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Identification of different serotypes of Foot and Mouth Disease Virus from Sylhet district, Bangladesh; by adoption and application of RT-PCR and mRT-PCR.

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ABSTRACT

Foot and mouth disease (FMD) is a major constraint for livestock in Bangladesh; as outbreak of FMD remains uncontrolled despite vaccination. Accurate and Rapid detection of FMD virus with its serotype in field samples is indispensible. So, molecular detection of FMDV was adopted using RT-PCR (reverse transcription- polymerase chain reaction) and mRT-PCR (multiplex reverse transcription-polymerase chain reaction). Ten (10) FMD suspected clinical samples from cattle of two different outbreak areas of Sylhet district of Bangladesh was collected. One set of universal primer (P32:P33) was used in RT-PCR for the detection of FMD virus regardless of their serotypes and a cocktail of primer mix (P38:P40:P74-77:P110) was used in mRT-PCR intending the identification of the serotypes A, O, C and Asia 1. Using universal primer sets 90% of the samples generated amplicon of expected size, indicating the samples containing FMD virus. By mRT-PCR, two serotypes, 'O' and Asia 1 were successfully, whereas type C and A were absent in this study. Out of the 9 viruses; 7 was identified as serotype 'O' and 2 were identified as Asia-1. Our study indicates that FMDV serotype 'O' and Asia-1 was circulating in the two upazilas (Sub-district) of Sylhet district during the study period. Our study also endorses that, RT-PCR and mRT-PCR can successfully be used for a dependable and rapid detection of FMD. However, presence and detection of 'O' and Asia-1 serotype of FMDV through this study and serotype A by other researchers emphasizes the critical need for use of trivalent vaccine in the field.

Key words: FMDV in Bangladesh, FMD diagnosis, FMD outbreak, RT-PCR and mRT-PCR.

INTRODUCTION

Foot-and-mouth disease (FMD) is a disease of all cloven-hoofed domestic animals. FMDV also affects more than 70 species of wild animals [10]. The disease FMD is classified under list A of animal diseases to be reported by the Office International des Epizooties (OIE) which, by definition, means that it has the potential for rapid and extensive spread within and between countries and can cause severe economic impact [2]. Infection with FMDV generally results by the rapid appearance of high rise of body temperature followed by formation of vesicles on the skin and mucous membrane; particularly on mouth, nose and inter-digital space of hoof [5]. Even after recovery from acute infection, most animals act as a carrier for the virus and the agent can be isolated from their esophagus and throat fluid after 2-3 years of post-infection [4].

The causal agent foot and mouth disease virus (FMDV) belongs to the genus *Aphthovirus*, under *Picornaviridae*. It is considered to be the most contagious infectious agent of domestic animals. It has extreme communicability and can spread rapidly through livestock populations and across continents. The disease is often transmitted from infected to apparently healthy susceptible animals through air or direct contact [13, 14]. FMD is difficult to control owing to its high contagious nature and existence of the virus as seven serotypes (A, C, O, Asia 1, SAT 1, SAT 2 and SAT 3), each of which comprises of numerous antigenic variants. Therefore, this disease has become an alarming threat to the livestock producers all over the world.

Outbreak of FMD may appear in a population of animals of a country as an epizootic or an enzootic forms. It is being considered as the most serious epizootic alarming disease in the world today. Almost all countries of the world are now on red alert to prevent entry of this disease by making serious restriction on the movement of man, animals and animal products (meat, milk, butter and cheese) from the enzootic countries.

FMD is one of the major constraints for livestock development in Bangladesh too. Among the infectious disease, FMD is considered as a serious problem of the cattle and other livestock population in this country. Studies conducted by Bangladesh Livestock Research Institute (BLRI) revealed the morbidity in cattle to be around 36%, in buffaloes 23% and in goats/sheep 5% and mortality rate, especially in calves, has been found to be about 51% in outbreak areas. Annual loss due to FMD has been estimated at about US $ 125 million per year in this country [20]. Sero-epidemiological investigation of this virus in cattle population indicated that four different types (A, O, C and Asia 1) of FMDV were prevailing in Bangladesh during 1960 to 1990 [9]. According to Islam [15], serotype A and 'O' of FMDV were prevailed in this country during 1996 to 2000. Recent reports on FMDV are not identifying serotype C in Bangladesh.

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Despite regular vaccination, outbreak of this disease has become a regular event throughout the country every year. Though the exact reason for frequent outbreak of the disease is not very clear, but it may be due to introduction of mutant virus, or inappropriate serotypes used for vaccine preparation or regular importation live animals from neighboring country. A significant number of cattle and buffaloes have been entering in Bangladesh from India every year either through authorized or unauthorized channels, which directly or indirectly serves a pathway for new virus introduction. Therefore, a reliable and rapid confirmatory diagnosis of FMD virus types is essential to formulate vaccine as well as to control the outbreak of the disease.

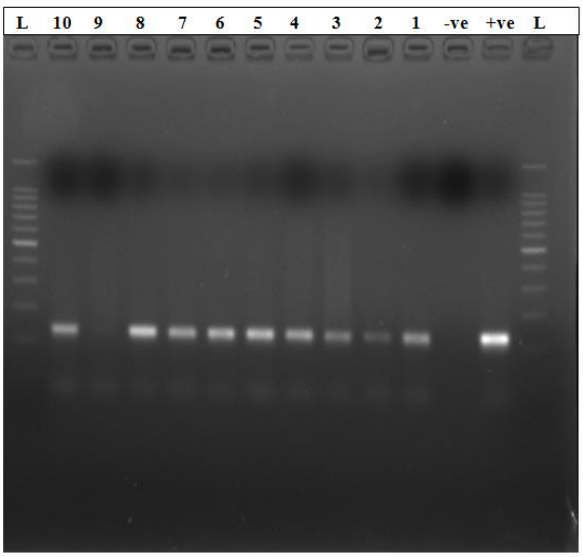

Fig.01. Agarose gel (1.5%) electrophoresis of PCR products (132 bp) obtained with primer pair P32:P33 using RNA extracted from FMD clinical samples. L: 100 kb Marker/Ladder, 1-10 Samples, -ve: Negative Control, +ve: Positive Control.

Nowadays diagnosis of FMD in field condition is not a difficult task. FMD can readily be diagnosed just by observing history and clinical signs in field conditions, although by this process; it is impossible to detect the serotypes of FMDV. In developing countries of Asia and Africa FMDV serotype diagnosis is complicated due to the presence of multiple serotypes and the high levels of antigenic variation of the indigenous types [7]. As a result, confirmatory diagnosis of FMDV either by serological or by molecular methods have no alternative. The laboratory diagnosis is usually made by enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT), virus neutralization test (VNT) etc [11, 17]. But all this tests are laborious, time consuming and in many cases, none specific. On the other hand, the molecular methods of diagnosis are more reliable and specific. Among the molecular methods, RT-PCR are being used routinely in many countries of the world as a highly sensitive, rapid and reliable means for detection and typing of FMDV [8]. Diagnosis of FMDV by RT-PCR procedures have been widely documented and have involved universal primers for the detection of FMDV regardless the serotypes [3] and multiplex RT-PCR has also been introduced [1] to identify the serotypes of circulating FMDV.

The present research has been designed to adopt RT-PCR and mRT-PCR for the identification of FMDV from field samples and also identification of Foot and Mouth Disease Virus (FMDV) circulation in Sylhet district, of Bangladesh.

Table.01 List of the primers, their sequences and size of PCR amplicon were used for the diagnosis of FMD virus from the field samples:

FMDV serotype	Primer label	Sequence(5´-3´)	Location	PCR products (bp)	Reference
All serotypes	P32	CAGATGCAGGAGGACATGTC	2B	132 bp	Vangrysperre and De Clercq, (1996)
	P33	AGCTTGTACCAGGGTTTGGC	2B		
O	P38	GCTGCCTACCTCCTTCAA	1D	402 bp	
C	P40	GTTTCTGCACTTGACAACACA	1D	596 bp	
Asia 1	P74	GACACCACTCAGGACCGCCG	1D	292 bp	
	P75	GACACCACCCAGGACCGCCG	1D		
	P76	GACACCACACAAGACCGCCG	1D		
	P77	GACACGACTCAGAACCGCCG	1D		
A	P110	GT(G:A:T:C)ATTGACCT(G:A:T:C)A TGCA(G:A:T:C)AC(G:A:T:C) CAC	1D	732 bp	Callens and De Clercq, (1997)

MATERIALS AND METHODS

The research work was conducted in the Department of Microbiology & Hygiene, Sylhet Agricultural University, Sylhet and Department of Pathology, Bangladesh Agricultural University, Mymensingh.

Sample Collection

Ten (10) vesicular fluid and tongue epithelium; as field samples were collected, among them five were from bulls & five were from cows, showing signs and lesions of Foot and Mouth Disease. Samples were collected from cattle of different outbreak areas of two upazila (Sadar & Goainghat) of Sylhet district of Bangladesh during the period from December

2011 to January 2012. The samples were initially preserved at -20^{0}C and later at -70^{0}C till processing.

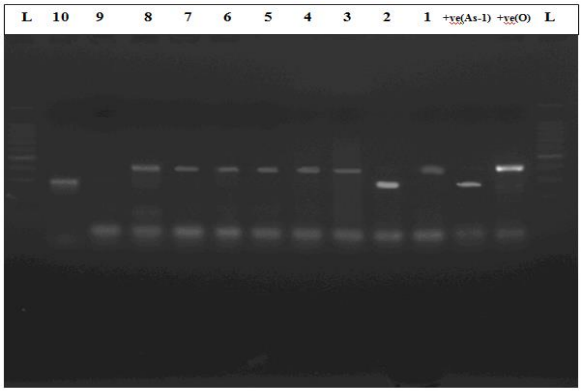

Fig.02. Agarose gel (1.5%) electrophoresis of PCR products (328bp) obtained with the primer pair P32:P33. The viral RNA was extracted from field cases of FMD; L: 100 kb Marker/Ladder, +ve(AS-1): Positive Control for Asia-1, +ve(O): Positive Control for O. Multiplex RT-PCR with P33/P38/P40/P74,P75,P76,P77/P110 Primer; 1-O Type(402 bp), 2-Asia-1(292 bp), 3- 'O' Type, 4-O type, 5-O type, 6-O type, 7- 'O' Type, 8-O type, 9-Negative, 10-Asia-1.

Processing of Vesicular fluid

Vesicular fluid was collected aseptically into PBS in screw-capped tube. The VF was centrifuged at 5000 rpm for 15 minutes. Supernatant was collected in fresh sterile tubes and used as inocula after antibiotic treatment.

Table 2: The thermal profile for the different primer sets:

Points	Name of Primers	
	P32:P33	P33:P38:P40:P74-77:P110
Reverse transcription	50^{o}C for 30 min	50^{o}C for 30 min
Initial PCR activation	95^{0}C for 10 min	95^{0}C for 1min
Number of cycles	30	35
Denaturation	95^{0}C for 1 min	95^0 for 15 min
Annealing	60^{0}C 1 min	60^{0}C for 1 min
Extension	60^{0}C 10 min	60^{0}C 10 min
Final extension	60^{0}C for 10 min	60^{0}C for 6 min
Hold at	4^{0}C	4 ^{0}C

Preparation of 20% suspension of tongue epithelium

Tissue was macerated with the mortar and pestle by adding PBS to make it 20% (w/v) suspension. Then it was centrifuged at 3000 rpm for 10 minutes. Supernatant was collected and stored at -70°C for future use after antibiotic treatment.

Extraction of RNA

Total RNA was extracted from each sample in the laboratory of the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh; using Qiagen RNeasy kit; the protocol provided by the Qiagenb® Netherlands were strictly followed. The RNeasy technology combines the selective binding properties of a silica-gel-based membrane with the high speed of microspin technology. High-salt buffer (Guanidine isothiocyanate, GITC) system allows up to 100µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Contaminants are efficiently washed away and high quality RNA is then eluted in 50µl of Nuclease free water.

RT-PCR

Selection and Synthesis of Primers for RT-PCR

The oligonucleotide primer for the detection of FMDV and FMDV serotypes were used from the 1D, 2B and 5 URT regions of the viral genome as published [8, 19]. Specifications of these primers are listed in Table 01. All oligonucleotides were synthesized by Rikaken® Co. Ltd. Japan.

Reaction Mixture (20 µl) for FMDV detection:

For the detection of FMDV in the sample, the RT-PCR using the universal primer set P32 and P33; 20 µl of reaction mixture was made for RT-PCR using Promega® one step RT-PCR Kit. The reaction mixture constituted of Nuclease Free Water-6µl, Master mix-12.5 µl, downstream primer (100 pmol)-0.5 µl, upstream primer (100 pmol)- 0.5 µl and Taq DNA polymerase-0.5 µl.

Reaction Mixture (20µl) for serotype determination of FMDV:

For serotype determination of the detected FMDV mRT-PCR (multiplex RT-PCR) was employed using 0.5 µl of each Upstream primers: P38, P40, P74, P75, P76, P77, (6pmol) and 0.5 µl of Consensus downstream primer (P33) (6pmol). 20µl reaction mixture was prepared using Promega® one step RT-PCR Kit; the other constituents were *Taq* DNA polymerase-0.5 µl, Nuclease Free Water-3µl, Master mix-12.5 µl

Procedure for RT-PCR

Preparation of reaction mixture

The whole process of the RT-PCR and mRT-PCR mixture preparation was done in PCR cooler to maintain the temperature. Required amount of the reaction mixture was prepared according to the number of sample by adding all the constituents as recommended in the sequential form as listed above. 20µl of reaction mix was dispensed to each PCR

tube. The tubes were labeled as needed. Later, 5µl extracted RNA template was added to the respective tube and mixed by pipetting; 5µl nuclease free water was added instead of RNA to the negative control tube. Then the PCR tubes were placed in the thermocycler programming the thermocycler as mentioned. After completing the RT-PCR, the PCR tubes containing RT-PCR products were taken out from the thermocycler and stored at 4°C in refrigerator until electrophoresis.

Electrophoresis

RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and examined under UV light using an image documentation system.

Documentation of the PCR product samples

After electrophoresis, the gel was taken out carefully from the gel chamber and the gel was gently washed in running water and placed on the UV transilluminator in the dark chamber of the Image Documentation System (Labortek, Germany). The UV light of the system was switched on; DNA bands were observed under UV light on the Transilluminator. The image was focused, acquired and saved in memory stick, as well as printed on thermal paper.

RESULTS

The research was conducted from December 2011 to January 2012; with a view to identifying circulating serotypes of foot and mouth disease virus in Sylhet. FMD affected cattle were examined for the vesicles at oral cavity and ulcerative lesion in the tongue, gums, lips and coronary band. Suitable samples were collected for laboratory analysis.

RT-PCR detection of FMDV

A total of 10 clinical samples (vesicular fluid and tongue epithelium) of 2 different upazila of Sylhet districts of Bangladesh were examined by RT-PCR using one set of universal primers (P32: P33). Of these 10 clinical samples, 9 (90%) appeared positive for FMDV in RT-PCR. RNA extracted from the clinical samples of FMD was tested a RT-PCR product of expected 132 bp was successfully amplified, while the negative sample failed to produce any DNA band. The results of amplification are presented in Fig. 01.

Table.03 Primer pairs for the differentiation of FMDV serotypes A, C, O, and Asia 1 and sizes of the resulting PCR products:

Primer sets / Serotypes	P33:P38	P33:P40	P33:P74-77	P33:110
FMDV-O	402bp	-	-	
FMDV-C	-	596bp	-	-
FMDV-Asia 1	-	-	292bp	-
FMDV-A	-	-	-	732bp

'-' = no amplicon

Multiplex RT-PCR (mRT-PCR) for serotyping of FMD virus

In the multiplex RT-PCR, it was possible to detect two different serotypes (O and Asia1) of FMDV in one RT-PCR reaction by using a cocktail of primer mix (Table 1). An FMDV strain can be identified as 'O' or Asia-1 if there is a PCR amplicon of 402 or 292 bp respectively. In the study, multiplex RT-PCR revealed two serotypes (O and Asia-1) of FMDV and DNA fragments of 402 and 292 bp were amplified for the 'O' and Asia-1 serotypes, respectively (Fig. 02); while absence of amplicon size 596bp and 732bp indicated the absence of serotype C and A in the tested samples.

DISCUSSION

In this experiment, gross studies were included to investigate the changes in tongue, muzzle and skin of coronary band of FMD suspected animals. The RT-PCR was performed for the detection of FMDV regardless the serotypes and multiplex RT-PCR was done for the identification of serotypes.

Pathological Investigation of Suspected Cattle

Gross investigation

Our study suggests that, the FMD affected cattle were easy to identify, because the lesion and clinical signs were so prominent. But in most of the cases the young calves developed little or on lesions at oral cavity or coronary area. This may be due to fact that the heart lesions, which is so massive that before developing oral or epithelial lesions the animal subjected to death. The findings of this study appeared similar with the previous findings made by [2, 6].

RT-PCR detection of FMDV

Rapid detection and identification of FMDV and its serotypes is important and essential both in animal health and vaccination programs. Molecular biology is providing extremely sensitive and specific tools for identifying and characterizing FMDV strains in clinical samples [16]. The molecular detection technique is rapid, accurate, highly sensitive and only small quantities of material are needed to perform the test. Detection of FMDV by means of RT-PCR has been described before [3, 19]. In this study, RT-PCR was adopted in order to detect FMD viral RNA in clinical samples, regardless of their serotypes.

Table.04: Result of tested samples:

Serotypes	Primer sets/	Samples									
		1	2	3	4	5	6	7	8	9	10
FMDV-O	P33:P38/ P40/P74,	402 bp	-	402 bp	402 bp	402 bp	402 bp	402 bp	402 bp	-	-
FMDV-Asia 1	P75,P76, P77/P110	-	292 bp	-	-	-	-	-	-	-	292 bp

'-' = no amplicon

In this study, the primer pair P33:P32 (product size 132 bp) were selected based on the conserved nucleotide sequences in 2B in all known FMDV types and used for the identification of FMDV regardless of their serotypes. The RT-PCR by using the primer pair P32:P33 successfully amplified 132 bp products from the field samples (Fig.1). The result of this study appeared similar with the previous findings [1, 8, and 19]. The specific oligonucleotide pair [19] was chosen because of its high efficiency of amplification of the target sequence with all seven serotypes and has no false positive reaction. The primer pair did not develop any reaction with other viruses such as SVDV, porcine enterovirus 1, CBV, EMCV or BVDV [19]. Amplifying this short 132 bp region permitted to use a high speed one step RT-PCR protocol, yielding a result within few hrs and thereby limiting the possibilities of non-specific reactions to occur.

Although all samples were collected from clinically positive cattle but 9 samples were showed positive in RT- PCR and the only 1 was negative. The exact cause of failure to detect the FMDV from remaining sample is not very clear. Faulty sampling, or absent of viral RNA in that sample during collection or the virus may mutate at that region from where the primer were selected might be the reasons. Moreover, in case of tongue epithelium, various kinds of disinfectants were used at low concentration (1% $NaHCO_3$) in the buccal cavity which destroyed the FMDV to a greater extent. Giridharan [12] have suggested that the exposure of specimens to higher environmental temperatures, incorrect pH or putrefaction of the specimens may lead to low concentration of virus or degrade the virus.

However, in this study, RT-PCR method showed a reliable, simple to perform and economic in terms of output. It is envisaged that this present evaluation will help in the future design of improved PCR protocols, for example nested PCR formats, to enhance the sensitivity of virus detection in the primary diagnosis of FMD [20].

Multiplex RT-PCR for Serotyping of FMD Virus

Serotyping is a fundamental part of FMD diagnosis; this helps in tracking back the origin of an outbreak, provides valuable information for epidemiological studies, and guides the choice of emergency vaccines. Identification of serotypes of FMDV by means of mRT-PCR has been described earlier [8, 12]. In this study, it was possible to differentiate four of the seven FMDV serotypes, i.e. O, A, C and Asia-1 in one amplification reaction by using mRT-PCR. This was possible by combining the downstream primer P33 (situated in the highly conserved 2B region, and common for all FMDV serotypes), with a type-specific upstream primer selected from the neighboring and more variable 1D gene (Table 1). An FMDV strain can be identified as A, C, 'O' or ASIA 1, if there is a PCR amplicon of 732, 596, 402 or 292 bp, respectively. In this study, the result of the multiplex RT-PCR revealed two serotypes (Asia1 and O) of FMDV and DNA amplicon of 292 and 402 bp were achieved for the Asia1 and 'O' serotypes, respectively (Fig. 2). The mRT-PCR took 2.30 hours and the results (From RNA extraction to electrophoresis) could be obtained within a single working day. This fast differentiation is useful for directing vaccine selection and in epidemiological studies [8].

In this study, mRT-PCR failed to detect serotype C and A in the field samples. This should be due to absence of serotype C and A in the study area or the collected samples was not containing the serotype. Findings of the present study clearly indicated that the molecular methods (RT-PCR and mRT-PCR) could be used as highly sensitive and specific for the serotyping of FMDV in a single reaction. This may enhance the precise detection of FMDV along with serotyping of the viruses from field cases. However study with more samples collected for a longer period of time could identify few other serotypes of FMDV.

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CONCLUSION

The research work was conducted for the adoption and application of RT-PCR & mRT-PCR, to the identification of circulating FMD virus in Sylhet district. A total of 10 samples (vesicular fluid & tongue epithelium) were collected from suspected FMD affected cattle of different outbreak areas of two upazila (Sadar & Goainghat) of Sylhet districts of Bangladesh were collected. RT-PCR and mRT-PCR were successfully adopted and standardized using the extracted RNA of reference FMDV. 10 samples were subjected to RT-PCR and mRT-PCR, where 9 (90%) samples were found positive for FMDV by RT-PCR. Out of the 9 FMD viruses 7 were detected as serotype 'O' and two were detected as serotype Asia-1 with the application of mRT-PCR. It can be concluded that FMDV serotype 'O' and Asia-1 was circulating in the study areas during the study period; and among the two serotypes 'O' was predominant in the investigated field samples.

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