

## Reverse transcription polymerase chain reaction (RT-PCR) based detection and serotyping of FMD Virus from field samples of Gazipur, Bangladesh, and adaptation of the virus in BHK-21 cell

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### ABSTRACT

The study aimed for the detection and serotyping of Foot and Mouth Disease virus (FMDV) circulating in Kapasia Upazila, Gazipur district of Bangladesh during 2013. Twelve samples comprising of tongue epithelium (n=8) and inter digital tissue (n=4) were collected from suspected cattle, and inocula were prepared. The inocula were inoculated into confluent BHK-21 cell line for virus propagation. After 3 subsequent passages; progressive cytopathic effects (CPE) specific for FMDV *i.e.*, rounding and flattening of cells, breaking down of the intercellular bridge and finally cell death (almost 100%) were observed; these were indicative of successful virus propagation in the cells. Viral RNA was extracted, and Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed using three sets of primers corresponding to the serotype 'O', 'Asia-1' and 'A', respectively. Out of the 12 samples, 10 (83.33%) were found to be positive for FMDV, and all of those were of serotype 'O'. It is concluded that FMDV serotype 'O' is circulating among the cattle of Gazipur district, Bangladesh.

### Keywords

BHK-21 cell, cytopathic effects, FMDV, RT-PCR

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### INTRODUCTION

Foot-and-mouth disease virus (FMDV) has seven serotypes and more than sixty subtypes belonging to

the family Picornaviridae and genus Aphthovirus. The infection is highly contagious, and can infect domestic and wild animals with cloven hooves. These along with continuous changing of the virus property make it difficult for the professionals in this field to combat the disease (Zabal *et al.*, 2013). FMDV is a non-enveloped, single stranded, positive sense RNA virus, approximately 8,500 bases in size surrounded by four structural proteins (VP 1-4) to form an icosahedral capsid (Rueckert, 1996). There are seven FMDV serotypes namely- 'O', 'A', 'C', 'SAT 1', 'SAT 2', 'SAT 3' and 'Asia 1'. Infection with one serotype does not confer immunity against another (OIE, 2009).

Foot and Mouth Disease (FMD), usually called Aphthous fever, is an acute, febrile, highly contagious and sometimes fatal viral disease of almost all the cloven-hoofed domestic animals including cattle, buffalo, sheep, goats and swine (OIE, 2009). FMDV also affects more than 70 species of wild animals (Fenner *et al.*, 1993). In animals with a history of vesicular disease with fever; the detection of FMDV in samples of vesicular fluid, epithelial tissue, esophageal-pharyngeal sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the detection of FMDV in the blood, heart or other organs in fatal cases. A myocarditis may be seen macroscopically (the so-called "tiger heart") in a proportion of fatal cases (OIE, 2009).

Outbreak of FMD causes severe economic losses to the livestock industries in terms of loss of draft power, meat and milk production, infant and adult animal mortality (Chowdhury *et al.*, 1993; Zinnah *et al.*, 2010; Belsham and Bøtner, 2015). As per the report of Chowdhury *et al.* (1993) in Bangladesh, the morbidity

due to FMD in cattle was around 35.5%, in buffaloes 23.3%, and in sheep 4.8%. On the other hand, mortality rate, especially in calves, had been found to be about 50.9% in outbreak areas. In Bangladesh, annual loss due to FMD has been estimated at about US \$125 million per year (Sil and Taimur, 2000).

Epidemiological investigation of this virus in cattle population indicated that four different types (A, O, C and Asia-1) of FMDV were prevalent in Bangladesh during 1960 to 1990 (Chowdhury et al., 1996). FMDV Serotype 'A' and 'O' were consistently present in Bangladesh during 1996 to 2000 (Islam et al., 2000). The recent studies indicated that three different types (A, Asia-1 and O) were prevalent in Bangladesh during 2007 to 2008 (Sil and Taimur, 2000; Zinnah et al., 2010; Nandi et al., 2013). Serotype 'O' was found to be responsible for 80% of the confirmed outbreaks, whereas 'Asia 1' and 'A' caused 12% and 8% outbreaks, respectively (Sarker et al., 2011; Hossen et al., 2014).

Several epidemiological and molecular studies have been conducted on FMDV in many countries (Edwards, 2004; Durand et al., 2008; Fernandez, 2008; Ryan et al., 2008; Huang et al., 2011; Hossen et al. 2014; Olabode et al., 2014) including Bangladesh (Islam et al., 2000; Howlader et al., 2004; Sarker et al., 2011); but there is no published data on molecular characterization with cell culture adaptation of FMDV in Gazipur region of Bangladesh, although this region has huge number of livestock population. Therefore, the present study was undertaken to identify the types of FMDV that are currently circulating in Gazipur, Bangladesh, so that the findings of the study can be used to adopt effective disease management and control strategies in Bangladesh.

## MATERIALS AND METHODS

**Study area:** FMDV samples i.e., tongue epithelium (T.E), and foot tissues (F.T) were collected from different villages of Kapasia Upazila (sub-district) under the district of Gazipur, during the period of January to November 2013.

**Sample collection and processing:** A total of 12 samples comprising of 8 tongue epithelia and 4 foot tissue from inter-digital space were collected aseptically from FMD affected cattle. The samples were collected in virus transport medium (VTM) containing 10,000 µg Streptomycin, 10,000 IU Penicillin and 25 µg Amphotericin B, and immediately transported in cool

condition to the Virology Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University for analysis.

The field samples were then homogenized with mortar and pestle separately, and 10% suspensions were prepared by adding sterile phosphate buffered saline (PBS). The suspension was then centrifuged at 5000 rpm for 15 min at 4°C, and the supernatant was collected. The collected supernatant was treated with 10,000 µg Streptomycin, 10,000 IU Penicillin and 25 µg Amphotericin B for 1 h (at 37°C), and then filtered with 0.22 µm filter. Sterility of the inocula was tested in fresh blood agar media and stored at -80°C for future use.

**Adaptation and propagation of virus in BHK-21 cell line:** The cells those were found as complete and confluent monolayer in the culture flask within 24 h of incubation were selected for infection with viruses. The growth media from the flask containing BHK-21 cell was removed and then the monolayer cells were washed with sterile PBS for 2 times. The inoculum was spread over the cell sheet by tilting at 37°C for about 60 min for better adsorption. Then, 5 mL of the maintenance media (2% heat inactivated FBS) was added in a 25 cm<sup>2</sup> flask and it was then returned to the incubator and kept at 37°C. The cells were examined twice daily under inverted microscope (Carl Zeiss®, Germany) until showing characteristic cytopathic effects (CPE) caused by FMDV. Various kinds of characteristic changes of cell rounding, swelling, breaking down of intercellular bridge and finally cell death indicated the presence of FMDV in the sample. In this way, all the 12 (100%; **Table 2**) samples were successfully passaged in BHK-21 cell line, and the infectious fluid (IF) could be harvested after 48 to 72 h of post-infection for further investigation for detection and serotyping of the FMDV by RT-PCR.

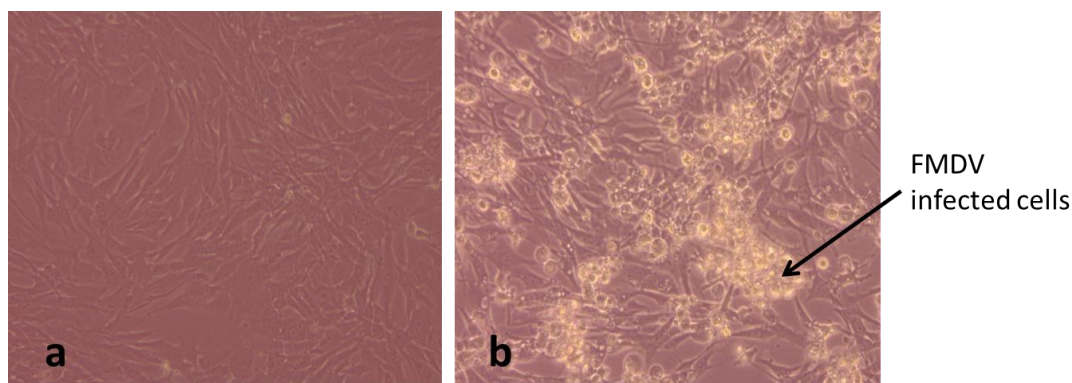
**Viral RNA extraction and RT-PCR:** Viral RNA was extracted from cell culture fluid using SV Total RNA Isolation System (Promega, USA), according to the instructions of the manufacturer. RT-PCR was carried out by Access RT-PCR system (Promega, USA) according to the manufacturer's protocol using specific primers (**Table 2**). The thermal profile used for cDNA synthesis was at 45°C for 45 min and at 94°C for 2 min for one cycle. For PCR amplification, thermal profile was used as initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 68°C for 2 min, for 40 cycles, and a final extension at 68°C for 7 min. After

**Table - 1:** List of the primers used for the detection of FMD virus serotypes.

Serotype	Primer	Sequence(5'-3')	Product size	Reference
O	FMDO F	ACCAACCTCCTTGATGTGGCT	1301-bp	Reid et al. (2000)
	FMDO R	GACATGTCCTCCTGCATCTG		
Asia-1	FMDAs1F	TACACTGCTTCTGACGTGGC	914-bp	Gurumurthy et al. (2002)
	FMDAs1R	GAAGGGCCCAGGGTTGGACTC		
A	FMDA F	TACCAAATTACACACGGGAA	866-bp	Reid et al. (2000)
	FMDA R	GACATGTCCTCCTGCATCTG		

**Table 2.** Detection of FMDV serotypes by one step RT-PCR.

Sample size	Sample adapted in cell culture	Serotype of FMDV positive by RT-PCR	No. of positive sample
Tongue epithelium (N=8)	Tongue epithelium 8 (100%)	FMDV serotype 'O'	8 (100%)
Foot samples (N=4)	Foot samples 4 (100%)	FMDV serotype 'O'	2 (50%)
<b>Total</b>			<b>10 (83.33%)</b>



**Figure 1:** FMDV propagation in BHK-21 cell line. a) Normal (uninfected) BHK-21 cell line, b) FMDV infected BHK-21 cell line. The infected cells become round and flat. The intercellular bridge was broken down, and finally the cells were died.

amplification, the amplicon was then visualized in 2% agarose gel stained with ethidium bromide after electrophoresis.

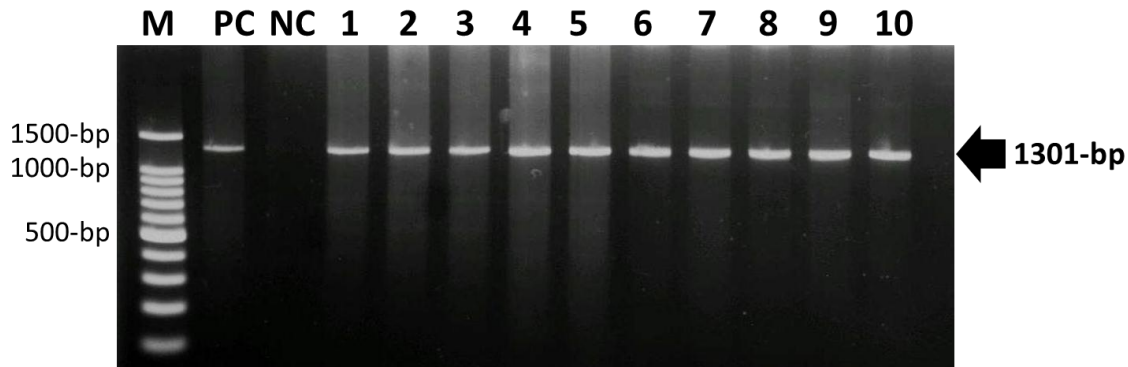
**Agarose gel electrophoresis:** The PCR products were analyzed in 2% agarose gel, stained with ethidium bromide and examined against UV light using an UV Solo-transilluminator (Biometra®, Germany). The positive sample was recorded based on the appearance of expected size of band in the gel (**Figure 2**).

## RESULTS AND DISCUSSION

Foot and mouth disease is a serious threat to livestock in Bangladesh and its economy. FMDV circulates in the country almost throughout the year, and the outbreak reaches to pick level in winter (Sarker et al., 2011). In

this study, 12 clinical samples were collected and subjected to BHK-21 cell line adaptation. Various types of established cell lines are used in different laboratories in the world like BHK-21, IBS-2 and others. Among these cell lines, BHK-21 is considered as the most sensitive one to FMDV (OIE, 2012). Here, we could adapt the FMDV field isolates in BHK-21 cell line. All the 12 samples exhibited characteristics cytopathic effect (CPE) e.g., rounding, swelling, clumping of the cells and break down of intercellular bridge (**Figure 1**).

These 12 cell culture supernatants corresponding to 12 samples were further analyzed for FMDV serotyping using RT-PCR (**Table 1**). RT-PCR is a reliable, rapid, highly sensitive and specific tool for the molecular detection of infectious agents including FMDV



**Figure 2:** RT-PCR image: Lane-M, 100-bp DNA marker; Lane-PC: positive control; Lane-NC: negative control; Lane-1 to 10: FMDV isolate 1 to 10 (1301-bp).

(Reid et al., 2000; Mehran et al., 2006; Hossen et al., 2014). RNA was extracted from the 12 tissue culture fluids that were found positive for CPE. Using RT-PCR with FMDV type specific primers, among the 12 samples, 83.33% (n=10/12) were found to be positive for FMDV (**Figure 2**); among these 10 samples, all (100%) were positive for FMDV serotype 'O' (**Table 2**).

In this study, serotype 'O' of FMDV was found circulating in Kapasia upzila under Gazipur district of Bangladesh. In another study, Hossen et al. (2014) examined 17 samples, of which 8 could be adapted in BHK-21 cell line; of these 8 samples, 6 were belonging to serotype 'O' and 2 were 'Asia-1'. Similarly, Loth et al. (2011) detected serotype 'O', Nandi et al. (2013) detected serotype 'O' and 'A', and Hossen et al. (2014) detected serotype 'O', 'A' and 'Asia-1' as the currently circulating FMDVs in Bangladesh. Our present study identified only FMDV serotype 'O', whereas FAO reported the existence of serotype 'O', 'A' and 'Asia 1' as the circulating FMDV in Bangladesh, Bhutan, India, Nepal, and Sri Lanka, however, serotype 'O' has been considered as the most dominant serotype (FAO, 2014). The failure to identify serotype 'A' and 'Asia-1' in this study might be due to small sample size.

## CONCLUSION

Tongue epithelium is found to be better as compared to foot tissue as a source of FMD virus for isolation. This study also confirms that BHK-21 cell line is highly sensitive for FMD virus isolation. Besides, RT-PCR can be used as an effective method for the detection of FMD virus serotype(s). Through this study, FMD virus serotype 'O' is confirmed to be circulated among cattle of Gazipur, Bangladesh.

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