattle showed various clinical signs, including raised body temperature, vesicular abrasion, oral pain, and vesicular lesions. 35 (29.1%) of the 120 cattle who were physically evaluated for six outbreaks displayed the normal clinical symptoms (Table 2). Lameness is caused by foot lesions, namely erosions on the coronary bands and interdigital spaces. This field inquiry did not yield any fatalities.

Table 2. *Sample Types Collected in Field Investigation and their Proportion.*

District	Kebele	No. sample	Tissue	Swab
Bishoftu	Danbi	5	(2) 40%	(3) 60%
Sululta	01	16	100%	-
Sebeta	02	4	100%	-
Dima Guranda	01	6	(3) 50%	(3) 50%
Bole	09	4	(1) 20%	(3) 80%
	Total	35	(26) 74.2%	(6) 25.7%

3.2. Virus Isolation

To test for CPE, 35 samples of processed and filtered viral suspensions were injected on the BHK-21 cell line. Effects such as cell lysis, aggregation formation, and separation from the culture tubes, which release the virus in the culture suspension, were seen. 24 (68.75%) of 35 clinical samples had CPE, while the remaining 11 (31.4%) samples did not. In contrast to the negative control, where the cell line was not detached, the BHK-21 cell line was detached from the tissue culture plate, aggregated, and dead cell suspended in the wells, indicating a positive result.

3.3. Detection of FMDV by Real-Time RT-PCR

The original samples from the 24 (68.75%) harvested tissue cultures that show CPE were used for real-time RT-PCR, whereas the 11 (31.4%) original samples were used for RNA extraction. By utilizing FMDV-specific primers in RT-PCR, 24 samples from cell suspension were found to be positive for the FMDV genome as opposed to the 11 (31.4%) original samples that were negative.

3.4. Detection of FMDV by Conventional PCR

The FMDV VP1 region was defined using conventional PCR on all 24 samples that had been detected as harboring FMDV by diagnostic PCR or RT PCR. 24 of them displayed a band with a width of roughly 328 bp on a 1.5% gel (Figure 2).

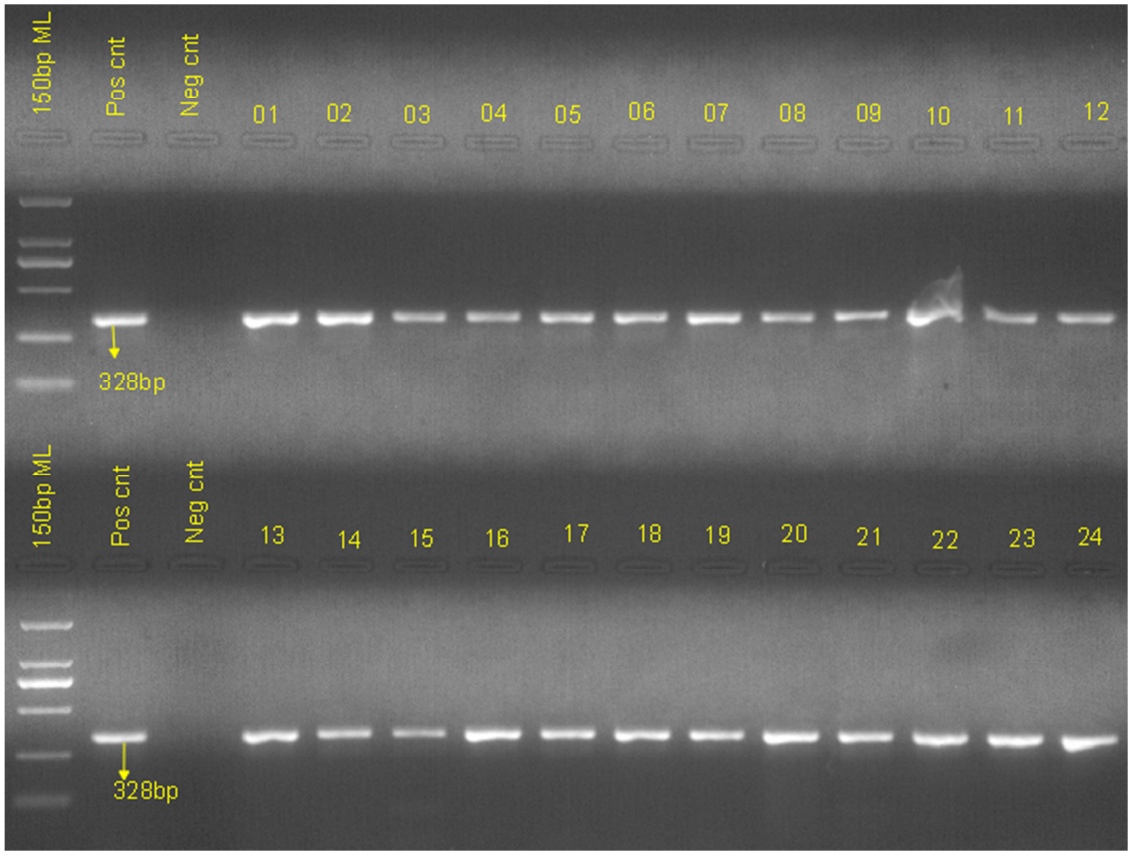

Figure 2. *Gel Electrophoresis Result of FMDV Genome Detected by RT PCR.*

Two primer sets, FMDV (F) and FMDV, focused on the 328 bp 5 UTR coding region of the virus (R). The numbers from 1 to 24 are examples of codes, and ML stands for the molecular ladder. All upper and lower coded 1-24 were positive amplified RNA product FMDV, while columns 1, 2, and 3 were molecular ladder, a positive control, and a negative control, respectively.

3.5. Identification of FMDV Serotype by Antigen Detection

This study discovered four serotypes from central Ethiopia using Sandwich ELISA: O, A, SAT-1, and SAT-2. Serotype O dominated, followed by serotypes A, SAT2, and SAT-1, in that order. Serotype 'A and O' samples came from Sebeta, 'O' samples came from Tafki, 'A and O' samples came from Dima Guranda, 'SAT-1 and SAT-2 samples came from Bishoftu,' 'O' samples came from Bole, and 'A and O' samples came from Sululta. In summary, this study's detection of SAT-1 and SAT-2 viruses in central Ethiopia resulted in the identification of 87.5 percent "O," 25.0 percent "A," 12.5% "SAT" viruses, and 12.5% "SAT-2" (Table 3).

Table 3. *Foot and Mouth Disease Virus Serotypes and Molecular Detection of the Genome.*

Sample collection site	No. sample	RT-PCR	Serotype
Bishoftu (01)	05	3	SAT-2 and SAT-1
Dima guranda	06	4	O and A
Sebeta	04	3	A and O
AA, Bole	04	3	O
Sululta	*16*	11	O
Total	35	24	

4. Discussion

Only 35 (29.1%) of the 120 clinically assessed animals were submitted to sampling in this study out of a total of 120 only 9 mouth swabs and 26 epithelial tissues were taken from this. Clinical symptoms that were seen included salivation, limpness, lesions in the mouth and nose, tongue ulcers, and dental pads on some of them. Lameness is brought on by foot lesions, namely erosions on the coronary bands and

interdigital areas. This outcome is consistent with earlier studies [21, 29, 30], which found that, respectively, 25.58%, 28.2%, and 28.8% of animals displayed clinical symptoms during an FMD outbreak.

This study also attempted to use diagnostic PCR to find the virus that was attacking the 3D genome. From a total of 35 samples, the FMD viral genome was found in 24 (68.57%) of them (16.11-30.03 cycle thresholds value), but not in 11 (31.4%). That bovine epithelial tissue samples had lower Ct values than oral swab samples may indicate a higher degree of viral RNA concentration is discovered in epithelial samples, according to Shimels T. [29] epithelium tissue 15.06 ct value and oral swab 30.19 ct value.

The WOAH reports that epithelial tissue samples contain more FMD viral RNA than vesicular and swab samples do. In this investigation, oral swabs had a greater 12.5% (n=3) ct value compared to epithelial tissues, which had a lower 29.16% (n=7) ct value. This result was consistent with the findings [32] of positive epithelial tissues in 22.2% (n=6) of samples from epithelial tissues and lower Ct values in 6.4% (n=6) of vesicular fluid samples and 7.4% (n=2) of swab samples, respectively.

The same result of 24 (68.57%) was obtained when the samples that tested positive with RT-PCR were rechecked with RT-PCR (samples show band about 328bp). This briefly demonstrates both approaches' accuracy and the fact that the samples tested positive for FMDV genomes. This conclusion is supported by earlier findings from [31] at Northern and Central Ethiopia, where (67.4%) samples were discovered to be positive for FMDV by convectional RT-PCR.

In this discovery, four FMD virus serotypes—O, A, SAT-2, and SAT-1—were isolated from the various outbreaks. Serotyping results showed that serotype 'O' (87.5%) and serotype 'A' (25%, respectively) viruses were the most prevalent in the research area. This is in line with the conclusions [5, 11, 18, 21], which reported serotype O as a dominant serotype in Ethiopia and throughout the world. [15] also stated that serotype 'O' is the dominant serotype circulating in the world. However, he disputes the findings [31], which claim that serotype 'A' was widely distributed in Ethiopia.

The second dominant circulating serotype in Sululta, Dima Guranda, and Sebeta town according to this study serotype was "A" (25%) [30, 21], who reported Serotype 'A in central Ethiopia, support this as well. Following serotype A, serotypes SAT-1 (12.5%) and SAT-2 (12.5%) were also discovered in the same outbreak. This is consistent with research [7, 34]). All suspect samples injected on BHK-21 confluent cell line 24 (68.5%) in this finding had a cytopahtic impact. Cell separation from the culture flask, aggregate development, rounding, and cell lysis were the telltale signs of this CPE. According to Huang [12], Negussie [21], and Tesfaye [31], the cytopathic effect was demonstrated by the rapid demise of the BHK-21 cell line, the infected cells' rounded and sloughing appearance, and their separation from the wall of the cell culture flask. The lack of CPE in some samples 11 (31.4%) could be due to the virus losing vitality during transportation and a delayed outbreak report.

5. Conclusion and Recommendations

In this observation, all questionable samples that were put into BHK-21 confluent cell line 24 (68.5%) displayed a cytopahtic effect. The hallmarks of this CPE included cell lysis, aggregation formation, rounding, and cell separation from the culture flask. The cytopathic effect was confirmed by the quick death of the BHK-21 cell line, the rounded and sloughing look of the infected cells, and their separation from the wall of the cell culture flask, according to Huang *[12]*, Negussie *[21]*, and Tesfaye *[31]*. A delayed outbreak report and the virus losing vitality during transportation may be to blame for the lack of CPE in some samples 11 (31.4%). We recommend that regular outbreak investigation encompassing all the regions and districts be done in order to identify the circulating serotype and that further sequence based analysis of the topotypes be done. This will help with the prevention and control of the diseases through vaccination.

Authors' Contributions

DN: created the study's concept and layout, took part in data gathering, laboratory analysis, and wrote the report. FW: Contributed to the drafting, editing, data interpretation, and revision of the manuscript. AA: Participated in the sample's molecular analysis in the lab. AM: I took part in sample preparation and laboratory virus serotyping analyses. Participated in the writing and editing of the manuscript were DS, TB, and BY. The final manuscript was read and approved by all writers.

Ethics Approval and Consent to Participate

The National Animal Health and Diagnostic and Investigation Center granted ethical approval (ethical clearance) (ARSERC 003/20, December 22, 2020), and the activities were carried out. Before beginning the activity, animal owners were also verbally queried and the best regard for the animals was only given if the owners were willing. Only veterinary professionals and those with extensive experience gathering clinical samples for foot and mouth disease were asked to participate in the activity.

Conflict of Interest

The authors declare that they have no competing interests.

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Availability of Data and Materials

All datasets that have led to the drawn deductions in the manuscript are herein presented in the paper.

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