

ORIGINAL ARTICLE

Outbreak investigation, molecular detection, and characterization of foot and mouth disease virus in the Southern part of Bangladesh

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ABSTRACT

Objective: The objective of the study was to investigate Foot and Mouth Disease virus (FMDV) outbreak in cattle in the Sarankhola Upazila under Bagerhat district of Bangladesh with isolation, identification, and molecular characterization of FMDV during April 2018.

Materials and Methods: This Upazila is located at southern border of Bangladesh and surrounded by mangrove forest Sundarban. The outbreak investigation team collected epidemiological data from outbreak location. In addition, the team collected a total of 30 (15 calves, 15 adult) tongue epithelial tissue samples from a clinically FMD-affected cattle. The confirmation of FMDV and its three serotypes (A, O, and Asia-1) was performed by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). An amplified product of the VP1 region of FMDV genome was sequenced by Sanger sequencing method after cultivation and reconfirmation of FMDV into the BHK21 cell line. Genetic variability was studied by constructing a phylogenetic tree.

Results: The investigation survey was carried out in overall 8,393 (8,393/15,580; 53.89%) cases including 3,050 (3,050/8,393; 36.34%) cases in calf and 5,343 (5,343/8,393; 59.77%) cases in adult cattle. The overall case fatality rate (CFR) was recorded as 2.27% (354/15,580) with significantly highest CFR in the calf (71.46%; 253/354) compared to an adult. The collected all 30 samples found with FMDV positive and mixed infection of all samples with serotype Asia-1 and serotype O were observed. In BHK 21 cell line, the eight FMDV positive samples showed a typical cytopathic effect during the third passage. Finally, DNA sequence data of two isolates found closely related with the isolates of bordering country India and Myanmar.

Conclusion: The investigation identified the risk factors involved in an outbreak of FMDV, namely, sharing the common paddy land after harvesting, no FMD vaccination, the interaction between cattle and wildlife, and cross bordering movement.

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Introduction

Foot and mouth disease (FMD) is the most widespread viral disease of cloven-hoofed animal and locally called as “Khura Rog” in Bangladesh [1–3]. It is caused by Foot and mouth disease virus (FMDV), a virus under genus Aphthovirus and family Picornaviridae with single-stranded and positive-sense RNA genome. An icosahedral capsid layer surrounded the viral genome that composed of four structural proteins, namely, VP1, VP2, VP3, and VP4 with 60 copies of

each protein. Totally seven immunologically distinct serotypes were identified including Types O, A, C, Asia 1, SAT 1-3 (South African territory) [4,5], and multiple subtypes circulating over the world [4].

In Bangladesh, serotype O was only circulated since 2000 [6] but the current study revealed that the three serotypes (A, O, and Asia-1) are being circulated in Bangladesh [7,8]. The prevalence of these three serotypes was 31% of both serotypes O and Asia-1, and 7% of serotype A, also

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found with mixed infection with serotype 7% O-Asia-1 and 2% serotype O-A [7]. Another researcher demonstrated that serotype O was responsible for 82% outbreak and its show dominance over serotype A and Asia-1 [9]. Three bordering countries of Bangladesh also circulated the same serotypes [10]. The largest border sharing country of Bangladesh is India where the prevalence of circulation serotypes O, A, and Asia-1 is 80%, 8%, and 12%, respectively [11].

FMDV is highly contagious and has a multi-route transmission capacity mostly direct contact with saliva, fomites, feeding, and watering places [12]. Also, it has transboundary potential that is responsible for the outbreak [13]. Livestock movements in inter-country are important factors for spreading the virus [14]. In Bangladesh, FMD prevalence was found in throughout the year but increases the incidence after two major festivals (Eid-ul-Fitarand and Eid-ul-Azha) [9]. It is the cause of increasing the animal movement throughout the country during this festival [15,16].

Now FMD is endemic in Bangladesh and outbreaks in every year [9,17]. It has a significant economic impact by reducing milk, meat production, and increasing calves mortality, also recovered animal losses in productivity [18,19]. Every year, the globally total impact of FMD is US\$ 6.5 to 21 billion for endemic countries and >US\$1.5 billion for FMD free countries [20].

In this study, an outbreak investigation was done and confirmation of FMDV infection by using clinical signs and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of the tongue epithelial tissue sample. Then find out the genetic relationship of outbreak FMDV by phylogenetic analysis. Finally, addressed the risk factors that are responsible for this outbreak.

Materials and Methods

Ethical approval

All the protocols used in the study were approved by the Institutional Animal Experimentation Ethics Committee of the Bangladesh Livestock Research Institute, Savar, Dhaka.

Study area

In April 2018, the FMD outbreaks in cattle were suspected in three Unions (Dhansagar, Khontakata, and Royenda) of Sarankhola Upazila of Bagerhat district. Sarankhola Upazila located at the south border of Bangladesh and surrounded by world largest mangrove forest Sundarban and bank of Balaswar River that is directly connected with Bay of Bengal (Fig. 1). It is located geographically at 22.2917°N 89.7917°E with a total area of 756.61 km². The Upazila has a cattle population of total 15,580 and there

is a conventional type of farming system. Where cattle are reared exclusively on an open field grazing pattern and general husbandry. Farmers have no experience in modern veterinary practices like vaccination and deworming technology. It has good access possibility to interact between wild animals and livestock due to the nearest Sarankhola Range of Sundarban.

Clinical sign and symptoms

The established clinical signs were observed to clinically suspect the FMD infection and following clinical signs were observed: primarily pyrexia up to 40°F, followed by the growth of vesicles on the whole oral cavity, including the tongue epithelium, gum, hard palate, dental pads, and muzzle, resulting in drooling of salivation. As vesicles developed on the teats of lactating cow, mastitis developed with other signs. Vesicles also developed on the inter-digital cleft and maggots grow when flies sited over it so that animal shows lameness. In the case of calf, high mortality with or without shows clinical symptoms.

Sample collection

Total of 30 tongue epithelial tissues of aphthous ulcers was collected from 30 (15 calves and 15 adults) clinically suspected cattle as 10 samples from each Union aseptically. The epithelial tissue samples were collected according to the guideline of OIE Terrestrial Manual [3]. Then the samples were shipped from the outbreak site to FMD Research Laboratory, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka in virus transfer media (VTM).

Preparation of VTM

The VTM prepared by Brain Heart Infusion Broth media with 1000,000 IU Penicillin G, Gentamicin, and Amphotericin B.

Epidemiological data collection

To consider the risk factors of outbreaks, a direct survey was conducted through pre-constructed questioner and data were collected from local people, farmers, and local veterinary service authority. Data were collected on the farming system, grazing pattern, housing, geographical location, animal interaction chances, wildlife and domestic animal relation, and animal marketing. Information on the population at risk, case, and case fatality rate also collected. The case fatality rate was calculated by the following formula:

$$\text{Case fatality rate} = \frac{\text{No. of death due to the disease}}{\text{No. of cases}}$$

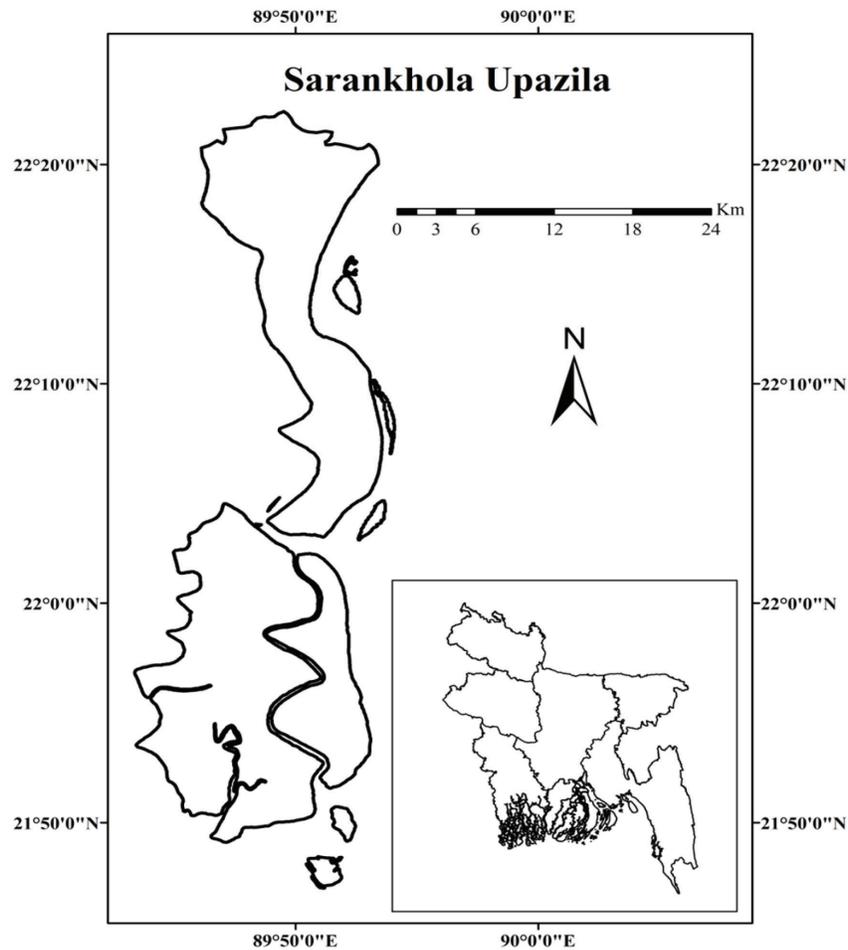


Figure 1. Map showing the outbreak area in Sarankhola Upazila of Bagerhat district of Bangladesh. The map was created by using Geographic Information Systems (GIS) software ESRI ArcGIS version 10.6.1 [42].

Table 1. List of oligonucleotide primers used for universal FMDV and serotyping of FMDV by RT-PCR.

Serotype	Primer name	Primer sequence (5' to 3')	Location	PCR products (bp)	References
Universal	1F	GCC TGG TCT TTC CAG GTCT	5'UTR	328	[40]
	1R	CCA GTC CCC TTC TCA GAT C	5'UTR		
O	P38	GCTGCCTACCTCCTTCAA	1D	402	
	P33	AGCTTGACCAGGGTTTGGC	2B		
Asia-1	P74	GACACCACTCAGGACCGCCG	1D	292	
	P33	AGCTTGACCAGGGTTTGGC	2B		
A	P110	GT(G:A:T:C)ATTGACCT(G:A:T:C)ATGCA (G:A:T:C) AC (G:A:T:C) CAC	1D	732	[41]
	P33	AGCTTGACCAGGGTTTGGC	2B		

Vaccination history

Farmers are not conscious of modern veterinary practices, vaccination, and deworming. So, there was no record of FMD vaccination in the outbreak area.

Detection of FMDV serotypes by RT-PCR test

An aliquot of epithelial tissue samples was ground by pestle and mortar with phosphate-buffered saline (PBS) solution and centrifuged at 3,000 rpm for 10 min to obtain

a 300 µl of supernatant fluid suspension. Then, the total genomic RNA was extracted by using QIAamp® Viral RNA kit (QIAGEN, Germany) following the manufacturer's protocol. For the detection of FMDV, all extracted RNAs were subjected to RT-PCR test using QIAGEN One-Step RT-PCR Kit with 10 pM/sample universal primers. The list of primers used and PCR thermal profile are listed in Tables 1 and 2, respectively. Then the differentiation of serotypes Asia-1, A, and O by using serotype-specific primers. The corresponding amplification site of VP1 gene was visualized by stained 1 µg/ml ethidium bromide into 2% agarose gel in gel electrophoresis. For the test verification, lab positive control of each serotype was used in every step. The GenBank Accession number of lab-positive control was KP119763 (Type-A), KP119758 (Type-O), and KP119757 (Asia-1).

Cell culture for isolation of FMDV

A total of 12 samples were selected for propagating into BHK-21 cell line. Epithelial tissue samples were ground by sterile pestle and mortar with an equal volume of sterilized PBS with antibiotics and then filtered through a syringe filter (0.2 µm). Then, 0.5 ml of filtered virus suspension was inoculated on the previously prepared BHK-21 monolayer cells containing 25 cm² tissue culture flasks. Then incubated at 5% CO₂ and 37°C temperature for 48 h. The cultured cells are observed twice for cytopathic effects (CPEs). Then remove the virus from infected cells by freeze-thawing three times and centrifuged the supernatant fluid at 5,000 rpm for 5 min. Finally, verified the presence of FMDV in the supernatant fluid by RT-PCR test.

Sequencing and phylogeny analysis

The amplified region of FMDV genomic RNA by RT-PCR was sequenced on both forward and reverse strands as previously described by Knowles et al. [21]. The SeqMan software (DNASar, Lasergene v.8) was used to assemble and verify the sequence data, and finally, nucleotide

sequences were aligned by using BioEdit version 7.2.5 [22] and Clustal W [23]. Then reconstruct the alignments by Maximum Likelihood phylogenetic tree with TN93+G+I model existing in MEGA X [24]. The robustness of the phylogenetic tree topology was verified with 1,000 bootstrap replicates.

Statistics

The record of the outbreak investigation, sample size calculation, FMD case, case fatality rates, and prevalence was analyzed by using Epi Info™ version 7 (Epi Info™, CDC, USA).

Results

On the basis of clinical signs and physical examination, overall 8,393 (53.87%) cattle were found FMD positive cases. Where 3,050 (36.34%) cases were in calf and 5343 (59.77%) cases were in adult cattle. Location wise FMD cases were found in 2,524 (54.98%), 4,501 (70%), and 1,368 (30%) in Dhansagar, Khontakata, and Royenda Union, respectively. The highest 1,890 (41.99%) FMD cases were found in calves in Khontakata and 1,843 (73.02 %) in adult in Dhansagar Union (Table 3).

In the outbreak area, the overall case fatality rates (CFR) on FMD were 354 (2.27%). But devastating CFR found in calves was 253 (71.46%). Total CFR in adult cattle was 102 (28.81%). The CFR was highest 43 (79.63%) in calves in Royenda and lowest 154 (68.44%) was in Khontakata Union. In the case of adult cattle, highest 72 (32%) and lowest 11 (20.37%) CFRs were in Khontakata and Royenda Union, respectively (Table 3).

A total of 30 randomly selected epithelial samples were collected from three locations of outbreak area for confirmatory diagnosis by RT-PCR test. At first, all the 30 samples were screened for FMDV by the universal primer of 5' UTR location of VP1 gene and found all samples are positive. Then differentiation of three serotypes (A, O, and Asia-1) made individually and found all 30 samples were positive

Table 2. PCR conditions for RT-PCR for both universal FMDV and serotyping of FMDV.

Sl. No.	Steps	Name of the primers	
		1F and 1R	P33, P38, P74, P110
1	Reverse transcription	50°C for 30 min	50°C for 30 min
2	Initial PCR activation	95°C for 15 min	95°C for 15 min
3	Number of cycle	30	35
4	Denaturation	94°C for 1 min	94°C for 15 sec
5	Annealing	55°C for 1 min	55°C for 1 min
6	Extension	72°C for 2 min	72°C for 30 min
7	Final extension	72°C for 7 min	60°C for 6 min
8	Hold at	4°C	4°C

Table 3. The FMDV outbreak investigation data.

Sl. No.	Location	Total cattle population	FMD cases			Case fatality rate		
			Calves (1–24 months)	Adult	Total	Calves	Adult	Total
1	Dhansagar	4590	681 (26.98%)	1843 (73.02 %)	2524 (54.98%)	56 (74.66%)	19 (25.33%)	75 (2.97%)
2	Khontakata	6430	1890 (41.99%)	2611 (58%)	4501 (70%)	154 (68.44%)	72 (32%)	225 (5%)
3	Royenda	4560	479 (35.01%)	889 (64.98%)	1368 (30%)	43 (79.63%)	11 (20.37%)	54 (3.95%)
	Total	15580	3050 (36.34%)	5343 (59.77%)	8393 (53.87%)	253 (71.46%)	102 (28.81%)	354 (2.27%)
	<i>p</i>		0.000*	0.001*		0.000*	0.001*	

*Significant at the 0.01 level.

for both serotypes Asia-1 and O but absent for serotype A (Table 4).

Eight out of 12 epithelial tissue samples were shown CPE during the third passage on BHK21cell culture (Fig. 2). The virus has also shown a positive result when RT-PCR was done from tissue culture fluid. Finally, these isolated three samples were considered for sequencing of the VP1 gene. The obtained sequences have been submitted in the NCBI GenBank under the accession numbers MK056240 (BLRI/BD/435.2), MH704868 (BD/BLRI/434.3), and MH699984 (BD/BLRI/435.4).

The phylogenetic tree was built by comparing the sequences with other sequences of Bangladesh and neighboring country collected from GenBank database (Fig. 3). All three isolates were found genetically closely related to isolates from the bordering country India and Myanmar. On the other hand, isolates BLRI/BD/435.2 (MK056240.1) and BD/BLRI/435.4 (MH699984.1) were closely related with isolate MG603265.1 (99%) from Myanmar and isolate AF390718.1 (70%) from India, respectively.

Discussion

The outbreak of FMD should be addressed at an early stage for effective control measures and prevent further transmission stated by Longjam et al.. The FMD virus can be transmitted very rapidly from the onset of shedding to surrounding areas due to its airborne transmission pattern [25].

The RT-PCR detected that all 30 clinical samples were tested for mixed infection with serotypes Asia-1 and Type O. Researchers demonstrated that the infection with multi serotypes of FMD virus increased the severity of infection as well as increased the mortality rates [26]. Serotypes A, Asia-1, and O are circulated in Bangladesh [6–8,27]. On the other hand, serotypes Asia-1 and O are more prevalent that is 31% and serotype A is less prevalent that is 7% [7].

Giasuddin et al. [7] recorded the highest (7%) prevalent in the mixed infection with serotypes Asia-1 and O than serotypes A and O in Bangladesh. On the other hand, Siddique et al. [9] revealed that serotype O was responsible for 82% outbreaks in Bangladesh and it is dominant over serotypes A and Asia-1.

According to the case report, it is confirmed that half (53.87%) of the cattle population of Sarankhola Upazila was infected with FMDV, resulting in massive outbreaks of FMDV. The same case was reported by Kouato et al. [28] as 54% case report of FMD outbreaks in Niger from September to October 2013.

The overall case fatality due to FMDV was 354 (2.27%) with no vaccination. The closely related CFR was demonstrated in Ethiopia at 2.8% by Rufael et al. [29] and 2.9% by Negussie et al. [30], and 1.4% in South-East Asia during 2000–2010 reported by Madin [31].

In the case of calves, the CFR was 2.48 times higher than adult focused on the outbreak investigation. It indicated that calves are more vulnerable to FMDV due to the virus can invade into the myocardium of a left ventricle, resulting in necrosis of myocardium. This necrotic myocardium looks like a typical shape of striping like tiger skin, commonly known as ‘tiger heart diseases’ [32]. Sometimes calves can die without any clinical signs due to myocardial dysfunction [33]. The calves were kept intensively with mothers so they are in close contact with fomites, mangers, and other feeding materials [34]. The statement of CFR strongly agreed with Rufael et al. [29] and Negussie et al. [30], they demonstrated 9.33 and 5.56 times more CFR in calves compared with the adult in Ethiopia. Radostits et al. [33] also concluded that the CFR in adult typically 2% and/or up to 20% in calves.

To find out the possible risk factors, questioner surveys were conducted, where the respondent local peoples revealed that FMD becomes epidemic during March–April months, due to after harvesting the paddy all cattle of

Table 4. Description of RT-PCR test results.

Sample ID	Location	Type	Sample type	Results				Comments
				Universal	Asia 1	O	A	
1	Dhansagar	Adult	Epithelium	+	+	+	-	Asia-1+O
2	Dhansagar	Calf	Epithelium	+	+	+	-	Asia-1+O
3	Dhansagar	Calf	Epithelium	+	+	+	-	Asia-1+O
4	Dhansagar	Adult	Epithelium	+	+	+	-	Asia-1+O
5	Dhansagar	Adult	Epithelium	+	+	+	-	Asia-1+O
6	Dhansagar	Calf	Epithelium	+	+	+	-	Asia-1+O
7	Dhansagar	Calf	Epithelium	+	+	+	-	Asia-1+O
8	Dhansagar	Adult	Epithelium	+	+	+	-	Asia-1+O
9	Dhansagar	Calf	Epithelium	+	+	+	-	Asia-1+O
10	Dhansagar	Adult	Epithelium	+	+	+	-	Asia-1+O
11	Khontakata	Calf	Epithelium	+	+	+	-	Asia-1+O
12	Khontakata	Calf	Epithelium	+	+	+	-	Asia-1+O
13	Khontakata	Calf	Epithelium	+	+	+	-	Asia-1+O
14	Khontakata	Adult	Epithelium	+	+	+	-	Asia-1+O
15	Khontakata	Adult	Epithelium	+	+	+	-	Asia-1+O
16	Khontakata	Calf	Epithelium	+	+	+	-	Asia-1+O
17	Khontakata	Adult	Epithelium	+	+	+	-	Asia-1+O
18	Khontakata	Adult	Epithelium	+	+	+	-	Asia-1+O
19	Khontakata	Adult	Epithelium	+	+	+	-	Asia-1+O
20	Khontakata	Calf	Epithelium	+	+	+	-	Asia-1+O
21	Royenda	Adult	Epithelium	+	+	+	-	Asia-1+O
22	Royenda	Calf	Epithelium	+	+	+	-	Asia-1+O
23	Royenda	Adult	Epithelium	+	+	+	-	Asia-1+O
24	Royenda	Calf	Epithelium	+	+	+	-	Asia-1+O
25	Royenda	Calf	Epithelium	+	+	+	-	Asia-1+O
26	Royenda	Adult	Epithelium	+	+	+	-	Asia-1+O
28	Royenda	Calf	Epithelium	+	+	+	-	Asia-1+O
29	Royenda	Adult	Epithelium	+	+	+	-	Asia-1+O
30	Royenda	Calf	Epithelium	+	+	+	-	Asia-1+O

surrounding villagers grazing freely in that paddy field. So, cattle shared the common grazing land and watering points with neighboring villages that increase the chances of an outbreak, the observation in agreement with Cleland et al. [35]. The farmers were not aware of FMD vaccination, and they did not vaccinate their cattle, which is one of the contributing risk factors for FMDV outbreak. To prevent the FMDV in endemic area vaccination by circulating serotypes is an effective tool to combat the outbreak [36]. The bovine immunoglobulin G1 (IgG1) can be released via milk from vaccinated cows that have lifesaving importance to reduce early calves mortality against FMDV [37].

In consideration of the geographical location of Sarankhola Upazila, it has a potential risk of FMDV

transmission from wildlife (ruminants, pigs) to cattle due to it is surrounded by Sundarban forest and close to Sarankhola Range of Sundarban forest [38].

A discussion with local villagers of outbreak area revealed that every week held a cattle market in Sarankhola Upazila. Where cattle interact with cattle of local sellers and traders those were from neighboring countries as cross-border cattle movement by water vehicles. The unsold cattle can carry the infection to the village that is the likely source of emerging serotypes of FMDV in Bangladesh [8]. FMDV has transboundary disease potential and involves in livestock trade pattern due to spreading through multiple routes and hosts [39,43].

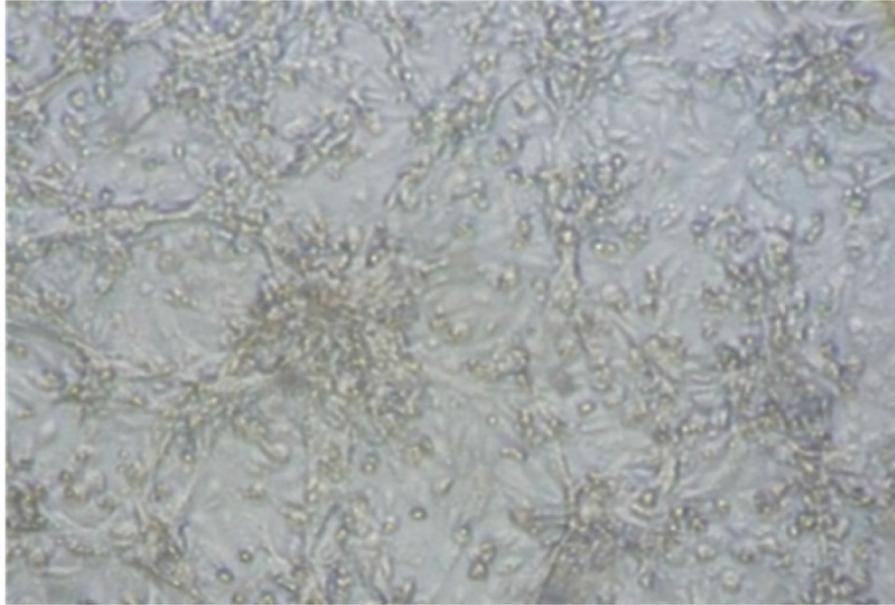


Figure 2. Syncytia formation of BHK-21 cells infected with Foot and Mouth Diseases Virus collected from clinical lesion, observed under 40× objective (Leica DMil, Germany).

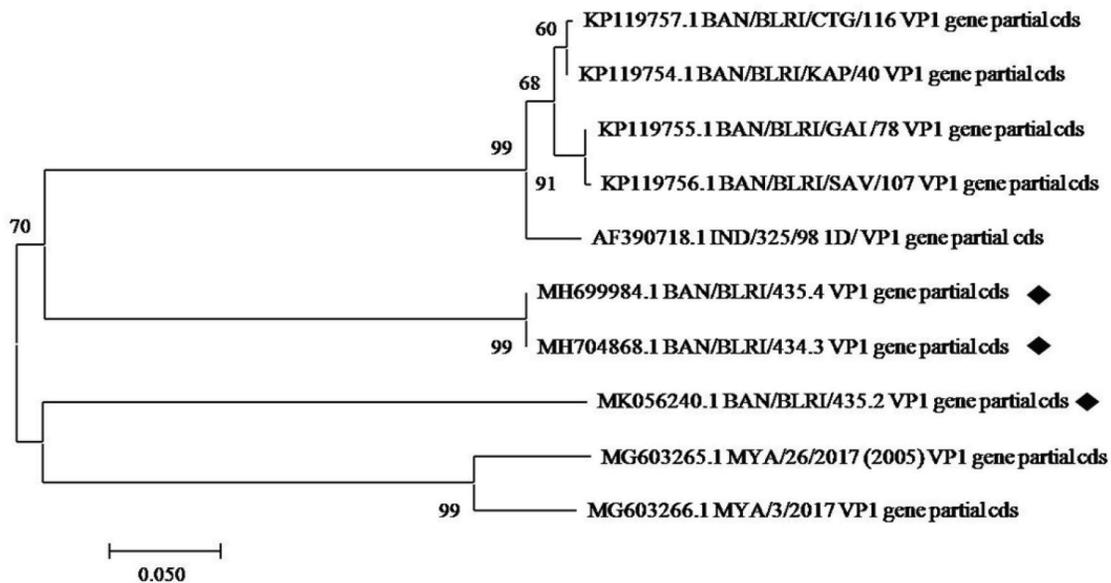


Figure 3. Neighbor-joining tree based on the partial virus protein VP1 coding sequence. The sequences generated for this study are marked with ◆ (filled diamond) symbol.

From the above outbreak investigation, it is recommended that farmers should be confirmed regular vaccination with trivalent FMD vaccine of circulating serotypes and good health management due to the endemic area [9]. Farmers also have to sensitize about the potential risk of FMDV transmission of cattle from wildlife and should avoid

sharing the common grazing land after harvesting paddy. To avoid transboundary transmission, unauthorized animal movement from neighboring countries should be stopped and strictly maintain quarantine protocols. It also recommended that unsold cattle should never be brought back to the farm and isolate the affected animal.

Conclusion

The investigation was conducted during the start of the outbreak of FMDV in Sarankhola Upazila of Bagerhat district, Bangladesh. The total case was found in 8393 and case fatality rate was 2.27%. The calves were more vulnerable than an adult and CFR of calves reached up to 71.46% that was highest CFR in any count. The investigation could not conclude the source of the outbreak but the risk factors and sequence analysis indicated that it could be from interaction with wildlife and cross-border animals of neighboring countries.

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Conflict of interests

The authors declare that they have no competing interests.

Authors' contribution

Md Zulfekar Ali and Eusha Islam designed the experiment and conducted the actual experiments. Both the authors took part in the analysis of data and drafted the manuscript. All the authors read and finally approved the publication of this research work.

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