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# Application of Reverse Transcription PCR for Detection of Foot and Mouth Disease Virus Type O in Infected and Recovered Animals

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**Foot-and-mouth disease (FMD) is a highly contagious and economically important disease of cloven footed domesticated and wild animals. Lot of works have been carried out to develop diagnostic tests in regard to this disease The present study was undertaken for detection of FMDV serotype 'O' in infected as well as in recovered animals by using sandwich ELISA, virus isolation in cell culture in BHK-21 cell line and reverse transcription PCR and the finding were compiled together to see the most sensitive test for detection of FMDV type 'O'. A total of 50 clinical samples comprising 40 tongue epithelium, 6 oral swab and 4 throat swab samples were subjected to sandwich ELISA where 20 were found positive for FMDV type 'O' (40%), where as all the swab samples were found to be negative for any serotype of FMDV. Sandwich ELISA positive samples and all the swab samples were subjected to virus isolation where only 11 samples showed CPE (36.66%). FMD virus serotype 'O' could be detected in all samples (100%), by RT-PCR using serotype 'O' specific primer. Therefore, the present study revealed that RT-PCR is most sensitive for the detection of RNA of FMD virus when compared to the results of the three tests.**

**Key words:** FMD, sandwich ELISA, CPE, RT-PCR.

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven hoofed domesticated animals (Alexanderson *et al.*, 2003) as well as many species of wildlife and is characterized by fever, lameness, salivation and vesicular lesions on tongue, feet, snout and teats. The disease has debilitating effects including weight loss, decrease in milk production and loss of drought power resulting in loss of productivity of affected animals for a considerable period of time. The etiological agent of FMD is a single stranded RNA virus of the genus Aphthovirus under the family Picorna viridae. The seven immunologically distinct serotype of FMD virus are A, O, C, Asia-1 and South African Territories (SAT) 1, 2 & 3. Multiple subtypes also occur within each serotype (Bachrach *et al.*, 1975).

Several conventional techniques such as complement fixation test (CFT) (Traub and Mohlmann, 1943; Longjam *et al.*, 2011), serum neutralization test (SNT) (Rweyemamu *et al.*, 1978) and enzyme linked immune sorbent assay (ELISA) (Have and Jensen, 1983; Periolo *et al.*, 1993; Longjam, 2011) are still in use for the detection of FMD virus in different clinical samples. Generally sandwich ELISA is carried out for the detection of specific FMDV antigens in different epithelial

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tissue suspensions which is usually accompanied by concurrent cell culture isolation where the infected samples show cytopathic effect in cell culture. With the introduction of several molecular techniques such as different polymerase chain reaction like RT-PCR (Mayer *et al.*, 1991; Amaral-Doel *et al.*, 1993; Hofner *et al.*, 1993; Rodriguez *et al.*, 1994; Marquardt *et al.*, 1995; Callens *et al.*, 1998; Fernandez *et al.*, 2008;), multiplex PCR( mPCR) (Giridharan *et al.*, 2005) in the diagnostic field of FMD, it is now-a-days easy to diagnose the disease in its very early stage because of the sensitivity and specificity of these molecular tools .The present study was undertaken for detection of FMDV serotype 'O' in infected as well as in recovered animals by using sandwich ELISA, virus isolation in cell culture in BHK-21 cell line and reverse transcription PCR .

## MATERIALS AND METHODS

### Source of samples

A total of 50 clinical samples used in the present study were taken from the repository of Regional Research Centre, All India Coordinated Research Project for Epidemiological Studies on Foot-and-Mouth Disease, Khanapara, Guwahati-22, which comprised of 40 tongue epithelial samples collected from FMDV infected animals and 10 oral/throat swabs, collected from recovered animals and were collected during the period of 2010 to 2012 from different North-Eastern regions. The tissue samples were properly preserved in glycerol phosphate buffer (PBS pH 7.2-7.6) and the oral/throat swabs were collected in maintenance media. All the samples were stored at -20°C.

### Preparation of sample materials

The tissue samples that were preserved in glycerol phosphate buffer (PBS pH 7.2-7.6) were used for preparing 10% tissue suspension (epithelium suspension) in PBS. Briefly, 100-200mg of sample (infected epithelium) was taken and washed in the PBS and triturated in a sterile pestle and mortar with the help of sterile sand particles using 2 ml PBS. The properly triturated materials were collected into a centrifuge tube and equal volume of chloroform was added. The content was centrifuged at 3500 rpm for 15 min. The clear supernatant was collected stored in sterile cryo vial at -20°C. The aliquots of supernatant were used for virus isolation, serotyping by Sandwich ELISA and RNA extraction for Reverse Transcription.

### Serotyping of field isolates by sandwich ELISA

In order to confirm the serotype of the isolates obtained from the repository and the infected cell culture fluids, were tested by Sandwich ELISA as per the bench protocol of Project Directorate on Foot-and-Mouth disease, IVRI, campus, Mukteswar, Uttarakhand. Type specific ( O,A,C and Asia1) anti-146S FMDV rabbit serum were used as coating serum. Type specific ( O,A,C and Asia1) anti-146S FMDV rabbit sera raised in guinea pig were used as tracing serum and the rabbit/goat anti-guinea pig immunoglobulin-HRPO conjugate were used as conjugate.

### Isolation of FMD virus in cell culture using BHK-21 clone 13 cell line

For isolation and propagation of virus from the clinical materials, BHK-21 (clone 13) cell line maintained at the All India Coordinated Research Project for Epidemiological Studies on Foot-and-Mouth Disease, Khanapara, Guwahati-22, were used Subculture of BHK-21 clone13 cell line was done as per standard procedure.

### Infection of cell monolayer

The epithelial suspension were used as inoculum for cell culture flasks (CORNING, New York, USA ) containing preformed complete monolayer of BHK-21 cells. The cell monolayer was washed once with the maintenance medium (Glasgow modified) and was then infected with 350 µl of supernatant with 50 µl of 1X antibiotic solution (Penicillin and Streptomycin). After an adsorption period of 60 min at 37°C the content was decanted off to remove unabsorbed virus. Finally, 2-5 ml of maintenance medium was added to each culture flasks and incubated at 37°C. The tubes were observed for next 48 hrs for cytopathic effect (CPE). Infected cell culture flasks were then harvested and were given two more blind passages in flask and harvested after a period of 24 to 36 hrs when they show CPE. The 25 $cm^2$ culture flasks (CORNING, New York, USA) containing preformed complete monolayer of BHK-21 cells were infected to obtain the working stocks of all isolates. Culture showing complete CPE were harvested by shaking and infected cell culture fluids were centrifuged (SIGMA- AVI, 3K30) at 3500 rpm for 15 mint at 10° C to remove all cell debris. The clear supernatants

containing virus were collected and stored in small aliquots (cryo vials) at (-80°C).

**Detection of FMD virus nucleic acid by polymerase chain reaction**

All the apparatus used for detection of FMDV were treated with DEPC (Di Ethyl Pyro Carbonate) to make them RNAase free .Samples found positive for serotype O in Sandwich ELISA were subjected in Reverse Transcription PCR for more accurate detection. Nucleic acid manipulation was done with RiboZOL™ RNA Extraction Reagent, (AMRESCO, USA.) RevertAid™ M-MuLv Reverse Transcriptase (MBI Fermentas Vilinus, Lithuania); Ribonuclease Inhibitor, Ribolock™ RNase (Fermentas). Primers used for reverse transcription PCR were NK-61; 5'GACATGTCCTCCTGCATCTG3' and DHP-13; 5'GTGACTGAACTGCTTTACCGCAT3'.

Isolation of RNA was done using RiboZol™ reagent. Again cDNA was synthesized using universal primer for FMDV i.e. NK-61. The Reverse Transcriptase PCR (RT-PCR) was done using the PCR master Mix (2X), Dream Taq™ Green. After mixing all the components PCR Master mix (2X) Dream Taq™ 12.5 µl, NK61 (10pmol/µl) 0.5µl, DHP13(10pmol/µl) 0.5µl, Nuclease free water 9.5µl and tamplate DNA 2.0µl in 0.2 ml PCR tube , the tubes were centrifuged at 3500 rpm for 30 seconds. The PCR tubes were kept in the thermal cycler (Applied Biosystems 2720 Thermal Cycler) for amplification of cDNA. The cycling conditions were denaturation 95 C for 15 min, followed by 35 cycles of template denaturation at 95 C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s followed by a cycle of final extension at 72°C for 10 min. The amplicons were subjected to the agarose gel electrophoresis using 2% agarose gel along with the suitable DNA marker.

## RESULTS AND DISCUSSION

Out of the total 50 samples subjected to sandwich ELISA , 20 (40%) epithelium suspension were found to be positive for serotype 'O', 14 epithelium suspension found to be positive for serotype 'A' and 6 epithelium suspension found to be positive for serotype 'Asia-1". All the swab samples were found to be negative for any serotype of FMDV. So in the present study it is found that sandwich ELISA is quite sensitive and specific for the detection of different serotypes of FMDV within short period of time. Pattnaik and Venkataramanan (1989) also reported that sandwich ELISA to be a sensitive test for detection of FMDV antigen in the epithelium suspension. The observation in the study is in well accordance with the previously reported poor efficiency of sandwich ELISA in detection of FMD virus antigen

**Table 1.** Compilation of results of sandwich elisa, virus isolation and reverse transcription PCR

| Subjected test | Total no of sample | Serotype o positive | Positive results |
|---|---|---|---|
| Sandwich elisa | 50 | 20 | 40.00 % |
| Virus isolation | 30 | 11 | 36.66 % |
| Rt- pcr | 30 | 30 | 100.00 % |

in oral swab samples where only in two samples the FMD virus antigen could be detected out of total 260 oral swab samples (Paixao *et al.*, 2008). Various factors like time of collection and condition during the transport of the samples has tremendous effect on the detection of antigen by sandwich ELISA. In the present study out of the 20 samples those were positive for serotype 'O' in sandwich ELISA, virus isolation was possible in 11 samples (55%). All the 11 samples were tongue epithelium suspension. However no virus could be isolated from the 10 numbers of swab samples. The findings indicated that in virus isolation out of 30 samples only 11 samples (36.66%) showed positive results for the presence of FMDV. In the first passage some of the samples showed cytopathic effect (CPE) 24 and 48 hrs of post infection (Fig. 1 and 2). All the infected tissue culture fluids of the first culture were used for the second passage. In the second passage, all the 11 samples showed CPE 24-48 hrs of post infection (Fig. 3 and 4). Again for confirmation third passage was done from each of

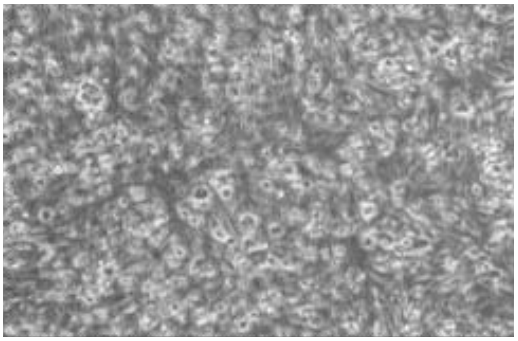

**Fig 1.** CPE at first passage, 24 hours of post infection

**Fig. 2.** CPE at first passage, 48 hours of post infection

**Fig. 3.** CPE at second passage, 24 hours of post infection

**Fig. 4.** CPE at second passage, 48 hours of post infection

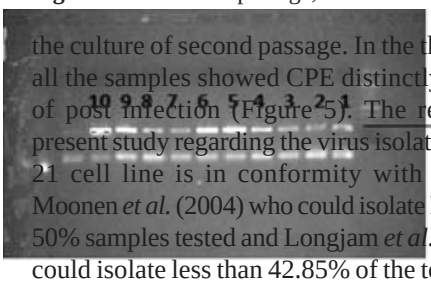


**Fig. 5.** CPE at third passage, 24 hours of post infection

the culture of second passage. In the third passage all the samples showed CPE distinctly just 24 hrs of post infection (Figure 5). The result of our present study regarding the virus isolation in BHK-21 cell line is in conformity with findings of Moonen *et al*. (2004) who could isolate FMDV from 50% samples tested and Longjam *et al*. (2011) who could isolate less than 42.85% of the total samples found positive for FMDV.

RNA extracted from all the 20 tongue epithelial suspensions which showed positive results in sandwich ELISA for FMDV serotype 'O'

**Fig. 6-7.** Agarose gel electrophoresis of rt-pcr amplified gene products of epithelium tissue suspensions showing FMDV o serotype specific band

and 10 swab samples which were found negative in sandwich ELISA, were all found positive for FMDV serotype O when subjected to RT-PCR. It indicated that all the samples (100%) were found positive for FMDV serotype 'O'. Specific product of 249bp was observed after agarose gel electrophoresis of RT-PCR amplicons (Fig. 6 and 7). The detail finding of all the three tests was compiled in the table 1.

Various factors like time of collection and condition during the transport of the samples has tremendous effect on the detection of antigen by sandwich ELISA. The inability to detect FMDV antigen in oral swab samples by sandwich ELISA might be due to the reason that, the samples were either not collected at the proper time of clinical manifestation or due to insufficient virus concentration in the samples. There are several factors that are responsible for the revival of the virus in the cell culture which may include susceptibility of the particular cell line, ability of the virus to adopt to grow in cell culture system, time of collection of the samples and conditions of collection or transport to maintain the infectivity of the virus particle present in the samples. As the present study showed that out of 30 samples only 11 were possible to isolate (36.66%), this may be due to the delayed collection of samples after onset of the disease or poor collection, storage or transport conditions. It is also said that FMDV from the samples collected after more than 10 days of appearance of the lesions were difficult to isolate by cell culture as it was attributed to the neutralization of infective particles by early formed antibodies on the onset of immune response in the host (Alexandersen *et al.*, 2003).

Diagnosis of picorna viruses by RT-PCR takes the advantage of the fact that up to half a million of virus genomes equivalent to 2-3 pg or 5-10% of total RNA can be produced per infected cell in culture, which can provides sufficient template for cDNA synthesis (Niedbalski *et al.*, 1998). The present observation was also similar to that of Parida *et al.* (2005) who observed that the detection of FMDV in virus isolation in persistent infection is difficult but can only be detected by RT-PCR. Again Moonen *et al.* (2004), found that during the first 100 days significantly more samples were positive by RT-PCR than by virus isolation, when they took the infected tissue samples from 17 numbers of cattle where 16 become carriers. The present results of the study also matched with the study carried out by Niedbalski *et al.* (1998) where they took the tongue and feet epithelium from 14 cattle infected experimentally and found that the tongue or feet epithelium of 13 cattle reacted positively by FMDV specific RT-PCR. Whereas nasal swabs collected from the same cattle group reacted negatively when collected 11-14days of infection.

Therefore the present study revealed that RT-PCR is most sensitive for the detection of RNA of FMD virus when compared to the results of the three tests. However to detect infected FMD virus particles, cell culture inoculation is unavoidable. The presence of FMD virus was detected by sandwich ELISA even in those samples from where virus could not be isolated. However, sandwich ELISA was not much sensitive enough to detect FMD virus in oral and throat swabs which may be because of very low concentration of FMD virus particle present in the swabs.

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