



## RT-PCR based detection and adaptation of foot and mouth disease virus serotype “A” in BHK-21 cell line

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### Abstract

The economic consequences of an outbreak of Foot and Mouth Disease (FMD) in Bangladesh is devastating. A sensitive, reliable and rapid diagnosis is crucial for the effective control of FMD. The present research was conducted for quick molecular detection and adaptation of FMD virus (FMDV) in BHK-21 (Baby Hamster Kidney) cell line. A two-step RT-PCR method was applied for the detection of the FMDV. Without prior adaption into BHK-21 cell culture, it is difficult to detect FMDV directly from the field samples by RT-PCR method. Samples were collected from the tongue epithelium (N=19) and inter digital tissues (N=4) of the suspected animals in Sujanager, Bera, Santhia of Pabna district and Kotowali, Trishal of Mymensingh districts of Bangladesh during the year 2014. BHK-21 cell subculture was done from a previously cultured bottle containing BHK-21 cells. Prepared inocula were inoculated into BHK-21 cell culture and incubated at 37°C for 24 h. After 36 h, cytopathic effects (CPE) were observed in BHK-21 cell line characterized by rounding and flattening of the cells, multinucleated giant cells formation, breaking down of the intracellular bridges and finally cell death indicated the presence of FMDV. Clear infectious BHK-21 cell culture fluid was collected and preserved at -20°C temperature for virus detection by RT-PCR with serotype specific primers. Viral RNA was extracted from the clear infectious cell culture fluid for cDNA synthesis and used for PCR. Out of 23 samples tested, 3(13.04%) were positive for FMDV serotype A. The findings of this study can be helpful for the selection of vaccine having specific FMDV type, and it may help in controlling FMD in Bangladesh.

**Key words:** BHK-21 cell line, cytopathic effect, FMDV, RT-PCR

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### Introduction

Foot and Mouth disease virus (FMDV) is a non-enveloped, single stranded, positive sense RNA genome, approximately 8,500 bases surrounded by four structural proteins (VP 1-4) to form an icosahedral capsid (Rueckert *et al.*, 1996). Seven serotypes of FMD virus such as O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1 and at least 65 subtypes have been identified (Rahman and Mozumder *et al.*, 1991; Le *et al.*, 2012; Olabode *et al.*, 2014). Infection with one serotype does not confer immunity against another (OIE, 2009).

The foot and mouth disease virus (FMDV) is the pathogen that causes Foot and Mouth disease (Carrillo *et al.*, 2005). FMD is one of the major threats for the development of livestock population in Bangladesh (Rahman *et al.*, 2011). 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 Botner, 2015). Annual loss due to FMD in Bangladesh has been estimated at about US\$62

million (FAO/OIE, 2012). 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 serotype O, A and Asia-1 are the currently circulating FMDV in Bangladesh as revealed by RT-PCR (Chowdhury *et al.*, 2016). Mixed infection having Type A and Asia-1 FMDV was also detected (Hossen *et al.*, 2014). 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).

Although every year vaccination is carried out throughout the country against FMD, still the disease outbreak occurs in many parts of Bangladesh (Sarker *et al.*, 2011). Recent report from FAO has recommended that surveillance and reporting of FMD needs to be improved throughout the country. Furthermore, all suspected clinical cases of FMD should be confirmed by laboratory examination (Mondal and Yamage, 2014).

Therefore, the present study was undertaken to identify the FMDV serotype “A” from Pabna and Mymensingh district of Bangladesh. It will help for the selection of vaccination, and strategies for the control of FMD in Bangladesh to improve the economy of farmers.

## **Materials and Methods**

**Location and period of the study.** The study was carried out with the help of Tissue culture laboratory, Virology Laboratory, Food Hygiene laboratory, and Molecular laboratory in the Department of Microbiology and Hygiene, Bangladesh Agricultural

University, Mymensingh. Period of the study was July, 2014 to June, 2015.

**Sample collection.** Samples were collected from the tongue epithelium (N=19) and inter digital tissue (N=4) of the suspected animals from Sujanagar, Bera, Santhia of Pabna district and Kotowali, Trishal of Mymensingh district of Bangladesh during the year 2014. Among 23 samples, 16 samples were collected from Pabna district and 7 samples were collected from Mymensingh district.

**Subculture of BHK-21 cell.** All the media and reagents were brought to room temperature before use. The growth media from the flask containing BHK-21 cell culture was removed. Then the monolayer cells were washed with sterile 1X PBS for 2 times. One milliliter (1 ml) of trypsin was added into the flask and mixed with the cell to detach the cells from the wall of the tissue culture flask. The flask was left in the incubator for 5 min. The tissue culture flask was shaken up and down smoothly to spread trypsin until detachment of the BHK-21 cell line. About 500  $\mu$ l growth media was added to the flask. Cell aggregation was broken by pipetting and depipetting. 10 ml of media containing cell was poured into 25 cm<sup>2</sup> flask. The flask was allowed to incubate at 37°C for 24 h.

**Adaptation of FMDV in BHK-21 cell culture.** The cells those formed confluent mono-layers 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 calls were washed with 1X sterile PBS for 2 times. One milliliter (1ml) of virus inoculum was added to the flask containing confluent monolayer of BHK-21 cells. The inoculum was spread over the monolayer cell by tilting for about 45-60 min for the establishment of better interaction. Then 10 ml of the maintenance media (1 $\times$ MEM supplemented with 2% heat inactivated fetal calf serum) was added in a 25 cm<sup>2</sup> flask and the vessel was returned to the incubator. Virus added flask was allowed to incubate at 37°C. The cells were examined twice daily under inverted microscope until show characteristic cytopathic effect (CPE) by Foot and Mouth disease virus (FMDV). The cells were examined under inverted microscope. FMD virus produced cytopathic effects (CPE) in the BHK-

21 cell at different intervals of post infection (24 to 96 h) which was characterized by the rounding and flattening of the cells, breaking down of the intracellular bridges and finally cell death which are characteristic findings of FMDV infected cells. Initially the growth of virus in BHK-21 cell line was lower, which increased after, subsequent 3 time's passage of infection into BHK-21 cell line.

**Viral RNA extraction and RT-PCR.** The infectious fluid containing FMDV was harvested after 48 h to 72 h of post infection. Viral RNA extraction from the infectious cell culture fluid was carried out according to the instructions of the manufacturer using SV Total RNA Isolation System (Promega, USA). Manufacturer protocol was followed for the conduction of RT-PCR using Access RT-PCR system (Promega, USA) and specific primers (Table 1). The thermal profile used for cDNA synthesis was

45°C for 45 min and 94°C for 2 min for one cycle. Thermal profile for PCR amplification was: initial denaturation at 94°C for 5 min, 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. PCR amplified products were separated electrophoretically on 2% agarose gel and stained with ethidium bromide after electrophoresis.

**Documentation of the PCR product.** After the PCR products electrophoresis at 100V for 35 min in TAE buffer on 2% agarose gel, the gel was subjected to ethidium bromide (0.6 mg/ml) for 10 min. The gel gently washed in running tap water and placed on the UV-transilluminator (Bio Rad, USA) in the dark chamber for the image documentation. The positive samples were recorded based on the appearance of expected size of band in the gel.

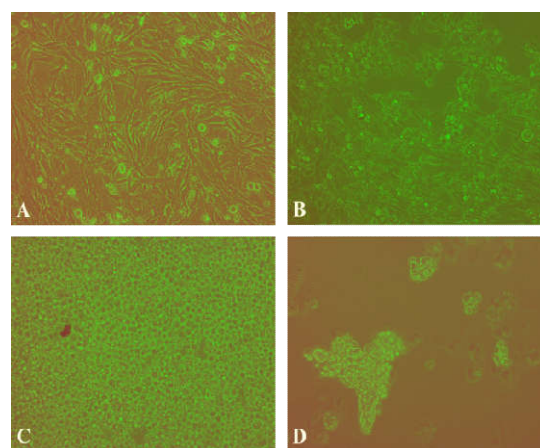
**Table 1.** List of the primers used for the detection of FMD virus serotype “A”

FMDV Serotype	Primer Designation	Sequence(5'-3')	PCR products size (bp)	Reference
A	FMDA F FMDA R	TACCAAATTACACACGGGAA GACATGTCCTCCTGCATCTG	866	Reid <i>et al.</i> , (2000)

### Results and Discussion

All the inocula were inoculated into BHK-21 cell line for the isolation of viruses. Out of 23 samples tested, 3 (13.04%) were found positive for FMDV serotype A (Table 2 and 3). The cytopathic effect produced by the inocula of FMDV in BHK-21 cells were manifested by the rounding and flattening of the cells, breaking down of the intracellular bridges and finally cell death which are characteristic findings of FMDV infected cells (Figure 1). The findings of cell culture following infection with FMDV were similar with the findings of Hossen *et al.*, (2014), Islam *et al.* (2000), Shahiduzzaman (2012) and Alam *et al.* (2015).

Rapid detection and identification of FMDV and its serotypes is important and essential both in animal health and control of FMD. Molecular biology is providing extremely sensitive and specific tools for identifying and characterizing FMDV strains in clinical samples (King, 2001).



**Figure 1.** **A:** Uninfected BHK-21 Cell (Elongated shape, Close packing of the cells, Little intercellular space), and **B, C and D:** FMDV infected BHK-21 cell showing cytopathic effect (CPE) (**B:** 1<sup>st</sup> passage: Initiation of infection and cell rounding started after 24 h of infection, **C:** 2<sup>nd</sup> passage: Almost 95% cell infected after 36 h of infection, **D:** 3<sup>rd</sup> passage: Almost 100% cell infected after 48 h of infection observed under 200X (Carl Zeiss, Germany).

## Detection and adaptation of FMD virus serotype A

The molecular detection technique is rapid, accurate, highly sensitive and only small quantities of material are needed to do the test. Detection of FMDV serotypes by means of RT-PCR has been described before (Alam *et al.*, 2015; Hossen *et al.*, 2014; Marupanthorn *et al.*, 2013; Le *et al.*, 2012; Loth *et*

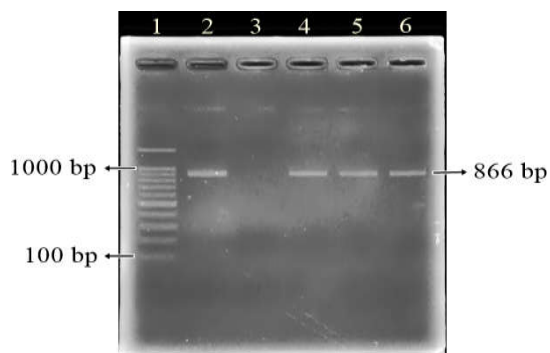
*al.*, 2011; Saeed *et al.*, 2011). We have performed the PCR after synthesis of cDNA from the extracted RNA of the FMD virus and found positive bands at 866bp after electrophoresis in 2% agarose gel which was specific for FMDV serotype "A" (Figure 2).

**Table 2.** Detected FMDV serotypes by two step RT-PCR

Sample size	Sample adapted in cell culture	Positive serotype of FMDV by RT-PCR	No. of positive sample
Tongue epithelium (N=19)	Tongue epithelium 19 (100%)	FMDV serotype "A"	3 (15.79%)
Foot samples (N=4)	Foot samples 2 (50%)	FMDV serotype "A"	0
<b>Total</b>			3 (13.04%)

**Table 3.** Name of the locality found positive for FMDV serotype "A"

Country name	Locality	Total no. of sample tested	Samples found positive For FMDV serotype "A"
<b>Bangladesh</b>	Pabna district (Sujanagar, Bera, Santhia)	16	3 (18.75%)
	Mymensingh district (Kotowali, Trishal)	7	0 (0.00%)
Total		23	3 (13.04%)



**Figure 2.** Image showing that Lane 1= 100 bp DNA marker, Lane 2=positive control, Lane 3= negative control, lane 4, 5 and 6=866 bp DNA band for FMDV serotype A following 2% agarose gel electrophoresis

### Conclusion

Present study proved that FMDV serotype "A" is present in the Pabna district of Bangladesh. RT-PCR technique followed by BHK-21 cell adaptation of virus is very sensitive method of FMDV isolation and identification and can be used for the detection

of FMD virus serotype. In future, the research findings may be used for the preparation of vaccine against FMDV.

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