Indian Journal of Animal Sciences **82** (3): 233–236, March 2012

Comparative evaluation of three diagnostic tests for detection of foot-and-mouth disease virus of Indian origin*

NEETA LONGJAM[1], A K SARMAH[2], RAJIB DEB[3], V K SAXENA[4] and TILLING TAYO[5]

Received: 24 January 2011; Accepted: 18 October 2011

ABSTRACT

In the present study, we have comparatively evaluated 3 diagnostic tests, viz. virus isolation, sandwich ELISA and multiplex PCR to detect foot-and-mouth disease virus serotype 'O' in clinical samples, isolated from Indian origin. Our results showed that multiplex PCR could detect FMD virus in the highest number of samples than rest of the two comparatively.

Key words: Foot-and-mouth disease virus serotype 'O', Multiplex PCR, Sandwich ELISA, Virus isolation

Foot-and-mouth disease (FMD) is a highly contagious disease affecting ungulates (Brooksby 1982), and major problems is its control in India (Tamilselvan *et al*. 2009). The virus exists in 7 types, viz. O, A, C, Asia1, SAT1, SAT2 and SAT3, but a large number of subtypes have evolved within each serotype (Pereira 1977). Only 4 serotypes, viz, 'O', 'A', 'C' and Asia 1 were ever recorded in India. In India, FMD control is implemented by regular vaccination and for effective control, outbreaks should be detected at an early stage and persistent infections should also be recognized to prevent further transmittance. Currently, the FMD diagnosis is done by virus isolation (VI), sandwich-ELISA (S-ELISA), liquid-phase blocking ELISA (LPBE), multiplex-PCR (mPCR), and recently DIVA test which is an indirect ELISA for detection of antibody against non structural proteins. Considering the world wide acceptance of these molecular tools for accurate diagnosis of FMDV, the present study was undertaken to compare the efficiencies of sandwich ELISA, multiplex PCR, and virus isolation in cell culture for detection of FMDV serotype 'O' originated from Northeastern hilly regions of Indian origin.

MATERIALS AND METHODS

The clinical materials comprised mouth/feet lesion i.e. tissue sample (70) and oral swab (20). For standardization of different methods, cell culture adapted vaccine strains (INDR2/75 for O, IND490/97 for A and IND63/72 for Asia 1) maintained at the Project Directorate on Foot and Mouth Disease, Indian Veterinary Research Institute (IVRI) Campus, Mukteshwar, Uttarakhand were used as controls in this study.

Table 1. List of oligonucleotide primers used for RT and mPCR

Primers	Sequence	Location	Size	Sense	Specificity	Reference
NK61	5'GACATGTCCTCCTGCATCTG3'	2B	20	-	FMDV	Knowles and Samuel (1995)
DHP9	5'GACCTGGAGGTYGCGCTTGT3'	1D	20	+	Asia 1	Giridharan *et al.* (2005)
DHP13	5'GTGACTGAACTGCTTTACCGCAT3'	1D	23	+	O	Giridharan *et al*. (2005)
DHP15	5'CAACGGGACGARCAAGTACTC3'	1D	21	+	A	Giridharan *et al*. (2005)
DHP16	5'GTGTCRGRTAACCAACACAC3'	1D	20	+	C	Giridharan *et al*. (2005)
O primer	5'CGGGTGACTGAACTGCTTTA3'	1D	20	+	O	PD on FMD. IVRI, Mukteswar
A primer 1	5'GCCATCCACGAGCTKCTCGT3'	1D	20	+	A	PD on FMD. IVRI, Mukteswar
X1-1D-492F	5'TTYAAYTACGGYGCTGTRAAGGCT G3'	1 D	25	+	Asia 1	PD on FMD. IVRI, Mukteswar

Sandwich ELISA for serotyping: Sandwich ELISA was done as per the bench protocol of Project Directorate on Foot-and-Mouth disease, IVRI, campus, Mukteswar, Uttarakhand.

Isolation of FMD virus in cell culture: BHK-21 (clone-13) cell lines maintained at the Project Directorate on Foot and Mouth Disease, Indian Veterinary Research Institute

* This work forms M.V. Sc. thesis work submitted by the first author.

Present address: [1,5]M V Sc, Ph D scholar (neetavet@gmail.com), Dept of Veterinary Microbiology, College of Veterinary Science and Animal Husbandary, Selesih, Aizawl, Mizoram.

[2]Professor, College of Veterinary Science, Assam Agricultural University Khanapara, Guwahati 781 022.

[3]M V Sc., North Joynagar, Agartala, West Tripura 799 001.

[4]Scientist, CSWRI, Avikanagar, Rajasthan 304 501.

campus, Mukteswar, Uttarakhand were used for isolation and propagation of virus from the clinical materials. Subculture of BHK-21 clone 13 cell line was done as per Rai (1980).

Detection of FMD virus nucleic acid by multiplex PCR: Samples found positive for serotype O and those in which no viral antigen were detected by sandwich ELISA were subjected in multiplex PCR for more accurate detection. Oligonucleotide primers for reverse-transcription (RT) and polymerase chain reaction (PCR) were commercially synthesized. Details of the oligonucleotide primers used in the study are given in Table 1. An aliquot of supernatant was used for RNA extraction using the TRIzol reagent (Invitrogen) as per the manufacturer's instructions. Reverse transcription of the viral RNA was performed using Reverse Transcriptase.

Statistical analysis: The statistical analysis of the generated experimental data was done as per standard procedure (Snedecor and Cochran 1994).

RESULTS

Detection of FMDV serotype in clinical materials by Sandwich ELISA: Out of the total 90 samples subjected to sandwich ELISA, 45 epithelium suspension (ES) were found positive for serotype 'O', 6 ES were positive for serotype Asia1 while the rest 39 samples were negative for any FMD virus serotype, which comprised 19 ES and 20 oral swabs. The corrected OD value of the ELISA positive samples ranges from 0.203 to 1.156, compared to the positive control well (2.386) and back ground well (0.01).

Virus isolation from clinical materials: During the present study, virus isolation was possible in 36 out of 84 samples. The 36 positive samples comprised 35 ES and 1 oral swab. At first passage in BHK-21 cells, none of the samples showed cytopathic effect (CPE). During the present study on virus isolation in cell culture, out of 84 samples, 10 ES showed CPE at third passage within 20 to 24 h post infection. Rest 26 samples showed CPE in fifth and sixth passage within 16 to 24 h of post infection. Out of the 26 samples, one sample belonged to oral swab and the rest 25 were of ES. The cell culture fluids of the samples that have showed characteristic CPE were subjected to sandwich ELISA for confirmation of the FMDV serotypes. Out of the 35 ES positive for virus isolation in cell culture, 34 belonged to FMDV serotype 'O'. The remaining one ES from where virus could be isolated was found to have serotype 'A' which was earlier found to be negative for any of the FMDV serotypes by sandwich ELISA. The only oral swab found positive for isolation of FMDV during the present study also was typed to be serotype 'O'.

Detection of FMD virus nucleic acid by multiplex PCR

Testing of reference sample: The amplicons were confirmed in 2% agarose gel by electrophoresis. The specific products size used are 249bp for serotype 'O', 376bp for serotype 'A' and 573bp for serotype 'Asia1' (Fig. 1).

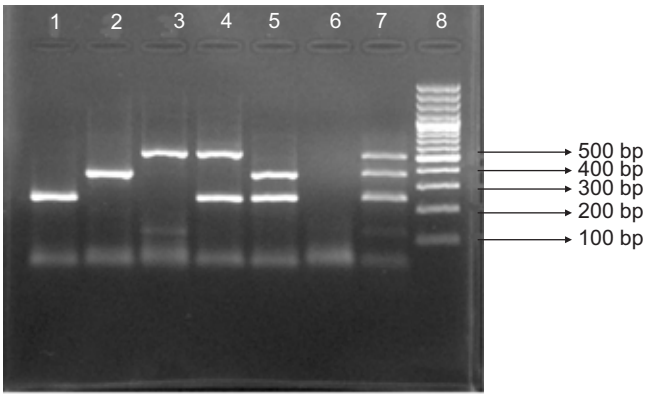

Fig. 1. Agarose gel electrophoresis of multiplex PCR amplified gene product of serotype O, A and Asia 1. Lane 1, O specific (249bp) band; lane 2, A specific (376bp) band; lane 3, Asia1 specific (573bp) band; lane 4, O and Asia 1 mix; lane 5, O and A mix; lane 6, negative control; lane 7, O, A and Asia1 mix; lane 8, ladder.

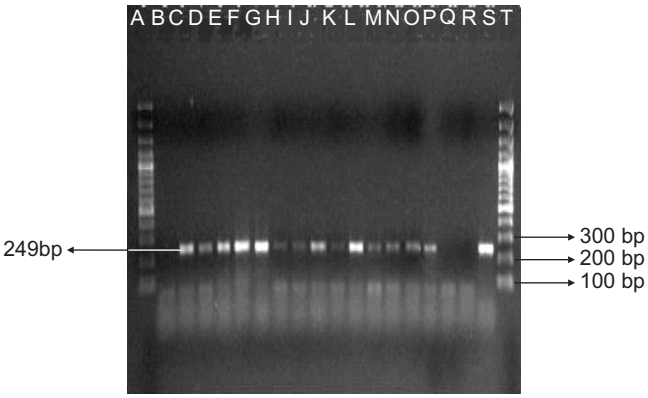

Fig. 2. Agarose gel electrophoresis of multiplex PCR amplified gene product of clinical materials showing serotype 'O' specific band. Lane A and T, ladder; lane B-Q, test samples; lane R, negative control; lane S, positive control.

Testing of clinical materials: Out of the 84 samples, 45 samples were found positive for FMD virus serotype 'O' while the rest 39 samples were found negative earlier by sandwich ELISA. Out of the 84 samples subjected to mPCR, 55 (65.48%) samples were found to be positive for FMDV serotype 'O' (249bp), while the rest 29 (34.52%) samples did not reveal presence of FMDV for any of the FMDV serotype (Fig. 2). Among the 55 samples positive in mPCR, 53 belonged to ES and only 2 oral swabs were found to have gene for FMDV serotype 'O'.

Comparison of sandwich ELISA, Virus isolation and multiplex PCR of FMD virus: A comparative result of all the tests is summarized in Table 2 . During the present study, out of 84 samples, 45 samples were found positive for serotype 'O' and 39 were found negative for FMDV by sandwich ELISA. However, by virus isolation technique, virus could be isolated from 36 samples while the remaining 48 samples did not reveal presence of FMDV. On the other hand, by multiplex PCR out of 84 samples, FMDV could be detected in 55 samples.

Table 2. Comparative result of sandwich ELISA, virus isolation and multiplex PCR for detection of FMD virus

Test	Nature of the samples	No. of samples tested	No. of samples	
			Positive	Negative
S-ELISA	Tissue	64	45	19
	Oral swab	20	0	20
	Total	84	45(53.57)	39
Virus isolation	Tissue	64	35	29
	Oral swab	20	1	19
	Total	84	36(42.85)	48
mPCR	Tissue	64	53	11
	Oral swab	20	2	18
	Total	84	55(65.47)	29

Figures in the parenthesis indicate percentage.

Statistical analysis

The four tests differed significantly at 1% level of significance. Probability (P) calculated between the four tests also gave the same result.

DISCUSSION

S-ELISA is being used for detecting the presence of FMDV antigen in the clinical materials from the lesions from infected animals. Despite being a rapid test it has lower analytical sensitivity and is not suitable for use with certain sample types (Hoffmann *et al*. 2009). The immunology based assay like ELISA may stand less sensitive in diagnosis of FMD when clinical materials are dispatched from distant places to the referral laboratories without maintaining proper transport condition which may lead loss of structural integrity of the viral proteins. It is now recognized that RT-PCR assay can play an important role for the rapid and accurate detection of FMDV in a wide range of clinical sample types. Multiplex PCR is a sensitive technique in which serotype specific primer are used for detection of FMDV nucleic acid in the clinical materials and is also a valuable tool for detection of FMD virus serotype (Giridharan *et al*. 2005).

In this particular study detection of serotype 'O' is being used as a model. Detection of antigen through ELISA in clinical materials depends on various factors like time of collection and conditions during the transport of samples. In this study FMD virus could be detected in none of the oral swab samples though in the materials from foot-and-mouth lesions, the antigen was detected. The inability to detect FMDV antigen in oral swab samples by S-ELISA might be due to the reason that, the samples were either not collected at the proper time of clinical manifestation (Prasad *et al*. 1992) or due to insufficient virus concentration in the samples.

Samples (84) comprising 45 positive for serotype 'O' and 39 negative for FMDV antigen of any serotype by sandwich ELISA were passaged in BHK-21 clone-13 cell line up to 6 passages. The presence of FMD virus specific cytopathic changes were considered positive and absence of cytopathic changes even after 6 passages were taken as negative. The percentage of FMD virus isolation from epithelial suspensions (54.68%) observed in the present study is in conformity with findings of Shaw *et al*. (2004) who could isolate FMDV from 58.4% samples. Prasad *et al*. (1992) could also isolate FMD virus from 62.9% from epithelial suspension.

In the present study, in 6 samples in which ELISA failed to detect FMD virus antigen, could be isolated in 6th passage. Though in sandwich ELISA 45 samples were found positive indicating the presence of viral antigen, only from 36 samples virus could be isolated in the cell culture. The samples from where virus isolation was not possible either due to the delayed collection of samples after onset of the disease or poor collection/transport conditions. Alexandersen *et al*. (2003) also reported that there is a lower possibility of virus isolation from the samples collected after more than 7–10 days of appearance of lesions and it was attributed to the neutralization of infected particles by the early formed antibodies on onset of immune response in the host.

The field isolates showed characteristic cytopathic effect (CPE) between third and sixth passages, which was completed by 18–24 h post infection. The present observation was in agreement with the findings of Goel and Rai (1985), who reported that the field isolates of FMDV could be adapted in BHK-21 clone 13 cell lines and they also recorded characteristic CPE between third and fifth passages. Mishra *et al*. (1995) also adapted FMDV field isolates to BHK-21 clone 13 cells in 3–7 serial passage. In their study, it was observed that vaccinated animals took more time (22–24 h) to produce complete CPE than the isolates from unvaccinated animals.

None of the oral swab samples were earlier found positive for FMDV by sandwich ELISA. The present observation confirmed the observation of Marvin and Barry (2004) that negative sandwich ELISA result of clinical samples has to be tested by virus isolation, followed by reconfirmation of cell culture fluid by sandwich ELISA.

In the present investigation, all the 84 clinical samples tested for virus isolation were subjected to mPCR using FMD virus serotype O, A and Asia 1 specific primers. During the present study, 55 (65.47%) samples were found to have nucleic acid of FMD virus serotype 'O'. Among the 55 positive samples, 53 (82.81%) samples belonged to tissue epithelium. The present observation was in agreement with the finding of Giridharan *et al*. (2005), who could detect FMD virus in 88.73% samples of which 52.81% were typed as serotype 'O'. They also reported that mPCR worked more efficiently on clinical samples compared to sandwich ELISA and was an efficient test to diagnose FMD virus in old as well as new samples. Out of 20 oral swabs subjected to mPCR in the present study, only 2 samples showed the presence of

FMDV specific nucleic acid of serotype 'O'. However those 2 oral swabs were negative for any of the FMDV serotype when subjected to sandwich ELISA earlier. Involvement of multiple serotypes of FMDV in the clinical materials could not be observed during the present study. However, Woodbury *et al*. (1994) observed the involvement of mixture of 2 or more serotypes of FMD virus in the clinical specimens. In the present study, one sample which was found positive for FMDV serotype by virus isolation, was found negative for nucleic acid of any FMDV serotypes in mPCR. This might be a reflection of inefficient RNA extraction, although inhibitors in the samples such as RNAses could not be ruled out (Callahan *et al*. 2002).

Our results showed that mPCR is more efficient on clinical samples as it could detect 82.81% type 'O' serotype out of 64 ES as compared to sandwich ELISA which could detect 70.31% FMDV serotype 'O'. This finding is in agreement with Giridharan *et al*. (2005). It was suggested that exposure of specimens to higher environmental temperature, incorrect pH or putrefaction in a poor quality specimen may lead to lowered virus or antigen concentrations, thus preventing the detection by ELISA (Giridharan *et al*. 2005). FMDV could not be detected in 20 oral swab samples screened by sandwich ELISA. This might be attributed to low concentration of antigen in the samples or inappropriate collection of samples. However, FMDV could be isolated from only 1 oral swab sample in BHK-21 clone 13 which was typed to be serotype 'O' by sandwich ELISA.

In the present study a significant difference at 1% level was observed between sandwich ELISA, virus isolation and multiplex PCR by employing Chi- square and probability tests. On the other hand, no significant difference was observed by critical difference analysis between sandwich ELISA and virus isolation; sandwich ELISA and multiplex PCR. While virus isolation and multiplex PCR differ significantly at 1% level of significant.

ACKNOWLEDGEMENTS

Authors are thankful to the Dean, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, India; and Director, Project Directorate on Foot and Mouth Disease, Indian Veterinary Research Institute (I.V.R.I.) campus, Mukteswar, Uttarakhand, India for providing all the facilities to carryout the studies.

REFERENCES

Alexandersen S, Zhang Z, Donaldson A I and Garland A J. 2003. Pathogenesis and diagnosis of foot-and-mouth disease. *Journal of Comparative Pathology* **129** (1): 1–36.

Brooksby J B. 1982. Portraits of viruses: foot-and-mouth disease virus. *Intervirology* **18**: 1–10.

Callahan J D, Brown F, Osorio F A, Sur J H, Kramer E, Long G W, Lubroth J, Ellis S J, Shoulars K S, Gaffney K L, Rock D L and Nelson W M. 2002. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal of the American Veterinary Medical Association* **220** (11): 1636–42.

Giridharan P, Hemadri D, Tosh C, Sanyal A and Bandhopadhyay S K. 2005. Development and evaluation of multiplex PCR for differentiation of foot and mouth disease virus strains native to India. *Journal of Virological Methods* **125**: 1–11.

Goel A C and Rai A. 1985. Growth curve plaque assay and inactivation studies of FMD virus subtypes O_5 O_1 and O_6 of Indian origin. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases* **6** (1): 16–28.

Hoffmann B, Martin B, Reid SM, Peter M, Oura CAL, Piet A, van Rijn Slomka M J, Jill Bank Ian H, Brown D J, Alexander Donald P and King A M. 2009. A review of RT-PCR technologies used in veterinary virology and disease control: Sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Veterinary Microbiology* **139**: 1–23.

Marvin J G and Barry B. 2004. Foot and Mouth Disease. *Clinical Microbiology Review* **17** (2): 465–93.

Mishra N, Rai D V and Pattnaik B. 1995. Strain differentiation of foot-and-mouth disease virus type 'Asia1'. *Indian Journal of Animal Sciences* **65** (3): 368–75.

Pereira H G. 1977. Subtyping of foot and mouth disease virus. *Developments in Biological Standards* **35**: 167–74.

Prasad S, Kumar A, Ahuja K L and Sharma R. 1992. Prevalence of foot-and-mouth disease virus types in North west India 1985–87. *Indian Journal of Virology* **81**: 49–52.

Rai A. 1980. Subtyping of foot-and-mouth disease isolate types O and Asia 1 recovered from field outbreaks in India. *Indian Journal of Animal Sciences* **50** (2): 164–68.

Shaw A E, Reid S M, King D P, Hutchings G H and Ferris N P. 2004. Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *Rev sci tech Off int Epiz* **23** (3): 1003–09.

Tamilselvan R P, De Sanyal A and Pattnaik B. 2009. Genetic transitions of Indian serotype O foot and mouth disease virus isolates responsible for field outbreaks during 2001–2009. *OIE/FAO Reference Laboratories Network Meeting* from 23rd to 27th November 2009, pp 11– 13, New Delhi.

Woodbury E L, Samuel A R, Knowles N J, Hafez S M and Kitching R P. 1994. Analysis of mixed foot-and-mouth disease virus infection in Saudi Arabia: prolong circulation of an exotic serotype. *Epidemiology and Infections* **112**: 201–11.