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Detection of Foot and Mouth Disease Virus (FMDV) in Cloven-hoofed Animals from Different Areas of Bangladesh

Swarna Reza[1*], Salina Malake[1], Abu Musa Al Asari[2], Mohammad Giasuddin[3] and Mohammad Showkat Mahmud[3]

Abstract

Foot and mouth disease (FMD) is considered as one of the key threats to livestock industries worldwide. This investigation reported the circulation and detection of FMDV in sheep, goats and cattle at different regions of Bangladesh and the major risk factors for occurrence of the Foot and Mouth Disease. For this, clinical samples were collected from tongue epithelium, tissue from inter digital space, saliva, feces and milk from sheep, goats and cattle suspected to be infected with FMDV. Four different areas of Bangladesh were selected for sample collection (Savar, Sirajganj, Bandarban and Chittagong). During the study period, all samples were subjected to RNA extraction followed by conventional one step RT-PCR amplification of the VP1 gene, which is the most variable region of FMDV genome. FMD positive isolates were subjected to multiplex RT-PCR using specific primer sets to differentiate FMDV serotypes. There is limited epidemiological data in Bangladesh defining the circulation of FMD virus in sheep and goats population. A total of 145 outbreaks from sheep (90) and goats (55) were reported at Savar area during winter, 2016. Morbidity rate was found 20% and 18.19% in sheep and goats respectively. Whereas, mortality rate was 2.22% and 1.81% in sheep and goats respectively. Detection of circulating FMDV serotypes and disease monitoring of animals entering Bangladesh are crucial components for an effective national FMDV control program in Bangladesh.

Keywords

Foot and mouth disease; Livestock; Investigation; Morbidity; Mortality

Introduction

Bangladesh is known as the most densely populated countries in the South Asia of the world. Livestock keeps important value in the economy of this country, considered to the backbone of agriculture. Foot-and-Mouth disease (FMD) is one of the most devastating viral diseases which is common in cattle, buffalo, sheep and goats, and is one of the threat in the economy of livestock industries [1]. Every year, Bangladesh damages of US$60–150 million for this devastating viral diseases [2]. The Clinical sign of this disease is characterized by fever and vesicular eruption in the mouth, muzzle, foot, teats and other hairless soft areas of the body and highly infectious and contagiousfor livestock animals [3] though humans are scarcely infected by foot-and-mouth disease virus [4]. FMD causes low mortality rate in adult animals but the destroying effect causes weight loss, decrease in milk production, reproductive failures and loss of draught power resulting in reduced productivity in livestock animals. The clinical diseases varies between species to species, breed of the animal affected, and serotype and strain of FMD virus (FMDV) [5,6].

Foot-and-Mouth disease is caused by Foot-and-mouth disease virus (FMDV) is a picornavirus, the prototypical member of the genus Aphthovirus of the family Picorna-viridae [7]. FMDV consists of a single-stranded, plus-sense RNA genome of approximately 8,500 nucleotides surrounded by a protein capsid [8]. The FMDV genome is classified into: (a) 5' untranslated region (5'-UTR) containing non-coding nucleic acids that made of many regulatory elements; (b) protein coding region (ORF) coding for both structural and nonstructural proteins; and (c) 3' UTR or non-coding region and has a poly A tail. There are four structural proteins namely VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A] and 8 non-structural proteins; L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D [9]. FMDV RNA-dependent RNA polymerase lacks proof reading ability, so the genome is subjected to a high rate of mutation. The virus exists as seven immunologically distinct serotypes that is O, A, C, Asia 1, Southern African Territories SAT 1, SAT 2 and SAT 3 with multiple subtypes within each serotype. Out of seven serotypes of FMDV, three different types (O, A and Asia-1) are prevalent in Bangladesh.

Materials and Methods

Sample collection:A total of 145 outbreaks from sheep (90) and goats (55) were reported at Savar area and a total 5 clinical samples were collected from Sheep and goats. 45 clinical samples were collected from cattle from Savar, Chittagong, Sirajganj and Bandarban during various FMD outbreaks in Bangladesh, 2016. Among 90 sheep 50 were females and 40 were male where female goats were 36 and male goats were 19.Samples preferably included tissue from the inter digital space (Hoof tissue), tongue epithelium, saliva, milk and feces. Samples were collected in the viral transport medium (VTM) containing 0.04 M phosphate buffer (pH 7.2-7.6), 1% phenol red, antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml), and equal volume of glycerol. The samples were transported to laboratory in portable cooler within 24 hrs and stored at -80 degree C until processed.

Preparation of Samples (Inoculum)

Piece of the tissue was removed out from the glycerol buffer, dried with sterile tissue paper. Approximately 1-2 gm tissue was weighted by an electric balance and homogenized with sterilized mortar and pestle. Add phosphate buffered saline (PBS) 5 times of the tissue weight measured to prepare 20% suspension. In case of saliva and milk sample, collected sample is mixed with PBS 5 times of the volume of sample to prepare 20% suspension. The suspension of each of the sample was then centrifuged at 3000 rpm for 10 minutes maintaining temperature at 4°C.The supernatant was collected in 50ml falcon tube, marked properly and stored at -80°C till further processing.

Extraction of Viral RNA

Viral RNA was extracted from each sample with RNeasy® Mini

***Corresponding author:** Swarna Reza, Department of Biotechnology &Genetic Engineering, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh, Email: reza.swarna@gmail.com

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Kit reagent (QIAGEN, Netherlands). After RNA extraction, the target sequence was amplified by using QIAGEN® one step RT-PCR kit (QIAGEN Inc., Netherlands). In this method reverse transcription and PCR are carried out sequentially in the same tube.

RT-PCR

The oligonucleotide primer for the detection of FMDV and FMDV serotypes were used from the 1D, 2B and 5' UTR regions of the viral genome as published [10,11]. More specification of these primers is listed in the Table 1. All oligonucleotide primers were synthesized by Sigma, USA. In uniplex PCR, only one set of primer is used by targeting a single gene. In this study uniplex PCR assays were performed to detect the 5'UTR, common for all serotypes. The samples were subjected to One Step RT-PCR using universal primers, IF and IR, which identified whether the virus belonged to FMD group or not. The primer pair IF and IR were selected with reference to the conserved sections of the 5'UTR of FMD virus genome and was intended for identification of all seven serotypes which amplifies a 328 bp fragment (11). RT-PCR amplified products were subjected to horizontal gel electrophoresis in 2% agarose gel in 1X TBE (Tris-borate EDTA) buffer at room temperature at 100 volt (50 mA) for 30 min Tables 2 and 3.

Results

A total of 145 cases from sheep (90) and goats (55) were recorded at sheep and goats farm of Savar area. Morbidity rate were found 20% (27) and 18.19% (10) in sheep and goats respectively (Figure 1). Whereas, mortality rate were 2.22% (2) and 1.81% (1) in sheep and goats respectively (Figure 1).

Total 5 (Table 4) samples were collected and tested for the presence of FMDV RNA. Four samples representing 80% of the total samples were confirmed as positive for FMD. FMD positive isolates were subjected to multiplex RT-PCR using specific primer sets. No isolates were positive for O, A, C, Asia 1 type (Figure 2a and b).

All of the isolates were subjected to PCR based detection of 5' untranslated region (UTR) of the FMDV genome using universal primer set 1F and 1R (QIAGEN One Step RT-PCRKit-QIAGEN, Netherlands). Total 45 samples of cattle were collected and tested for the presence of FMDV RNA (Table 5). Twenty eight samples representing 62% of the total samples were confirmed as positive for FMD (all PCR data not provided). Total twenty seven (27) samples were collected from Sirajganj region and 63% isolates were positive for FMD. Four samples were collected from Savar and and 100% isolates were positive for FMD, Four (4) samples were collected from Chittagong region and 75% isolates were positive for FMD. Out of the ten (10) samples collected from Bandorban region 40% were FMD positive.

RT-PCR using specific primer sets (Table 1) made possible to detect different serotypes of FMDV. In this study two serotypes; O type and Asia1 type have been identified. Among these isolates serotype O and Asia 1 accounts for about 50% and 36% of the total isolated positive samples respectively (Figure 3a). Four (14%) samples

Table 1: Primers used in this study for the detection of specific genes in FMDV.

FMDV Serotype	Primer name	Sequence (5' to 3')	Location	References
All serotypes	1F	GCCTGGTCTTTCCAGGTCT	5'UTR	[10]
	1R	CCAGTCCCCTTCTCAGATC	5'UTR	
	P33*	AGCTTGTACCAGGGTTTGGC	2B	
O	P38	GCTGCCTACCTCCTTCAA	1D	[12]
C	P40	GTTTCTGCACTTGACAACACA	1D	
Asia 1	P74	GACACCACTCAGGACCGCCG	1D	
	P75	GACACCACCCAGGACCGCCG	1D	
	P76	GACACCACACAAGACCGCCG	1D	
	P77	GACACGACTCAGAACCGCCG	1D	
A	P110	GT(G:A:T:C)ATTGACCT(G:A:T:C) ATGCA (G:A:T:C) AC (G:A:T:C) CAC	1D	[9]

*P33 primer reverse (downstream) for all.

Table 2: Composition of reaction mixture for FMDV simplex PCR and multiplex PCR.

Component concentration	Volume/reaction (simplex PCR)	Volume/reaction (multiplex PCR)
RNase-free water	13.5 µl	11.5 µl
5x QIAGEN OneStep RT-PCR Buffer	5.0 µl	5.0 µl
dNTP Mix (containing 10 mM of each dNTP)	1.0 µl	1.0 µl
Forward Primer (100 pmol/µl)	1.0 µl	-
Reverse Primer (100 pmol/µl)	1.0 µl	-
Upstream Primer: P38 (100 pmol/µl)	-	0.5 µl
Upstream Primer: P40(100 pmol/µl)	-	0.5 µl
Upstream Primer: P74 (100 pmol/µl)	-	0.5 µl
Upstream Primer: P75 (100 pmol/µl)	-	0.5 µl
Upstream Primer: P76 (100 pmol/µl)	-	0.5 µl
Upstream Primer: P77 (100 pmol/µl)	-	0.5 µl
Upstream Primer: P110 (100 pmol/µl)	-	0.5 µl
Consensus Downstream Primer: P33 (100 pmol/µl)	-	0.5 µl
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 µl	1.0 µl
Template RNA	2.5 µl	2.5 µl
Total volume	25.0 µl	25.0 µl

Table 3: The thermal profile for the one step RT-PCR with different primmer sets.

Points/Steps	1F:1R	P33: P38: P40: P74: P110
	(Simplex PCR)	(Multiplex PCR)
Reverse transcription	50˚C for 30 min	50˚C for 30 min
Initial PCR activation	95˚C for 15 min	95˚C for 15 min
Number of cycles	30	35
Denaturation	94˚C for 1min	94˚C for 15 sec
Annealing	55˚C for 1min	55˚C for 1min
Extension	72˚C for 2 min	72˚C for 2 min
Final extension	72˚C for 7 min	60˚C for 6 min
Hold at	4˚C	4˚C

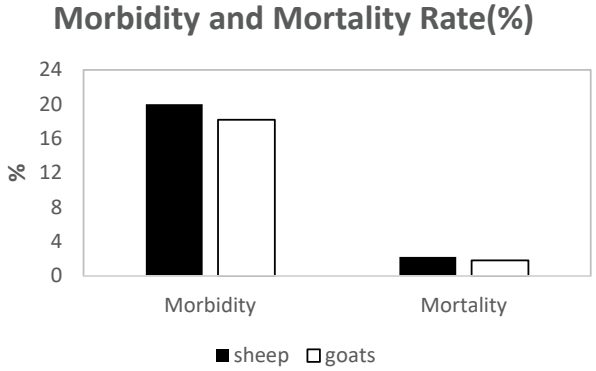

Figure 1: Morbidity and mortality rate of sheeps and goats.

Table 4: Positive isolates of FMDV in Sheep and Goat from Savar.

Region/ District	Total sample	Positive sample	Seotyping			Non typing	% of positivity
			O type	A type	Asia1 type		
Savar	5	4	_	_	_	4	80

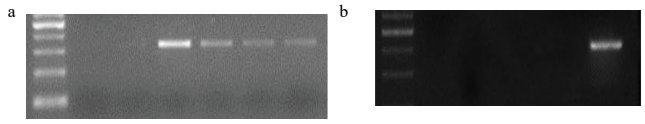

Figure 2: 2a Simplex RT-PCR products of FMDV (5 UTR) after 2% agarose gel electrophoresis. Lane 1= DNA marker (100 bp), Lane 2= Negative control, Lane 3= Negative result, Lane 4= Positive control. Lane 5, 6, 7 = Positive result (328 bp). (b) FMD positive isolates were subjected to multiplex RT-PCR using specific primer sets . 2b: Lane 1= DNA marker (100 bp), Lane 2= Negative control, Lane 3,4,5= Negative result, Lane 6 = Positive control (O Manisa, Inactivated Antigen, IAH PIRBRIGHT LABORATORY, UK).

Table 5: The distribution of samples and their diagnostic RT-PCR test status of Cattle.

Region/ District	Year of sample collection	Total Samples	Positive Samples	Serotypes			Non typing	% of posi-tiviy
				O	Asia-1	C		
Sirajganj	2016	27	17	7	10	-	-	63%
Savar	2016	4	4	-	-	-	4	100%
Chittagong	2016	4	3	3	-	-	-	75%
Bandarban	2016	10	4	4	-	-	-	40%
Total		45	28	14	10		4	62%

were found to be non-typed. In this study FMDV type C was not found among the tested clinical samples (Figure 3b).

The impact of seasonal variation on the prevalence of foot and mouth disease in sheep and goats and cattle was observed. In this study, FMD outbreak was observed in winter in Sheep and goats. During winter, five samples were collected from savar and from which 80% isolates were FMD positive. Out of 5, 31 and 14 samples collected in summer, monsoon and winter, respectively, 1, 24 and 7 samples were found positive. So prevalence was highest in monsoon (77%) and lowest in summer (20%) (Table 6). Prevalence of FMD virus was varied among male and female animal. Out of 17 male animal 9 found positive and 23 were positive in 33 female animal, comprising percentage 53 and 70 respectively (Table 7).

During sample collection the age of the sheep and goat were recorded. All suspected sheep and goat were young. Animals were categorized into three age-groups and tested for the presence of FMD virus. Prevalence was highest (76.92%) in age group-2 (2-4 years), intermediate (33.33%) in group-1 (<2 years) and lowest (25%) in old animals constituting age group-3 (Table 8). During the study period, out of 30 and 20 samples collected from indigenous and cross breed, respectively, 22 and 10 samples were found FMDV positive. The percentage of FMD affected animal in both indigenous and cross breed is 73.33% and 50% respectively (Figure 4). During sample collection vaccination history of the suspected animal was recorded. Susceptibility of the animal to FMDV was varies and depended on vaccination profile. In this study 20 and 30 samples from vaccinated and non-vaccinated animal and 8 and 24 samples were found FMDV positive (Table 9).

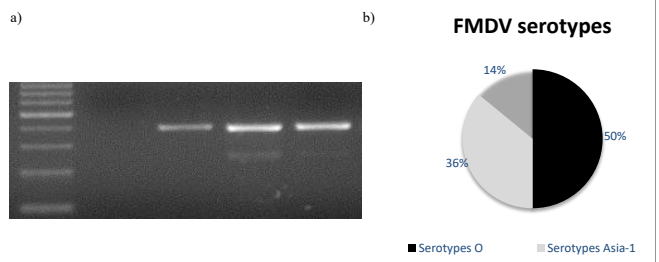

Figure 3: (a) Electrophoresis of RT-PCR products from cattle sample on 2% agarose gel stained with ethidium bromide. Lane 1: 1 kb plus DNA ladder (Invitrogen, USA), lane 2: Negative control, lane 3, 4: Positive Samples, lane 5: Positive control (O Manisa, Inactivated Antigen, IAH PIRBRIGHT LABORATORY, UK). (b) FMDV serotypes.

Table 6: Association between outbreak of FMD and seasonal influences.

Season	Summer (March-June)	Monsoon (July-October)	Winter (November-February)
Total samples	5	31	14
FMDV positive samples	1	24	7
FMDV positive samples(%)	20	77	50

Table 7: Prevalence of FMD in both sexes of animal.

FMD suspected animal	Sex	Total samples	Positive samples	Positiveisolates per 100 samples (%)
Sheep and goats	Male	2	1	50
	Female	3	3	100
Cattle	Male	15	8	53
	Female	30	20	67
Total	Male	17	9	53
	Female	33	23	70

Table 8: FMD variation in different age categories.

Age group	Description	Total samples	FMDV positive samples	%
1	Young (<2years)	11	5	45.45
2	Adult (Between2 and 4 years)	33	25	75.75
3	Old (Above 4years)	6	2	33.33
Total		50	32	64

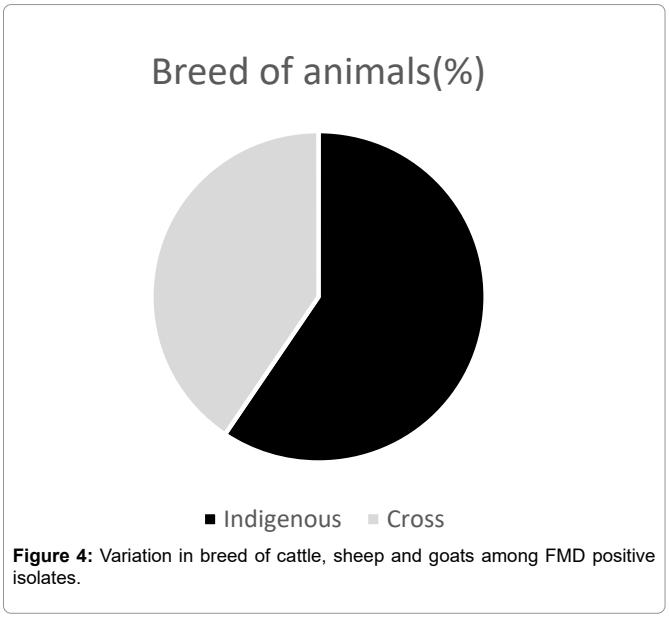

Figure 4: Variation in breed of cattle, sheep and goats among FMD positive isolates.

Table 9: Correlation between FMD outbreak and vaccination profile.

Sample from	Total Sample	Positive Sample	%
Vaccinated Animal	20	8	40
Non-vaccinated Animal	30	24	80
Total	50	32	64

Discussions

Economically devastating foot and mouth disease causes several economic losses throughout the world. Completely eradication FMD is not possible yet through conventional vaccine as highly variable antigenicity was found in all serotypes. Sheep and goats are highly susceptible with FMDV by the respiratory route. The virus mostly infects by direct contact with infected animals. Infection can also happen mucous membranes in contact with contaminated food and through abrasions on skin. The virus can persist for up to 3.5 years in cattle, up to nine months in sheep and up to four months in goats [13].

In our study, 80% sheep and goats were confirmed as positive for FMD and 62% cattles were positive for FMD(Tables 4 & 5). Clinical signin characterized byinappetance, panting, pyrexia (≥ 40°C), lesions on the feet and mouth, fever, and viremia [14-17]. It has been reported, however, up to 25% of infected sheep may fail to form lesions, and an additional 20% may be seen only one lesion. However, the clinical signs and symptoms of FMD may be influenced by the virus strain and breed of animals [18].

In each outbreak, the number of animals at risk, age and sex category of animals affected and number died due to FMD were recorded to determine morbidity and mortality. The 20% and 18.19% morbidity of sheep and goats in the infected herds reported in our study (Figure 1) was higher than the morbidity of 4.8% previous reported in Bangladesh [19] in sheep and goats. This wide difference in morbidity could be due to differences in age composition of herds as the disease's morbidity is known to be higher in young calves [20] and could also be due to other factors. Higher number of morbidity rate causes loss of milk production of the livestock animal though mortality rate is lower.

Although most papers report that males are more prone to be affected both mild [21-24] and severe [25], FMD but surprisingly, serologic evidence does not support this finding. In this study, female animal suspected with FMD showed the highest prevalence. Females (70%) were found more susceptible than males (53%) (Table 6). This result is different from Sarkar et al. who showed that prevalence of FMD was significantly higher in males (36.36%) than females (16.06%) [26]. The result of the study is not also supported by Mannan et al. who also found that susceptibility to FMDV infection is higher among male cattle (35.77%) than female cattle (15.97%) [27].

Outbreaks of FMD do not occur uniformly throughout the year across Asia. In Fukuoka, Japan, for example, weekly numbers of FMD cases have been reported to evaluate with average temperature and humidity [28] but in North China . May through July are the months with highest incidence in temperate regions of Asia. It is not clear whether prediction of outbreaks is possible to depend on temperature. In our study we found, highest occurrences in wet season monsoon and lowest in dry season summer (Table 8). [29] virus decline during dry seasons than during wet seasons, which could illustrate the relation of FMD and seasonality.While any relationship between climate and FMD is not clear, speculations include a lower FMD incidence because of decreased social contact during winter [30] and in contrast, increased social contacts during winter have been thought to facilitate the spread of droplet-borne diseases [31]. Prevalence of FMD varies among different age groups of animals. In this study, the susceptibility rate among the suspected animal was highest (75.75%) in adult (2-4 years) and lowest (33.3%) in old animal (>4 years). The young ones (<2 years) were in an intermediate position having 45.45% susceptibility (Table 8). This may be attributable to the young animal being herded in homestead areas and hence having less chance of exposure. Those animals aged >4 years may have acquired the infection from multiple serotypes and/or infections.Mannanet al., reported similar findings, showing maximum prevalence among the adults (34.18%) [27]. So, these findings are similar to the findings of present study. Although there is a small variation in the outcome, this might be due to the small size of the collected sample and the sensitivity of the detection method.

In the present study, breed specific prevalence depicted that, FMD was observed affecting mostly indigenous animal (73.33%) and less effect in crossed breed (50%) (Figure 4).The higher occurrences of the disease in indigenous compared to cross breeds might be due to the suboptimum management systems implemented on indigenous livestock animal as they were supplemented with minimum inputs due to their low production and body weight gain. The higher level of incidence might be due to higher frequency of close contact with infected animals of the nearby farmers whichfacilate to spread out FMD virus [32,33]. The present result is similar with Sarkar et al. and Mannanet al., who reported higher incidence and prevalence of FMD in indigenous breed than cross breed [26,27].

FMD virus is highly mutagenic, thus it is difficulties to select

vaccine against it is the huge variation between, and even within, serotypes. Due to the multiple serotypes of FMDV in circulation, identification of the serotype affecting any one region is required in order to select the most appropriate for inclusion in a vaccine preparation. This means FMD vaccines must be highly specific to the strain involved. Moreover, vaccination only gives temporary immunity that works only months to years. In the current study, FMD was observed affecting mostly non-vaccinated animal (80%) and less effect in vaccinated animal (40%) (Table 9).

Conclusion

Present study has confirmed that the occurrence of FMD in sheep, goats and cattle in different parts of Bangladesh and its association with variation of the seasonal effect, age, sex and breed is significant. Most risk group for outbreak was also reported in the current study. The results of this study could add value to minimise the FMDV outbreak from those areas.

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Conflict of Interest

There is no conflict of interest.

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Author Affiliations

Top

[1]*Department of Biotechnology &Genetic Engineering, Jahangirnagar University,Savar, Dhaka-1342, Bangladesh*
[2]*Department of Microbiology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh*
[3]*Animal Health Research Division, Bangladesh Livestock Research Institute, Savar, Dhaka-1341, Bangladesh*