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Research Article

DETECTION OF ANTI-NON STRUCTURAL PROTEIN ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE VIRUS IN THE BOVINE POPULATION OF HARYANA DURING FMD CONTROL PROGRAMME IN THE YEAR 2012

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ABSTRACT

The non structural protein ELISA (NSP-ELISA) was used to detect the antibodies against 3AB3 NSP of foot-and-mouth disease virus (FMDV) in the bovine serum samples collected from different districts of Haryana. The NSP-ELISA as developed and standardized using *E. coli* expressed recombinant r3AB3 by the Project Directorate on FMD, Mukteswar, Uttarakhand was used in the present study. Serum samples from a total of 4200 randomly selected animals from all the 21 districts (200 from each district, 100 each from cattle and buffalo) of Haryana [eight under FMD-Control Programme (FMD-CP, launched in 2003-04) Pre-Phase XV and 13 under extended FMD-CP, launched in 2010-11, Pre-Phase V] were analyzed. Of the 4200 serum samples, 76 (1.809%) serum samples were found positive for anti-3AB3 NSP antibodies. The serum samples from eight FMD-CP districts of Haryana demonstrated anti-3AB3 NSP FMDV antibodies in 3% (48/1600) animals. On the other hand, the serum samples from extended FMD-CP districts demonstrated anti-3AB3 NSP antibodies in 1.077% (28/2600) animals. Higher virus activity was recorded in Bhiwani, Jhajjar and Sirsa (all FMD-CP districts). Significantly higher proportion of cattle (2.85%; 60/2100) had r3AB3 specific antibodies compared to buffaloes (0.76%; 16/2100).

Keywords: Foot-and-Mouth Disease, FMD-control programme, Non-structural Proteins, r3AB3 NSP-ELISA, Haryana

Foot-and-Mouth disease (FMD) caused by FMD virus (FMDV) is one of the most important viral diseases of ungulate animals causing heavy economical losses to livestock industry on account of direct losses in the form of productivity and indirect losses in the form of restriction on international trade of livestock and their products including their germplasm. The disease causes heavy economic losses to the tune of more than 4 billion US dollars per year (Venkataramanan *et al*., 2006). Several nations have attained FMD-free status through stamping out policy and/ or systematic mass vaccination programme and enjoy economic benefits from international trade in animals and livestock products (Kakker and Sharma, 2012).

In order to participate in the international trade, absence of FMDV circulation in the animals and their products in a particular region needs to be proved. Therefore, it is necessary to differentiate between the FMDV infected and vaccinated animals as the current OIE guidelines recommend the differentiation of infected and vaccinated animals (DIVA strategy) if vaccine has been used for control and eradication of FMDV (OIE, 2004). There are three basic tools for DIVA strategy for the FMDV: i) virus isolation from oesophageal-pharyngeal fluid (OPF), ii) detection of viral nucleic acid by polymerase chain reaction (PCR) and iii) non structural protein (NSP) serology. The first two methods are not suitable for the DIVA strategy because of low virus titer, intermittent nature of virus recovery and the possible presence of neutralizing antibodies in OPF. So, the third method, i.e. NSP serology is the most suitable method for the DIVA strategy. Antibodies to NSPs of FMDV are considered to be indicators of FMDV infection for discrimination of infected animals from vaccinated ones (De Diego *et al.*, 1997; Sorensen *et al.*, 1998; Bruderer *et al.*, 2004; Clavijo *et al.*, 2004a). Enzyme-linked immunosorbent assay (ELISA) based assays can be used to detect anti-NSPs antibodies in carrier cattle (Moonen *et al.*, 2004). Several recombinant NSPs of FMDV viz. 2B, 2C, 3A, 3AB, 3B, 3ABC and 3D have been used in NSP-ELISAs (Clavijo *et al*., 2004b; Brocchi *et al*., 2006). Of these, 2C, 3A, 3ABC or its derivatives such as 3AB3 based NSP-ELISA tests

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have emerged to be the most reliable indicators and appear to produce conclusive evidence of previous infection (De Diego *et al*., 1997; Mackay *et al*., 1998), whether or not the animals have also been vaccinated. Antibodies against 3ABC have been detected up to 395 days post infection in both cattle and sheep (Sorensen *et al.*, 1998). Although, the literature on 3AB3 NSP-ELISA is sparse (Silberstein *et al*., 1997; Sorensen *et al*., 1998; Yakovleva *et al*., 2006; He *et al*., 2010), it has been demonstrated that the antibody response against 3AB3 could be used as a reliable serological marker for DIVA (Chung *et al*., 2002).

FMD-Control Programme (FMD-CP) was launched in India during 10th Five Year Plan in selective regions to create FMD free zones through mass immunization of cattle and buffaloes. In Haryana, initially eight of the 21 districts namely Bhiwani, Fatehabad, Hisar, Jhajjar, Jind, Rohtak, Sirsa and Sonepat were covered under FMD-CP. The remaining 13 districts of Haryana were also covered for mass FMD vaccination with the help of resources from Central pool: Assistance to States for Control of Animal Diseases (ASCAD) till 2010. The FMD-CP was extended to cover whole of Haryana during 2011, thereby replacing the annual mass vaccination under ASCAD by biannual FMD vaccination. A total of 14 phases of mass FMD vaccinations have been carried out since the start of the programme i.e., January 2004 till October 2012 at a regular interval of 6-8 months in FMD-CP districts and 11 phases of vaccinations in 13 districts under ASCAD (seven)/ extended FMD-CP (four) in Haryana.

The Regional Research Centre (RRC) on FMD, Hisar is actively participating in implementation of FMD-CP by providing logistic support in the form of surveillance and sero-monitoring work in Haryana. The present communication describes detection of antibodies against 3AB3 NSP of FMDV from the bovine serum samples collected from all the 21 districts of Haryana during the FMD-CP Pre Phase XV (eight districts) and extended FMD-CP Pre Phase V (13 districts).

MATERIALS AND METHODS

Serum Samples: A total of 4200 random serum samples of bovines (2100 each from cattle and buffalo) received at the RRC of All India Co-ordinated Research Project on FMD (ICAR), Department of Veterinary Microbiology of this university were used for the detection of anti-3AB3 NSP antibodies against FMDV. These samples were pre-vaccination serum samples from all the 21 districts (200 from each district) of Haryana before the start of FMD-CP Phase XV (eight districts, Pre- Phase XV, 1600 samples) and extended FMD-CP Phase V (13 districts, Pre- Phase V, 2600 samples) from both cattle and buffaloes (10 each of cattle and buffalo from ten villages of each district).

r3AB3 NSP-ELISA: The r3AB3 NSP-ELISA was performed to demonstrate antibodies against NSP of FMDV using *E. coli* expressed r3AB3 as developed and standardized by the Project Directorate on FMD (PDFMD), Mukteswar, Uttarakhand (Mohapatra *et al*. 2011). The r3AB3 NSP-known positive and known negative sera were received from the Central FMD Laboratory, Mukteswar, Uttarakhand. Briefly, 96 well ELISA plates (Nunc Maxisorp, Denmark) were coated @ 50µl/well r3AB3 NSP (~ 40 ng/well) diluted in coating buffer (Sigma) and incubated at 4^{0}C overnight. The plates were then kept in an incubator at 37^{0}C for 15 min. After washing thrice with washing buffer, 1:20 dilution of test sera made in diluent buffer @ 100 µl/well were added in the wells along with reference positive and negative sera. The plates were incubated for 1h at 37^{0}C in a plate shaker. After incubation, the plates were washed thrice with a soak period of 3 min each. The reaction of different sera with r3AB3 NSP was detected by addition of 50µl/well optimally diluted rabbit anti-bovine IgG peroxidase (1:2000 in diluent buffer) and incubated for 1h at 37^{0}C in a plate shaker. Finally, after washing five times with a soak period of 5 min each, 50µl of freshly prepared substrate solution of Orthophenylene-Diamine (OPD)/ H_2O_2 was added to each well and plates were left in dark for the development of colour. The reaction was stopped by adding 50µl/well 1M H_2SO_4. The optical density (OD) of the wells was measured using ELISA reader (TECAN, Austria) at 492nm. The test was considered valid provided the mean absorbance of the positive control wells was not less than 0.8. Likewise, the test was rejected if the mean absorbance of the negative control serum was >0.3. The OD in background control wells should was <0.01.

The final result for each test serum was expressed as the percent positivity (PP) value, calculated by dividing the OD of the test serum by that of the positive control serum and then multiplying with 100. The result was interpreted as 3AB3 NSP positive, if PP value was more than 40%; and negative, if PP value was less than 40%.

RESULTS AND DISCUSSION

Of the 4200, a total of 76 (1.809%) serum samples from both cattle and buffaloes were found positive for anti-3AB3 NSP FMDV antibodies; the district-wise distribution is depicted in Table 1. Animals from FMD-CP districts (eight districts, Pre- Phase XV) of Haryana demonstrated anti-3AB3 NSP FMDV antibodies in 3.00% (48/1600) animals (Table 2). On the other hand, the animals from extended FMD-CP districts (13 districts, Pre- Phase V) demonstrated anti-3AB3 NSP antibodies only in 1.077% (28/2600) animals (Table 2, Fig. 1). The difference in the anti-3AB3 NSP antibodies profiles among FMD-CP and extended FMD-CP districts indicated that animals from the later demonstrated slightly lower positivity despite the fact that animals in FMD-CP districts had received 14 rounds of vaccinations where as animals from extended FMD-CP districts had received only 11 rounds of vaccinations. However, the higher level of anti-3AB3 NSP reactivity in FMD-CP districts might be attributed to the sporadic cases of FMD recorded in Bhiwani, Jhajjar and Rohtak districts (all FMD-CP districts) during previous year i.e. February and March 2012 (Anon, 2012) whereas no sporadic case of FMD was recorded in districts under extended FMD-CP during previous year.

Table 1
Animals showing anti-3AB3 NSP antibodies against FMDV in Haryana

Region	District	No. of samples	
		Tested	Positive (%)
Northern	Panchkula	200	1 (0.50)
	Ambala	200	0 (0.00)
	Yamunanagar	200	4 (2.00)
	Karnal	200	0 (0.00)
	Kurukshetra	200	3 (1.50)
	Kaithal	200	1 (0.50)
	Panipat	200	1 (0.50)
	Total	1400	10 (0.714)
Central	Sirsa	200	21 (10.50)
	Fatehabad	200	2 (1.00)
	Hisar	200	2 (1.00)
	Jhajjar	200	13 (6.50)
	Jind	200	4 (2.00)
	Rohtak	200	0 (0.00)
	Sonipat	200	1 (0.50)
	Total	1400	43 (3.07)
Southern	Bhiwani	200	5 (2.50)
	Mohindergarh	200	3 (1.50)
	Rewari	200	10 (5.00)
	Gurgaon	200	1 (0.50)
	Mewat	200	0 (0.00)
	Faridabad	200	0 (0.00)
	Palwal	200	4 (2.00)
	Total	1400	23 (1.642)
	Grand Total	4200	76 (1.809)

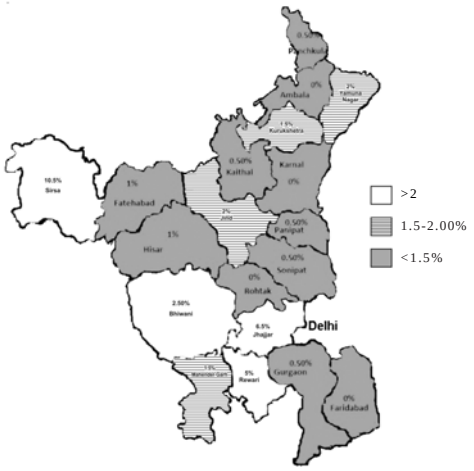

Fig 1. Animals showing anti-3AB3 NSP antibodies against FMDV in Haryana.

It is worthwhile to mention that the seven districts (Panchkula, Ambala, Yamunanagar, Karnal, Kurukshetra, Kaithal and Panipat) in northern part of Haryana demonstrated very low percentage of 3AB3 positive animals (0.714%, 10/1400). On the other hand, the districts, (Sirsa, Fatehabad, Hisar, Jhajjar, Jind, Rohtak and Sonipat) in central part of Haryana had 3.07% (43/1400) 3AB3 positive animals (Table 1). In this region, highest virus activity was recorded in Sirsa and Jhajjar (10.5 and 6.5%, respectively). The virus activity in Jhajjar district may be attributed to sporadic cases of FMD recorded during March 2012 (Anon, 2012). However, the districts in southern part of Haryana, i.e. Bhiwani, Mohindergarh, Rewari, Gurgaon, Mewat, Faridabad and Palwal had 1.642% (23/1400) anti-3AB3 positive animals (Table 1). In this region, highest virus activity was recorded in Rewari and Bhiwani (5 and 2.5%, respectively). The

Table 2
Animals showing anti-3AB3 NSP antibodies against FMDV in FMD-CP (Pre-Phase XV) and extended FMD-CP (Pre-Phase V) districts of Haryana

Districts	No. of serum samples	
	Tested	Positive (%)
FMD-CP (8)	1600	48 (3.00)
ASCAD (13)	2600	28 (1.077)
Total	4200	76 (1.809)

Table 3
Species-wise distribution of animals showing anti-3AB3 NSP antibodies against FMDV in Haryana

Programme (Districts)	Number of serum samples showing anti-3AB3 NSP antibodies			
	Cattle		Buffaloes	
	Tested	Positive (%)	Tested	Positive (%)
FMD-CP (8)	800	39 (4.87)	800	9 (1.12)
ASCAD (13)	1300	21 (1.61)	1300	7 (0.53)
Total	2100	60 (2.85)	2100	16 (0.76)

virus activity in Bhiwani district may be attributed to sporadic cases of FMD recorded in this district during March 2012 (Anon, 2012). Since southern districts of Haryana share border with Rajasthan, higher virus circulation in these districts stresses the need for extension of FMD-CP at least in the districts of Rajasthan bordering Haryana in order to further minimize virus circulation in Haryana. Further, the higher anti-3AB3 NSP reactivity in these districts as well as Sirsa (in northern Haryana) may also be due to uncontrolled nomadic herd movements in Haryana from adjoining states like Rajasthan as the maximum number of anti-3AB3 NSP positive animals have been observed in border districts to Rajasthan.

Species-wise analysis of the data revealed that significantly higher proportion of cattle (2.85%; 60/2100) had anti-3AB3 NSP FMDV antibodies compared to buffaloes (0.76%; 16/2100, Table 3) suggesting FMDV circulation to be more in cattle than buffaloes. All the four FMD outbreaks recorded during 2009 and 2010 were in four districts of Haryana involving cattle only (Kakker and Sharma, 2012). It is not clear whether this difference was attributed to species determinants for comparative virus susceptibility or due to better protection in buffaloes following vaccination. However, there are evidences that after launch of FMD-CP in Haryana, most of the sporadic cases of FMD have been recorded in cattle only. Further, the post-vaccinal immune response was better in buffaloes compared to cattle during different phases of vaccination (Anon, 2010, 2011, 2012).

As a result of bi-annual vaccination under FMD-CP in Haryana a declining trend in the number of anti-NSP FMDV positive animals has been observed. Using 3A NSP-ELISA, Kumar *et al*. (2007) detected 31 and 18% anti-3A NSP FMDV positive animals before first (start of FMD-CP) and before fourth phase of FMD-CP, respectively which further reduced to 12% (Anon, 2009) before eighth phase of FMD-CP vaccination. There was species difference in prevalence of anti-3AB3 NSP antibodies between cattle (2.85%) and buffalo (0.76%). These results are in accordance with the results of Kumar *et al*. (2007) who also observed the species difference on the development of anti-NSP antibodies in cattle and buffaloes. However, Kumar *et al*. (2007) observed higher percentage of anti-3A NSP antibodies i.e., 35.2 and 14.81% in cattle and buffalo, respectively in 3A NSP-ELISA. One possible explanation of the species difference may be that cattle develop more prominent clinical signs than buffaloes, which in turn, may be correlated to higher replication of the virus and hence a higher prevalence of anti-NSP FMDV antibodies in cattle. The anti-3A NSP FMDV antibodies have been reported to exist upto 510 dpi in cattle (Kumar, 2006). Though persistent infection may also occur in buffaloes, persistence of anti-3A NSP FMDV antibodies is less compared to cattle.

The NSP serology has great advantages over other tests for DIVA strategy like its high throughput and persistence of antibody response against NSPs for longer duration. However, the NSP serology for DIVA may have limitations in differentiating the carrier animals from those which had mere contact with FMDV and have completely eliminated the virus following infection, as serum antibodies against NSPs will be produced in both cases. By definition, the FMDV carrier animal is one in which virus persists beyond 28 days particularly in OPF (Sutmoller *et al.*, 2003). Therefore, the identification of an infected carrier in true sense, requires virus isolation (Zhang and Alexandersen, 2003), viral genome detection by RT-PCR (Clavijo *et al.*, 2004a), and/ or IgA detection in OPF (Parida *et al.*, 2006) along with the application of NSP serology.

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