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New approaches for post-vaccination surveillance of foot-and-mouth disease

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Summary. – Foot-and-mouth disease (FMD) is a contagious and important transboundary disease of cloven-hoofed animals and ruminants. In ruminants, an animal is considered as a foot-and-mouth disease virus (FMDV) carrier if a live FMDV/FMDV RNA is obtained from the oro-pharyngeal fluid (OPF) beyond 28 days after infection. These carrier animals may pose a risk for causing outbreaks in healthy animals. Moreover, it is important to conduct serosurveillance to know the virus circulation. In the present study, an ELISA was developed using field samples to detect FMDV specific secretory IgA antibodies. These samples were also tested for the presence of FMDV RNA using quantitative real-time PCR (qRT-PCR). It was found that more carrier animals were detected by IgA ELISA in comparison to qRT-PCR. Thus, IgA ELISA is an important tool to detect FMD carriers. An ELISA based on detection of antibodies against FMDV 2B non-structural protein (NSP) was also used to confirm the results obtained from screening of $3AB_3$ NSP ELISA. These two new approaches (IgA ELISA and 2B ELISA) form important tools for detection of carriers and virus circulation, respectively, during FMD eradication program.

Keywords: foot-and-mouth disease virus; carriers; IgA; 2B non-structural protein; $3AB_3$ non-structural protein

Introduction

Foot-and-mouth disease (FMD) is an important transboundary disease of ruminants and cloven-hoofed animals. It is caused by foot-and-mouth disease virus (FMDV) which belongs to the family *Picornaviridae* and the genus *Aphthovirus.* In ruminants, irrespective of vaccination status an asymptomatic, persistent infection can be established after recovery from FMD infection. An animal is considered as carrier when FMDV can be recovered in oropharyngeal scrapings after 28 days post-challenge (Sutmoller *et al.,* 1968). The existence of the carrier state has a significant impact on FMD control program (Parida *et al.*, 2007). Carrier animals may pose a risk of new outbreaks and spreading of the virus (Dawe *et al.*, 1994a,b; Hedger and Condy, 1985). There is a strong correlation between the circulating humoral antibody titre against FMDV and protection against the virus (McCullough *et al.*, 1992; Oh *et al.*, 2012). The first virus neutralizing antibody is IgM, appearing after 3–4 days of infection/vaccination, peaking after 10–14 days and thereafter declining (Golde *et al.*, 2011). IgG appears 4–7 days post infection/vaccination and becomes the major virus neutralizing antibody after 2 weeks (Collen *et al.*, 1989; Salt *et al.*, 1996). The titre of IgG1 was reported to be higher than IgG2 (Salt, 1993a). IgM followed by IgA and IgG are the major antibody subclasses found in the upper respiratory tract (Salt, 1993a). IgM and IgA mediated neutralizing activity has been observed in the pharyngeal fluid after 7 days of virus exposure (Francis *et al.*, 1983).

The oro-pharyngeal IgA responses have been studied in FMDV-vaccinated and infected cattle followed by demonstration that parenteral administration of conventional FMD vaccine does not elicit any IgA antibody in OPF (Archetti

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Abbreviations: CV = coefficient of variation; FMD = foot-and-mouth disease; FMD-CP = FMD control program; FMDV = FMD virus; NSP = non-structural protein; OPF(s) = oro-pharyngeal fluid(s)

et al., 1995; Francis *et al.*, 1983; Moonen *et al.*, 2004; Parida *et al.*, 2006; Salt, 1993a). Earlier, studies have been done in experimental animals to detect carriers by IgA ELISA (Pacheco *et al.*, 2010b; Parida *et al.*, 2006). Thus, IgA test has a considerable potential for the detection of sub-clinical infection in vaccinated cattle and for identification of persistently infected cattle following the application of a vaccinate-to-live policy (Biswal *et al.*, 2008). In the present study, IgA ELISA for the detection of anti-FMDV IgA in FMDV infected carrier animals was developed as a new and better approach for the detection of FMDV carrier animals, in comparison to the detection of FMDV RNA by qRT-PCR in field samples. In this study, IgA ELISA have been used to detect carriers in field samples. In India, established test recombinant $3AB_3$ ELISA (Mohapatra *et al.*, 2011) is used for serosurveillance of FMD and virus circulation. Also, a recombinant 2B NSP ELISA was used to confirm the results obtained from $3AB_3$ screening test, as an indicator of virus circulation in buffalo population.

Materials and Methods

Sample collection for IgA ELISA and qRT-PCR. In the present study, two types of clinical materials were procured: nasal samples and oro-pharyngeal fluids (OPFs). Thirty buffalos were selected randomly from different parts of Haryana state, India for the collection of nasal fluid and OPF. Nasal fluids were collected from the vestibule of nostril by using a $1/6^{th}$ portion of regular size cotton tampons pre-dampened by the addition of 0.5 ml of PBS with pH 7.5 and held by forceps. In the laboratory, approximately 1–2 ml of nasal fluid sample was extracted from each tampon by compression within the barrel of a syringe (Parida *et al.*, 2006). These nasal samples were tested for the presence of IgA antibody against the FMDV. OPFs were collected by using a probang sampling cup (Sutmoller *et al.*, 1968). The OPFs collected were tested for the presence of IgA antibody against FMDV and also for the presence of FMDV RNA. For negative set of samples, a total of 11 nasal and 11 OPF samples were collected from the place where FMDV has not been recorded for least 6 years. For positive set of samples, 11 nasal and 11 OPF samples were collected from 11 clinically FMDV infected animals for more than 28 days. These animals were clinically positive. These samples were used in deciding the cut-off value of IgA assay.

Serum samples for detection of antibodies against 2B and 3AB non-structural proteins. A total of 800 buffalo serum samples from 8 districts of Haryana, India under FMD control program (FMD-CP) were procured from Regional Research Center on FMD, LLRUVAS, Hisar, India. These samples were collected from the field after vaccination of buffalos with trivalent FMDV vaccine under FMD-CP in India.

Sample processing for IgA ELISA. For testing in IgA ELISA, OPF and nasal samples were processed. The OPF samples were centrifuged at 8,500 x g for 5 min at 4°C, supernatant was serially diluted two-fold starting with 1:2 dilution in blocking buffer. Nasal and OPF samples were also diluted in blocking buffer.

Indirect sandwich ELISA for detection of IgA antibodies. An indirect sandwich ELISA was developed to detect anti-FMDV IgA antibodies from OPF and nasal samples. Briefly, 96 well plates (Nunc Maxisorp™, ThermoFisher Scientific, USA) were coated with rabbit hyper immune sera diluted 1:6,000 in carbonate-bicarbonate buffer, pH 9.6 and were incubated overnight. The rabbit hyper immune sera were obtained from FMDV antigen detection kit, kindly provided by Project Directorate FMD (PDFMD), Mukteshwar, India. These hyper immune sera were serotype specific i.e. raised against a particular FMDV serotype (serotype O, serotype A). Next day, the plates were washed thrice with washing buffer (PBS with 0.5% Tween-20). After this, 50 µl of viral antigen (serotype O – 1:15 dilution and serotype A – 1:15 dilution) diluted in blocking buffer were added in separate wells and the plates were incubated at 37°C for 1 h. The FMDV antigens used in this study were from FMDV antigen detection kit, kindly provided by Project Directorate FMD (PDFMD), Mukteshwar, India. After washing thrice, 50 µl of diluted positive control, negative control, nasal and OPF samples were added into each well. The plates were incubated for 1 h at 37°C. After washing thrice, anti FMDV IgA in the samples were detected by adding 50 µl of diluted anti-bovine IgA-HRPO conjugate (Bethyl Laboratories, USA) to each well. The plates were incubated at 37°C for 1h and then washed thrice. Finally, 50 µl of freshly prepared substrate solution of O-phenylene diamine (OPD)/H_2O_2 (Sigma-Aldrich) was added to each well and plates were left in dark for development of the color. The reaction was stopped by adding 50 µl 1 mol/l sulphuric acid to each well. The optical density of the wells was measured using ELISA reader (Tecan, Austria) at 492 nm.

Interpretation of IgA ELISA. The final result of each test serum was expressed as the percent positivity (PP) value.

$$\text{OD (corrected)} = [\text{OD of sample}] - [\text{OD of antigen control}]$$

$$\text{Percent of positive control (PP)} = \frac{[\text{OD (corrected) of test serum}] \times 100}{[\text{OD (corrected) of positive control serum}]}$$

Interpretation of 2B and $3AB_3$ ELISA. The results were interpreted according to the method standardised by Project Directorate FMD, Mukteshwar, India. For $3AB_3$ ELISA, the samples were considered positive when the PP value was more than 40% and negative when PP value was less than 40%. For 2B NSP ELISA the samples were considered positive when PP value was more than 50% and negative, when the PP value was less than 50%.

Statistical analysis of ELISA results. To determine the performance and diagnostic parameters (specificity and sensitivity), non-parametric ROC curve analysis was performed in MedCalc statistical program (Belgium) using method suggested by DeLong and colleagues (DeLong *et al.*, 1988). The reference variable for infected animals showing clinical lesions and in-contact animals was indicated as one. For naïve animals from FMD free areas, the

reference variable was taken as zero considering the fact that there is no virus circulation/infection. A common cut-off value was decided for both nasal and OPF IgA tests. The cut-off was chosen as the OD value showing 100% specificity along with the least compromise in sensitivity.

Detection of antibodies against $3AB_3$ and 2B NSP of FMDV. For detection of anti-2B and anti-$3AB_3$ antibodies recombinant 2B and recombinant $3AB_3$ were used in indirect-ELISA format. Both 2B and $3AB_3$ are *Escherichia coli* expressed recombinant proteins. Recombinant 2B was kindly provided by PDFMD, Mukteswar, India. Details of 2B expression can be found in earlier published study (Biswal *et al.*, 2014). $3AB_3$ ELISA was performed using ELISA kit kindly provided by PDFMD, Mukteshwar. The $3AB_3$ and 2B NSP-ELISA was performed in Central FMD Laboratory, Mukteswar, Uttarakhand. Details of $3AB_3$ can be found in earlier studies by Mohapatra and colleagues (Mohapatra *et al.*, 2011). In each test, a positive and a negative control was used. Serum samples from animals infected with FMDV after 28 days post infection and showing OD value 1.2–1.5 were chosen as positive control. Serum samples collected after 3 days post infection having OD value 0.2–0.3 were taken as negative control. Briefly, ninety-six well ELISA plates (Nunc Maxisorp, Denmark) were coated with 50 µl/well each of $3AB_3$ and 2B NSP proteins (50 ng of purified recombinant protein per well) diluted in coating buffer, pH 9.6 and incubated at 4°C overnight. Next day, the plates were put in incubator at 37°C for 15 min before use. The plates were washed three times with washing buffer (PBS with 0.5% Tween-20). Subsequently, test sera and controls were diluted 1:20 in diluent buffer and 50 µl/well were added. The plates were incubated for 1 h at 37°C in a plate shaker. The plates were washed three times with a soaking period of 3 min each. To detect the reaction of different sera with $3AB_3$ and 2B NSP, 50 µl/well optimally diluted (1:3,000 in diluent buffer) rabbit anti-bovine IgG/peroxidase (Sigma-Aldrich) was added and the plates were incubated for 1 h at 37°C in a plate shaker. The plates were again washed three times with a soaking period of 5 min. 50 µl of freshly prepared substrate solution of OPD/H_2O_2 (Sigma-Aldrich) was added to each well and the plates were kept in dark for the development of color. The reaction was stopped by adding 50 µl/well of 1 mol/l sulphuric acid. The optical density of the wells was measured using ELISA Reader (Tecan, Austria) at 492 nm.

Isolation of FMDV RNA from OPFs and FMDV vaccine. Isolation of RNA was done according to the method described earlier (Chomczynski and Sacchi, 1987) with slight modifications. Briefly, a volume of 400 µl of OPF and vaccine were mixed with 600 µl TRIzol (Invitrogen, USA) in 2 ml microcentrifuge tubes and mixed vigorously by vortexing. Then, 180 µl of chloroform were added to each tube, vortexed and kept at room temperature for 12–15 min. Samples were centrifuged at 12,000 x g for 15 min and the aqueous phase supernatant was transferred to a fresh microcentrifuge tube (1.5 ml). An equal volume of isopropanol was added, mixed by vortexing and kept at -20°C overnight for RNA precipitation. Next day, the mixture was centrifuged at 12,000 x g for 20 min to pellet the precipitated RNA. The supernatant was carefully discarded without disturbing the pellet. The RNA pellet was washed using 70% ethanol by centrifugation at 7,500 x g for 5 min. The pellet was air-dried, and the RNA was dissolved in 20 µl of nuclease free water. RNA samples were stored at -70°C untill further use.

One step qRT-PCR. qRT-PCR was carried out in 7500 Standard Real-time PCR cycler (Applied Biosystems, USA) using Titan One Tube RT-PCR kit and Taqman probe (Sigma-Aldrich). Reactions were performed in 20 µl volume reaction mixture. Also, no template control was used. RNA extracted from commercially available FMD vaccine was used as positive control. Primer sequence and conditions used to amplify 3D region of FMDV was according to Callahan and colleagues (Callahan *et al.*, 2002). The template was subjected to following cycling conditions: 50°C for 2 min: 1 cycle; 95°C for 10 min: 1 cycle; 95°C for 15 s, 60°C for 1 min: 50 cycles. The primer and probe sequence: forward primer 5'-ACTGG GTTTT ACAAA CCTGT GA-3', reverse primer 5'-GCGAG TCCTGC CACG GA-3' TaqMan® probe: TCCTT TGCAC GCCGT GGGAC. The results of one step qRT-PCR were analyzed according to the standard of OIE (OIE 2009), that is Ct value of 40–50 is considered as borderline, Ct value of less than 40 is considered positive and Ct value less than 20 is considered strong positive.

Results

Determination of cut-off values to estimate diagnostic sensitivity and specificity for IgA ELISA

Eleven known positive animals and eleven known negative animals were tested to calculate the cut-off value through non-parametric ROC analysis for nasal and OPF IgA ELISA. Based on the best balance between sensitivity and specificity of the test using both FMDV serotype O and A antigens the cut-off value chosen was 0.45 optical density (OD). A common cut-off was decided for detection of carriers infected with both serotypes (O and A) from both nasal and OPF samples (Table 1).

The sensitivity and specificity for detection of carriers infected with FMDV A serotype using nasal samples was 81.82% and 100%, respectively. The sensitivity and specificity for detection of carriers infected with FMDV A serotype using OPF samples was 70% and 100%, respectively (Table 1). One animal's OPF sample was found positive by qRT-PCR. Same animal's nasal and OPF sample were positive in IgA ELISA to detect FMDV serotype O infection. Nine nasal samples and 9 OPF samples were found to be positive for FMDV. Eleven samples (5 nasal fluids and 6 OPF samples) were found positive for carriers infected with FMDV serotype O. Seven samples (4 nasal fluids and 3 OPF samples) were found positive for carriers infected with FMDV serotype A (Table 2).

Table 1. Diagnostic sensitivity and diagnostic specificity of IgA ELISA for detection of FMDV carriers from nasal and OPF samples of buffalos infected with FMDV O and A serotypes

Sample	Serotype	Sensitivity	Specificity	LR+	LR-
Nasal	O	90.91	100.00	11.00	0.091
OPF	O	90.91	100.00	11.00	0.091
Nasal	A	81.82	100.00	09.00	0.18
OPF	A	70.00	100.00	07.00	0.30

LR+: likelihood ratio positive; LR-: likelihood ratio negative.

Table 2. Details of buffalo samples collected for IgA ELISA

Sample	Type of sample	Clinical signs /Clinical status	+ve by IgA ELISA (pos./tested)	+ve by qRT-PCR (pos./tested)	Serotype O/A
Field samples	Nasal	Unknown	09/30	0/11	5/4
	OPF	Unknown	09/30	1/11	6/3
Negative samples	Nasal	-ve	0/11	0/11	
	OPF	-ve	0/11	0/11	
Positive samples (collected after 28 days of infection)	Nasal	+ve	11/11	0/11	
	OPF	+ve	11/11	0/11	

pos./tested: number of samples found positive after testing/total number of samples tested.

Table 3. 2x2 contingency table for number of samples found positive by 2B and $3AB_3$ ELISA

	$3AB_3$ ELISA	Positive	Negative	Total
2B ELISA	Positive	15	3	18
	Negative	49	733	782
	Total	64	736	800

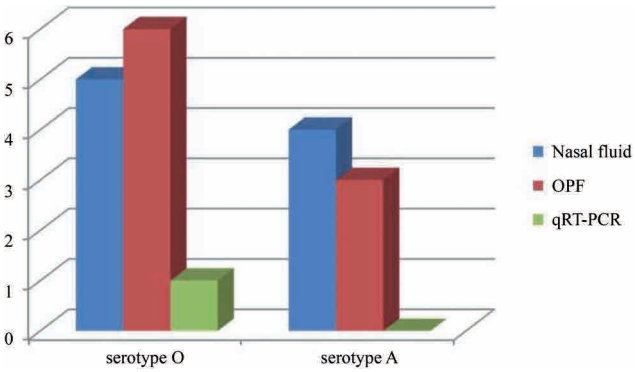

Fig. 1

Number of samples positive by IgA ELISA (OPF and nasal samples) and qRT-PCR (OPF sample)

Nasal fluid and OPF indicates number of samples positive by IgA ELISA. qRT-PCR indicates number of OPF samples found positive by qRT-PCR.

Detection of FMDV carriers by IgA assay

Eleven nasal and eleven OPF samples were tested by the IgA test developed. It was found that all 11 nasal and 11 OPF samples were found positive by IgA test. The clinical status and outcome of IgA test is shown in Table 2. Out of 30 tested samples, 5 nasal fluids and 6 OPF samples were found positive for anti-FMDV IgA against serotype O. Four nasal fluids and three OPF samples were found positive for anti-FMDV IgA against serotype A (Table 2, Fig. 1). IgA ELISA, percentage coefficient of variation (% CV) was analysed for positive and negative controls. The intrassay % CV and interassay % CV was below 5% and 10%, respectively.

Detection of virus circulation by 2B and $3AB_3$ ELISA

Serum samples from 800 randomly selected buffalos from eight districts of Haryana under FMD-CP were analyzed. In both 2B and $3AB_3$ ELISAs % CV was analysed for positive and negative controls. The intrassay % CV and interassay % CV was below 5% and 10%, respectively. In $3AB_3$ ELISA we have obtained 64 positive and 736 negative samples. Total of positive samples by 2B ELISA was 18 and 782 samples were negative. Out of these, 64 serum samples were found positive for anti-$3AB_3$ NSP antibodies and 18 serum samples were found positive for anti-2B NSP antibody (Table 3). Fifteen serum samples were positive for both anti-$3AB_3$ NSP antibodies and anti-2B NSP antibodies. The percentage of seropositivity found by $3AB_3$ was 8% (64/800) and by 2B

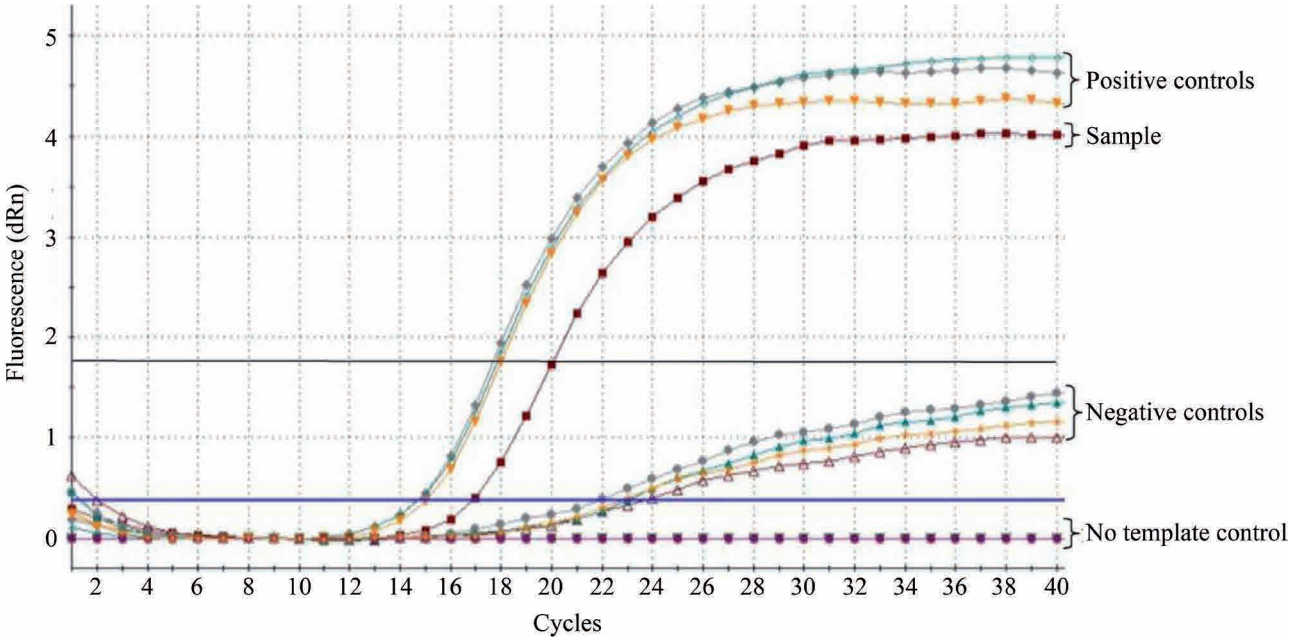

Fig. 2

Real-time PCR graph

Positive control (3 paralel samples), negative control (4 paralel samples), no template control (one sample) and one positive OPF sample obtained from field outbreak.

was 2.25% (18/800). However, after considering 2B as the confirmatory test the percentage positivity by combining $3AB_3$ and 2B was found to be 1.87% (15/800).

Detection of FMDV carrier buffalo by qRT-PCR

Thirty OPF samples were tested by one step qRT-PCR and one sample was found positive with the Ct value of 20 for the presence of FMDV (Fig. 2). The samples used as positive control were strong positive with a Ct value of 18. Samples used as negative controls were negative without any Ct value. Similarly, no template control (NTC) was also negative and didn't show any Ct value (Fig. 2).

Discussion

FMD is a major constraint to international trade as it prohibits export of animals and animal products from countries having FMD. In countries where FMD is endemic, the disease is controlled by biannual vaccination whereas in the countries free from FMD, stamping out (e.g. UK and France in 2001) or emergency vaccination (e.g. the Netherlands in 2001 and Korea in 2010) is practiced. If a live FMDV/FMDV genome is detected in the OPF after 28 days after infection the ruminant is considered as a carrier (Salt, 1993a; Sutmoller *et al.*, 1968, 2003). There is a possibility of developing the carrier state in ruminants following clinical and /or subclinical disease, irrespective of vaccination status (OIE, 2005; Salt, 1993b, 2004). There is 45% chance of immunized animals becoming virus carriers after a contact challenge (Cox *et al.*, 2006a; Parida *et al.*, 2006). There are few reports suggesting that carrier animals can transmit the FMDV under field conditions (Dawe *et al.*, 1994a,b; Hedger and Condy, 1985). However, as carriers may be considered a risk for transmitting infection, they must be identified by post-vaccination surveillance to be free of infection and to regain the FMD-free status for the purpose of international trade (Parida *et al.*, 2005).

Parenteral administration of FMDV antigen through vaccine does not generate mucosal antibody response, however it can be produced only through persistence of virus and its replication in the mucosal region of oropharynx. Detection of IgA in mucosal fluid is an indicator of persistent infection (Archetti *et al.*, 1995; Moonen *et al.*, 2004; Parida *et al.*, 2006; Salt *et al.*, 1996). It has also been reported that during the persistence of FMDV, the virus replicates in the pharyngeal lymphoid tissues of nasopharynx (Arzt *et al.*, 2010, 2011; Pacheco *et al.*, 2010a). Mucosal antibody response does not persist on the mucosal site in the absence of ongoing antigenic stimulus (Shewen *et al.*, 2009). It was found that anti-FMDV IgA antibodies present on the mucosal surface are an indicator of antibody response to FMD infection by the mucosal B cells but not due to migration from plasma

(Archetti *et al.*, 1995). In an experimental setting it was observed that a higher neutralizing antibody titer was present in nasal fluid than in oral fluid of both vaccinated and unvaccinated carrier animals (Archetti *et al.*, 1995; Kaaden and Matthaeus, 1970; Maddur *et al.*, 2008; Matsumoto *et al.*, 1978). Thus, secretory IgA from oropharynx, nasal and oral fluids are potent indicators of FMDV carrier.

In this study, 11 samples each from nasal fluids and OPFs were tested. By using the ROC analysis, a cut-off value of 0.45 was determined for IgA ELISA using nasal and oral fluids. At 0.45 cut-off, specificity of nasal and OPF IgA ELISA for detection of carriers infected with FMDV serotype A and serotype O was 100. Hence, in the present study 0.45 was considered as cut-off value and at this cut-off value the nasal IgA ELISA was found to be more sensitive with the sensitivity of 81.82% than oral IgA ELISA (sensitivity = 70%) for detection of carriers infected with FMDV serotype A. Both, in nasal fluids and OPFs, IgA ELISA was found equally sensitive (90.91%) at the cut-off value 0.45 for detection of carriers infected with FMDV serotype O. Virus or viral genome can be recovered from OPFs and from the scrapings of epithelial cells of the dorsal and ventral soft palate, dorsal pharynx and anterior oesophagus by qRT-PCR and also by virus isolation (Kitching, 2002). In the present study only one OPF sample was found positive by qRT- PCR for the presence of FMDV RNA, whereas a number of samples were found positive for the presence of anti-FMDV IgA in the nasal and oral fluid. This is in agreement with the previous study (Parida *et al.*, 2005) where FMDV in carriers could not be detected from the probang samples at any time point of sample collection. The successful detection of virus or viral genome in the probang samples is dependent on several other factors such as subsequent handling of samples, serial sampling procedure and the skill of the operator (Kitching, 2002). Hence, finding of the present study also supports the finding of Parida and colleagues (Parida *et al.*, 2006) that the presence of anti-FMDV IgA antibody in mucosal fluid is an indicator of oro-pharyngeal replication of FMDV. Due to unavailability of FMDV, vaccine sample and FMDV antigen was used to extract the FMDV RNA which was further used as positive control in qRT-PCR. In the present study, the IgA ELISA was developed as a proof of principle to detect carriers after FMD outbreaks. We were able to collect eleven nasal and eleven OPF samples from eleven animals as the disease was reported in those specific eleven animals only. However, in future we aim to validate the IgA assay with more negative and positive samples.

In India, FMD control program (FMD-CP) was launched in 2004 nationwide, including Haryana. In order to participate in international trade of animals and animal products, absence of FMDV circulation in the animal population needs to be proved. It has been documented that vaccinated animals may become carrier with or without showing clinical signs (Barnett *et al.*, 2004). It has been reported in experimental settings that, following contact challenge, 45% of vaccinated animals developed persistent FMD infection (Cox *et al.*, 2006b; Parida *et al.*, 2006). Since carrier animal poses risk for transmitting infection to susceptible populations, it is important to clearly identify them by post-vaccination surveillance to confirm them free from infection and, therefore, to regain the FMD-free status for the purpose of international trade (Parida *et al.*, 2005).

To regain the FMD free status without vaccination it is crucial to differentiate infected animals from vaccinated animals (OIE, 2005). This shows the importance of conduction of large-scale post outbreak serosurveillance to detect antibodies for FMDV NSPs. Particularly, in areas having low prevalence it is essential that the tests should have good specificity to interpret true positive results (Sorensen *et al.*, 1998). In India, under FMD-CP $3AB_3$ ELISA (Mohapatra *et al.*, 2011) is used for FMD serosurveillance. The 2B ELISA as a confirmatory test in conjunction with $3AB_3$ ELISA has already been validated in previous studies (Biswal *et al.*, 2014). Thus, in this study since $3AB_3$ ELISA is used as a standard test in Indian conditions, hence, 2B ELISA was tested in conjunction with $3AB_3$ ELISA to know the virus circulation in Haryana state of India. In the present study, $3AB_3$ protein and 2B protein were evaluated in an indirect ELISA format and 2B ELISA was validated as the confirmatory test to $3AB_3$ assay. The percentage positivity by 2B assay was found to be 2.25% whereas by $3AB_3$ assay it was 8%. By using 2B as confirmatory test the seropositivity was found to be 1.87 %.

To conclude, the two new approaches mentioned in the above work have potential to detect virus carriers and virus circulation. These approaches are potent tools to expedite the process of FMD eradication in endemic countries with more confidence. Recently, Jain and colleagues (Jain *et al.*, 2018) have developed a gold nanoparticle (GNP) based test for detecting FMD serotype specific antibodies. Based on this concept an IgA assay can also be developed for rapid diagnosis of FMD carriers. Moreover, IgA assay can detect carriers on individual basis which can be separated from healthy animals and thus reduces the risk of disease transmission.

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