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Systematic follow-up investigation of NSP seroreactors and in-contact cattle and buffaloes for foot-and-mouth disease virus using probang sampling

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Abstract

Background Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of cloven-hoofed animals. In India, the FMD Control Program has been ongoing for the last two decades. A 3AB3 nonstructural protein (NSP)-based indirect ELISA test is used for population serosurveys to differentiate between infected and vaccinated animals (DIVA). In the present study, a systematic follow-up investigation of the NSP seroreactors and in-contact bovines was carried out from rural cohorts as well as an organized farm in Haryana, India to identify the carrier or neoteric animals. No FMD outbreak was reported from Haryana, a Northern state of India in 2022 and NSP reactivity has also consistently been under 10% for the last five years (2018–2022).

Results Bovines from ten villages of district Hisar, Haryana, demonstrated 5.3% (20/377) [cattle (11.3%; 12/106) and buffaloes (3.0%; 8/271)] FMDV 3AB3 NSP reactivity. Out of those 20 NSP reactors, nine months later, two buffaloes were randomly screened. Both were found negative for NSP reactivity as well as for FMDV in oropharyngeal fluid (OPF) by reverse transcription-multiplex polymerase chain reaction (RT-mPCR) using 1D/2B gene-specific primers. Further screening was done in a herd of regularly vaccinated cattle ($n = 11$) of an organized farm with no history of FMD outbreaks for more than a decade. All the susceptible animals were vaccinated with FMD + Haemorrhagic septicemia + Black Quarter combined oil adjuvanted vaccine. An NSP reactivity of 36.7% (4/11) in cattle calves 2–4 months after vaccination indicated either the exposure of animals to FMDV or the presence of residual NSPs in the vaccine. None of the OPF samples collected twice from these cattle at intervals of 36–44 days were found to be positive for FMDV with RT-mPCR. The observed NSP seropositivity could be linked to either false positive reactions or evidence of past exposure and virus elimination during OPF sampling. Nearly all animals exhibited protective antibody titers ($\geq \log_{10} 1.65$) against the structural proteins of FMDV serotypes O, A, and Asia-1 by Solid Phase Competitive ELISA (SPCE) indicating the effectiveness of vaccination.

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Conclusion The present study provided a preliminary follow-up investigation to assess the status of NSP seroreactors to establish the circulation of FMDV in the animal population, if any, so that the effectiveness of the ongoing vaccination program could be assessed and potential disease-free zones could be identified.

Keywords Foot-and-mouth disease virus, Reverse transcription- multiplex polymerase chain reaction, Oropharyngeal fluid, 3AB3 non-structural protein enzyme-linked immunosorbent assay, Differentiation between infected and vaccinated animals, Solid phase competitive ELISA

Introduction

World Organization for Animal Health (WOAH)-listed foot-and-mouth disease (FMD) is a highly contagious, economically devastating vesicular disease of cloven-hoofed animals such as cattle, buffalo, pig, sheep, goat, camel, and more than 70 wildlife species [1]. The etiological agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* of the family *Picornaviridae*. Out of the seven FMDV serotypes (O, A, C, Asia-1, SAT1-3) found across the globe, only three serotypes, i.e., O, A, and Asia-1, are prevalent in India [2]. FMDV consists of approx. 8.4 kb positive sense, single-stranded RNA genome having a single long open reading frame that is translated into a polypeptide, which is proteolytically cleaved into four structural proteins (SPs: VP1, VP2, VP3, and VP4) and ten nonstructural proteins (NSPs: L, 2 A, 2B, 2 C, 3 A, 3B1-3, 3 C and 3D) [3]. India is considered to be endemic for FMD virus (FMDV) with the prevalence of infection reported from many parts of the country [2, 4]. During the severe incidence scenario, the total farm-level economic loss due to FMD in cattle and buffaloes in India was estimated up to USD 3159 million (INR 221,110 million) [5] while it was USD 6.0 million (INR 350.4 million) in Haryana during 2013-14 [6] which reduced to USD 0.15 million (INR 12.64 million) during 2017–2020 [7].

In ruminants, approximately 50–60% of animals after recovering from the acute stage of infection may have infectious FMDV persisting in the oropharynx beyond 28 days post-infection. These animals then become persistently infected despite regular vaccination and are referred to as carriers, whereby live virus or viral RNA may continue to be recovered from oropharyngeal fluid (OPF) for up to six months or more [8, 9]. Although most animals eventually recover from FMD, the disease can be fatal, especially in young animals. Some infected ruminants turn asymptomatic carriers but the possibility of transmitting the virus to other susceptible animals remains arguable. Another type of subclinical neoteric (temporally acute) infection refers to the vaccinated animals undergoing the acute phase of infection shedding substantially greater quantities of virus in oral- and nasal secretions than the carrier animals [10]. The sites of FMDV replication in persistently infected carrier animals have been examined by the detection of FMDV RNA using reverse transcription-polymerase chain reaction (RT−PCR) in OPF collected using probang cups [11] or by virus isolation in sensitive cell culture systems such as goat tongue cell line (ZZ-R 127), fetal porcine kidney cell line (LFBK-αvβ6) with primary bovine thyroid cells (BTY) being most effective [12, 13]. However, FMDV cannot be detected in regular oral or nasal swab samples collected from cattle during persistent infection [14]. Therefore, studies on FMDV carriers with no apparent clinical disease may provide useful information about silent carriers which may be a potential risk of outbreak in regions with endemicity.

Many FMD-endemic countries, including India, are participating in the OIE-endorsed Progressive Control Pathway for FMD (PCP-FMD), which facilitates the stepwise process toward FMD control and achieving eradication [15]. Detection of antibodies against FMDV NSPs in ELISA is used in population serosurveys as an indicator of exposure/virus circulation to differentiate between infected and vaccinated animals (DIVA) [16]. Demonstration of NSP antibody-free status in the population is a prerequisite for declaring disease-free zones and progressively regaining FMD-free status [17]. In India, the Indian Council of Agricultural Research-National Institute on FMD (ICAR-NIFMD), has adopted a sampling plan for FMDV sero-surveillance wherein young animals aged 6–18 months are surveyed to detect antibodies against FMDV NSPs to resolve the issue of false positives arising from repeated vaccinations with vaccines containing residual amount of NSPs [18].

Several NSPs of FMDV have been utilized as recombinant expressed antigens to detect anti-NSP antibodies in animals exposed to the virus or vaccinated with vaccines containing residual NSPs. The antigen of choice has been 3ABC polypeptide and its derivative recombinant fragments such as 3 A, and 3AB among others [16, 19]. The protein r3AB3 as an antigen has been widely implemented in India for DIVA screening in FMD surveillance. The r3AB3-based indirect ELISA has been tested to screen FMD-infected serum for up to 900 days post-infection [18]. Moreover, NSP ELISA based on 3ABC polypeptide has been shown to detect anti-NSP antibodies up to 233 dpi in experimentally infected cattle [20]. Also, another 3ABC ELISA has been used for long-term follow-up of a vaccinated farm in case of outbreak and monitored up to 1118 days post-outbreak [21].

The susceptible livestock population in Haryana has been vaccinated against FMDV serotypes O, A, and

Asia-1 under the FMD Control Programme (FMDCP) run by the Government of India since 2003-04 [2]. After the launch of the National Animal Disease Control Programme (NADCP) [presently Livestock Health and Disease Control Programme (LHDCP)] in 2019, the cattle and buffaloes of Haryana were vaccinated biannually against FMD + Haemorrhagic septicaemia (HS) combined oil adjuvanted vaccine [7]. No FMD outbreak was reported from the state of Haryana, India during 2022, and NSP reactivity was consistently under 10% for the last five years (2018–2022). Ideally, a follow-up investigation of NSP-seropositive animals through probang sampling is carried out in a region reporting no/low incidence of FMD. Consequently, ICAR-NIFMD has endorsed probang sampling from Haryana (in addition to Telangana and Andaman & Nicobar Islands) [7]. Active surveillance of animals without apparent clinical signs of FMD along with molecular epidemiological studies is needed to understand FMDV transmission and circulation within distinct geographic regions [10]. The present investigation was conducted as a systematic follow-up of NSP seroreactors by testing OPF of bovines for FMDV to establish virus circulation in the sample population.

Materials and methods

Sample collection

The present study involved the collection of blood and OPF samples from bovines of district Hisar, state of Haryana, India. The sampling population from rural cohorts included small-holding marginal farmers having 2–5 animals in a mixed system of rearing through grazing as well as stall feeding. The sampling from cattle at the organized farm pertained to a large herd maintained under regulated movement within the farm, with no unverified entry or addition of animals to the herd without vaccination, ensuring a check on virus entry.

Rural cohort

A total of 377 serum samples from 6 to 18 month-old cattle ($n = 105$) and buffaloes ($n = 272$) were collected from ten villages of district Hisar, Haryana during May 2022 (Fig. 1). The samples were collected using a two-stage stratified random sampling plan generated at reasonably high confidence (0.95%) using the epi-calculator under NADRES v2 (https://nivedi.res.in/Nadres_v2/Epical/stratified/random_sampling.php). The plan was jointly prepared by the ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI),

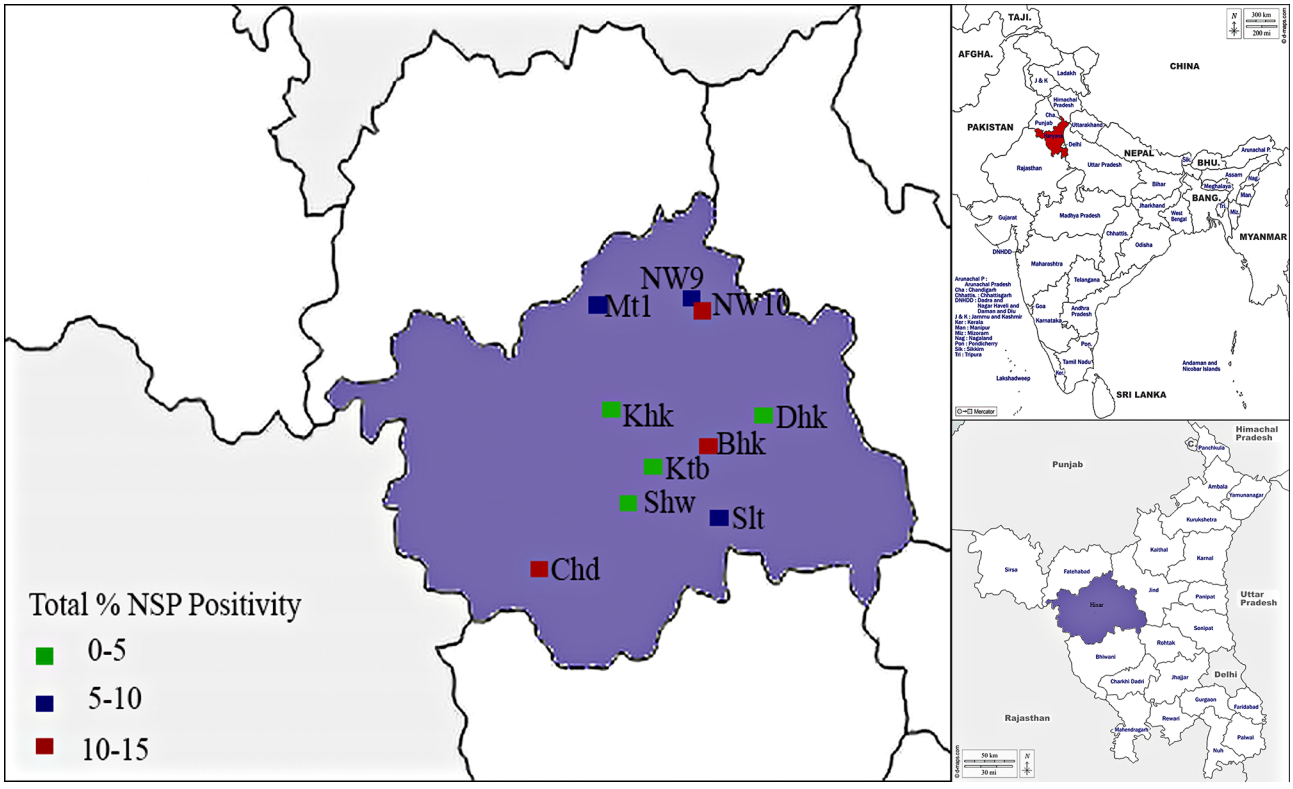

Fig. 1 Geographical distribution of the ten villages sampled in the study from the rural cohort. The range of foot-and-mouth disease virus non-structural protein percent positivity (Green: 0–5%; Blue: 5–10%; Red: 10–15%) for each village of district Hisar (shown in violet), state Haryana (shown in red), India. [Villages: Bhk: Bhaklana; Chd: Chaudhariwas; Dhk: Dharamkheri; Khk: Khokha; Ktb: Kutubpur; Mtl: Matloda; NW9: Narnaund Ward 9; NW10: Narnaund Ward 10; Shw: Saharwa; Slt: Sultanpur].
Source: https://d-maps.com/carte.php?num_car=4185&lang=en and https://d-maps.com/carte.php?num_car=8646&lang=en

Bengaluru, and ICAR-NIFMD, Bhubaneswar [18]. The OPF samples from NSP reactor buffaloes ($n = 2$) of village Bhaklana, were collected for FMDV detection as per the standard protocol [22]. The blood samples were also collected from the same animals to detect antibodies against FMDV SPs and NSPs.

Organized cattle farm

Paired blood samples (with & without anticoagulant) and OPF were collected from 6–18-month-old cattle ($n = 16$) of an organized farm at Hisar, Haryana. The first sampling was done randomly, 2–4 months after the animals were vaccinated with commercially available FMD + HS + Black Quarter (BQ) combined oil adjuvanted vaccine, followed by the second sampling at 36–44 days later. The vaccine contained FMDV serotypes O, A, and Asia-1 (potency: ≥ $3PD_{50}$ per dose), HS (upon challenge 2 out of 3 cattle should survive), and BQ antigens (upon challenge 4 out of 6 guinea pigs should survive).

The blood samples (with anti-coagulant) were brought from the farm under cold chain and analyzed within 24 h of collection. The serum was separated from blood samples (without anticoagulant) using standard procedures and stored at -80 °C till further use. The OPF samples were immediately stored in liquid nitrogen after collection and transported to the lab as per the biosecurity protocols for further processing.

Detection of antibodies against 3AB3 NSP of FMDV

The serum samples were screened for the presence of antibodies against 3AB3 NSP of FMDV using the indirect ELISA kit provided by the ICAR-NIFMD, Bhubaneswar [16]. The absorbance was measured at 492 nm with a reference at 620 nm using an ELISA reader (BMG LabTech, SPECTROstar^{Nano}). A sample producing an OD value more than the fixed cutoff ratio {(test serum sample mean OD/positive control serum mean OD) x 100, i.e., percent positivity value or PP value ≥ 40%} was considered positive for FMDV. The test was considered valid if the mean corrected absorbance of the positive control wells was greater than 0.8 and the PP value of the supplied negative control serum and background control were less than 20% and 10%, respectively. Additionally, the OD values in each of the duplicate wells of the positive control should not differ by more than 20% from the mean OD of the duplicate wells.

Solid phase competitive ELISA (SPCE) for Estimation of antibodies against FMDV structural proteins

The Solid Phase Competitive ELISA (SPCE) having 90% and 100% diagnostic sensitivity and specificity, respectively, was utilized to estimate the antibodies against the SPs of FMDV serotypes O, A, and Asia-1 [23]. The absorbance was measured at 492 nm using an ELISA reader (BMG LabTech, SPECTROstar^{Nano}) with reference at 620 nm. The SPCE test was considered valid provided the antigen and background controls showed OD values not less than 0.8 and not more than 0.1, respectively.

The percent inhibition in each well was calculated in relation to the antigen control (mean of six wells) using the formula:

$$\text{Percent inhibition} = 100 - \frac{Mean\ OD\ of\ sample - Mean\ OD\ of\ C_c}{Mean\ OD\ of\ C_o - Mean\ OD\ of\ C_c} \times 100$$

where C_c = conjugate control; C_o = antigen control.

The $\log_{10}$ value of the test sample dilution showing 35% inhibition was taken as the antibody titer of a given sample. Serum samples with antibody titers ≥ $\log_{10}$ 1.65 were considered to have a protective immune response against FMDV.

Molecular detection of FMDV

The RNA extraction was carried out from OPF samples using TRIzol LS reagent (Invitrogen™) according to the manufacturer's instructions followed by RT-multiplex PCR (RT-mPCR) for detection as well as typing of FMDV using 1D/2B gene-specific primers [24].

Blood parameters using a blood analyzer

The blood samples containing anticoagulants were analyzed within 24 h of collection using a blood analyzer. White blood cells (WBC; millions/mm^3), red blood cells (RBC; millions/mm^3), packed cell volume (PCV; %), mean corpuscular volume (MCV; fL), hemoglobin (Hb; gm/dl), platelets (millions/mm^3), mean platelet volume (MPV; fL), platelet crit value (PCT; %), platelet distribution width (PDW; µm), mean corpuscular Hb (MCH; Pg), MCH concentration (MCHC; %), red cell distribution width (RDW; %), lymphocytes (%), monocytes (%) and granulocytes (%) were measured. The blood parameters of seropositive and seronegative cattle were compared statistically using a one-way ANOVA test with SPSS software.

Results and discussion

FMD transmission and persistence in various domestic ruminants and susceptible livestock have been studied over the past several decades. In the context of India, cattle and buffaloes hold economic significance as the mainstay of milk production. The dynamics of the carrier state and its resolution over a period remain elusive despite extensive surveillance efforts and periodic assessments. In a consistently vaccinated population, the dynamics of virus transmission, especially in the carrier state are of vital importance to achieve the elimination of the virus. The situation is further aggravated in cases of superinfection with distinct serotypes. Thus, emphasizing the

importance of persistently infected carriers as potential mixing vessels for FMDV [25].

The present investigation was conducted to detect persistent or subclinical neoteric FMDV infection. NSP seroreactors and in-contact animals of the rural cohorts as well as organized cattle farm of district Hisar, Haryana (India) were systematically investigated. The state of Haryana has a total geographical area of 44,212 square km with a total livestock population of up to 7.11 million wherein cattle and buffalo populations are up to 1.93 and 4.37 million, respectively [26]. After the commencement of FMDCP in 2003-04 through mass vaccination of cattle and buffaloes, there was a remarkable decline in FMD outbreaks in Haryana. The economic cost evaluation revealed the impact of FMDV vaccination under the FMDCP in India with progressive control in disease prevalence. A subsequent decrease in economic burden was observed in states implementing the program compared to those that did not [5]. In 2022, the overall seropositivity for FMDV NSP was 5.3% (20/377) among bovines, with 11.4% (12/105) in cattle and 2.9% (8/272) in buffaloes, across ten villages in the Hisar district of Haryana (Fig. 1; Table 1). These findings closely mirrored the state-wide NSP prevalence of 5.1% (356/7044) among bovines, with cattle at 11.4% (239/2095) and buffaloes at 2.4% (117/4949), across all 22 districts of Haryana [27].

No FMDV NSP reactivity was observed in the samples of both cattle and buffaloes from three villages (Khokha, Dharamkheri, and Kutubpur) of district Hisar as well as in cattle of villages Saharwa and Bhaklana (Table 1). Although no NSP positivity was observed in buffaloes of three areas (Chaudhariwas and Narnaund Ward 9 &10), the reactivity was more than 25% in cattle of Chaudhariwas, Narnaund Ward 9, and Matloda, indicating virus activity in these areas. The distance between Narnaund and Matloda is around 30 km while it is more than 60 km from Chaudhariwas. The previous round of FMD + HS combined oil adjuvanted vaccination in the livestock of Haryana was carried out during November-December 2021, i.e., six months before collecting the serum samples for FMDV sero-surveillance, thus ruling out the possibility of DIVA reactivity due to residual amount of NSP in the vaccine, if any. If the animal receives vaccination with an inactivated purified NSP-free polyvalent FMD vaccine but remains unexposed to FMDV infection, it does not trigger an anti-NSP immune response in the host's body. Consequently, NSP antibodies serve as an indicator of infection. This differential induction of anti-NSP antibodies is exploited as DIVA ELISA to discriminate between infected and vaccinated animals. Nevertheless, seropositivity in NSP ELISA could be a scar of past infections; therefore, in some cases, the presence of NSP antibodies

Table 1 Location-wise foot-and-mouth disease virus non-structural protein seropositivity in cattle and buffaloes of district Hisar, Haryana (India)

S. No.	Village/ Locality	Total cattle + buffalo population [26]	Species (Population) [18]	Serum Samples Tested	Species-wise NSP positive samples	Species-wise NSP positivity (%)	NSP positive samples/ Total tested	Total NSP positivity (%)
1.	Khokha	1229	Cattle (289)	8	0	0	0/38	0
			Buffalo (940)	30	0	0		
3.	Dharamkheri	1617	Cattle (369)	9	0	0	0/38	0
			Buffalo (1248)	29	0	0		
5.	Kutubpur	1770	Cattle (478)	10	0	0	0/38	0
			Buffalo (1292)	28	0	0		
7.	Saharwa	2247	Cattle (555)	9	0	0	1/38	2.6
			Buffalo (1692)	29	1	3.4		
8.	Sultanpur	3980	Cattle (390)	4	0	0	2/38	5.3
			Buffalo (3590)	34	2	5.9		
10.	Narnaund Ward 9	281	Cattle (57)	7	2	28.6	2/36	5.5
			Buffalo (224)	29	0	0		
12.	Matloda	3954	Cattle (879)	8	2	25.0	3/38	7.9
			Buffalo (3075)	30	1	3.3		
14.	Chaudhariwas	2437	Cattle (737)	11	4	36.4	4/38	10.5
			Buffalo (1700)	27	0	0		
16.	Bhaklana	2071	Cattle (350)	6	0	0	4/38	10.5
			Buffalo (1721)	32	4	12.5		
18.	Narnaund Ward 10	848	Cattle (747)	33	4	12.1	4/37	10.8
			Buffalo (101)	4	0	0		
Total			**Cattle (4851)**	**105**	**12**	**11.4**	**20/377**	**5.3**
			Buffalo (16483)	**272**	**8**	**2.9**		

may not suggest the presence of the virus in the host at the time of sampling. Additionally, false positives in NSP ELISA may arise in the case of a repeated vaccination regimen with the FMDV vaccine containing residual amounts of NSP antigens [16, 28, 29]. Owing to the vast host diversity of FMDV, the possibility of maintaining the virus circulation in susceptible hosts by other species like pigs, goats, sheep, bluebuck (nilgai), yak, and mithun cannot be ruled out [30–32]. The molecular vigilance for FMDV thus becomes critical in the livestock scenario of mixed animal holdings of small and large ruminants. The significance of carrier animals in virus transmission and their potential to give rise to a new wave of infection in susceptible in-contact animals has thus far remained debatable [33], albeit they hamper the prospects of trade in livestock and livestock products as per current FMD policies globally [14, 28, 34].

The onset of clinical signs in FMD is associated with high levels of FMDV in blood and nasal fluid whereas the virus is first detectable in OPF during infection. OPF remains the sample of choice for detecting the FMDV genome by RT-PCR or by virus isolation in sensitive cell culture [12] for screening carriers and neoterics [9, 10, 12, 14, 35]. As per the guidelines of ICAR-NIFMD, Bhubaneswar, probang/OPF sampling was recommended from at least 10% of NSP-positive animals. Two out of 20 buffaloes from village Bhaklana, district Hisar, were found negative for NSP antibodies when tested nine months after the initial screening. The initial NSP seropositivity in ELISA could be linked to either exposure of animals to FMDV in the past and its elimination at the time of OPF sampling, or it could be due to false positive reactions. Serum samples from both buffaloes exhibited protective antibody titers ($\geq \log_{10}$ 1.65) against FMDV serotypes O, A, and Asia-1 (Table 2), which showed the effectiveness of FMDV vaccination in field conditions.

The RNA extracted from OPF samples of both buffaloes was found to be negative for FMDV when subjected to RT-mPCR (Supplementary Figure S1). A recent study conducted over 12 months with repeated OPF sampling at 30 dairy farms in Pakistan found that vaccinated Asian buffalo (*Bubalus bubalis*) can harbor significant subclinical FMDV circulation even in the absence of clinical symptoms. Multiple introductions of new FMDV serotypes and lineages were demonstrated on all the farms using RT-PCR on OPF samples without any clinical case of FMD [11]. Such a study emphasizes the role of serotype-specific virus detection in OPF samples to establish the carrier status of animals. Much remains to be understood about buffaloes as hosts and carrier animals for FMD. Moreover, the potential evolution of FMDV in the case of co-infected persistent carriers into novel strains or topotypes with better replication kinetics demands a concerted approach toward screening and elimination of the virus [2, 36].

Table 2 Buffaloes of rural cohort showing antibody titres against foot-and-mouth disease virus structural proteins in solid phase competitive ELISA

S. No.	Tag No. of animal	Log_{10} antibody titers against FMDV serotype		
		O	A	Asia-1
1.	100396-646900	1.65	1.65	2.25
2.	100397-943743	1.95	2.25	1.95

There is a need for establishing small-scale experimental data and reliable reconstruction of its implications toward transmission dynamics at higher scales such as farms, villages, countries, and transboundary regions [37–39]. Keeping this in view, a systematic follow-up investigation of NSP sero-reactor and in-contact animals was carried out in a regularly vaccinated herd at an organized cattle farm in Hisar. During the first as well as resampling after a gap of 36–44 days, 36.7% (4/11) of cattle were found positive for 3AB3 NSP antibodies against FMDV using NSP ELISA (Fig. 2; Table 3). The PP values of NSP-positive animals vaccinated two months (cattle ID: S434 and S445) and four months (cattle ID: 105 L and 111 L) before the first sampling were between 53–57%, and 43–44%, respectively. On average, the NSP positivity increased by 4.6–21.7% in the animals vaccinated six days before the second sampling. The PP values decreased by 7.4 and 13% in two animals (S434 and S445, respectively) not vaccinated before the second sampling, indicating either the exposure of animals to FMDV in the past or repeated immunization of animals with FMDV vaccine containing residual NSPs [28, 29].

The effectiveness of vaccination on the farm under study was evident from the fact that the cattle ($n = 10$) immunized with FMD + HS + BQ oil adjuvanted vaccine, 2–4 months before the first sampling, exhibited protective antibody titers ($\geq \log_{10}$ 1.65) against FMDV serotypes O, A and Asia-1 (Table 4). The only animal (211 S) exhibiting non-protective antibody titers ($< \log_{10}$ 1.5) against all three FMDV serotypes was seven months old at the time of the first sampling and was primo-vaccinated just six days before the second sampling. The same animal was also found to be NSP negative by DIVA ELISA (Table 3). There was no history of FMD outbreak on the farm for more than a decade as evidenced from the available records and no clinical FMD was observed in any of the animals.

Golde et al. reported that the antibodies in FMD-vaccinated hosts exhibited a rise in titer to partial protection and a dramatic decrease in virus shedding by the fourth-day post-vaccination, whereas complete protective titer was observed at day seven [40]. Moreover, seroconversion to 3ABC NSP antibodies has been observed at about six to eight days in cattle following exposure

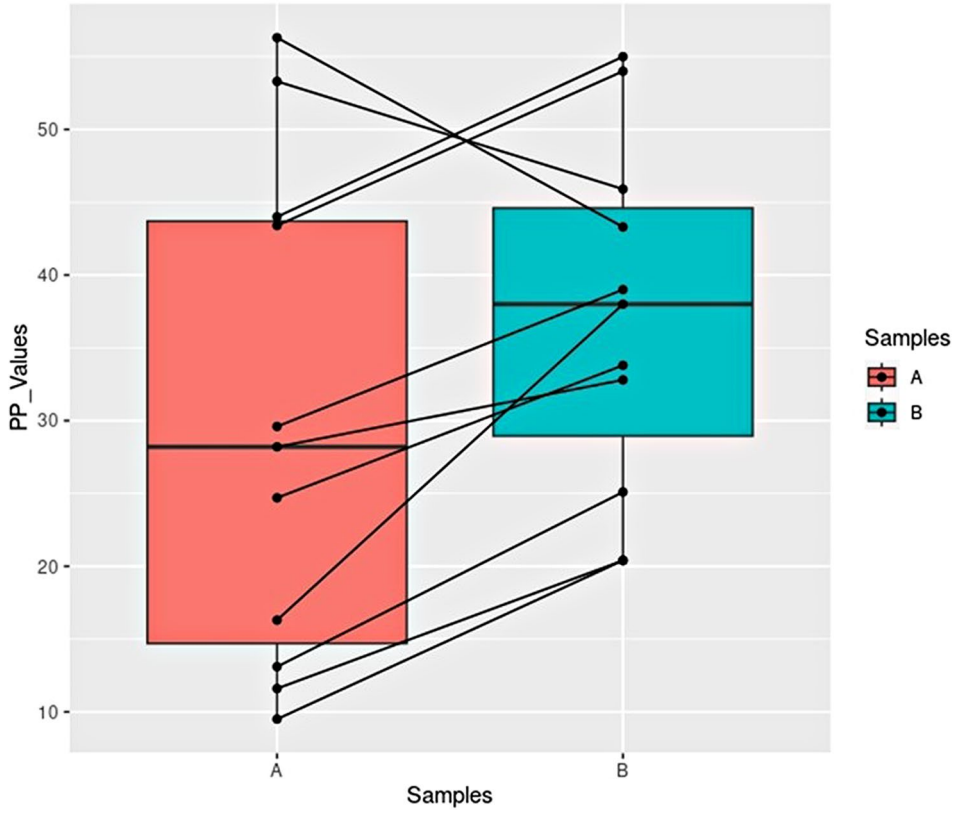

Fig. 2 Comparative trend of foot-and-mouth disease virus non-structural protein seropositivity at first (**A**: red colour) and second (**B**: green colour) sampling in cattle at an organized farm (Percent Positivity ≥ 40% was considered positive)

Table 3 Sero-reactivity against foot-and-mouth disease virus 3AB3 non-structural protein antigen using indirect ELISA in cattle at an organized farm

S. No.	Tag no.	Age (in months) at the time of first sampling	NSP reactivity during first sampling (PP values[#])	NSP reactivity during resampling (PP values)	ΔNSP values
1.	211 S[*]	07	Negative (09.5)	Negative (20.4)	10.9
2.	127 L[±]	11	Negative (28.2)	Negative (32.8)	4.6
3.	126 L[±]	12	Negative (16.3)	Negative (38.0)	21.7
4.	120 L[±]	15	Negative (24.7)	Negative (33.8)	9.1
5.	111 L[±]	16	Positive (43.4)	Positive (54.0)	10.6
6.	107 L[±]	17	Negative (29.6)	Negative (39.0)	9.4
7.	105 L[±]	18	Positive (44.0)	Positive (55.0)	11
8.	117 L[±]	18	Negative (11.6)	Negative (20.4)	8.8
9.	99 L[±]	18	Negative (13.1)	Negative (25.1)	12
10.	S445[$]	11	Positive (56.3)	Positive (43.3)	13
11.	S434[$]	14	Positive (53.3)	Positive (45.9)	7.4

[#] PP value ≥ 40% was considered positive

[*] Animal was primo-vaccinated six days before the second sampling

[±] Animals were vaccinated four months before the first sampling and six days before the second sampling

[$] Animals were vaccinated two months before the first sampling

[41]. However, indirect ELISA based on 2B antigen has reported the detection of NSP antibodies 10 days post-infection [16, 42].

The RNA extracted from all the OPF samples ($n = 11$) from the organized cattle farm was found to be negative for FMDV by RT-mPCR. Although some of the animals exhibited antibodies against the 3AB3 NSP of FMDV in ELISA (Table 3), none of the OPF samples was found to be positive for FMDV suggesting the NSP antibodies could be either false positives induced due to residual

Table 4 Cattle from an organized farm showing antibody titters against foot-and-mouth disease virus serotypes using solid phase competitive ELISA

S. No.	Tag no. of cattle	First sampling			Resampling		
		Log_{10} antibody titers against FMDV serotype					
		O	A	Asia-1	O	A	Asia-1
1.	211 S	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5
2.	127 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
3.	126 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
4.	120 L	> 2.4	1.95	1.95	1.95	1.95	2.25
5.	111 L	> 2.4	2.25	2.25	> 2.4	> 2.4	> 2.4
6.	107 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
7.	105 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
8.	117 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
9.	99 L	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4	> 2.4
10.	S445	> 2.4	2.25	2.25	> 2.4	> 2.4	2.25
11.	S434	2.25	2.25	2.25	2.25	2.25	2.25

NSPs in the vaccine (FMD + HS + BQ oil adjuvated) or due to silent circulation of virus in the herd without clinical signs. Future studies employing additional confirmatory tests, such as virus isolation or whole-genome sequencing, would be beneficial in distinguishing true carriers from false-positive NSP reactors.

In the present study, animals were not introduced to the farm without checking on FMD history; thus, it is unlikely that NSP positivity was observed to be attributed to the scar of past infection as suggested in NSP studies [16, 19]. This indicated that none of the animals ($n = 11$) could be identified as carriers or neoterics for FMDV. It is evident from past studies that unvaccinated animals shed a higher load of virus than vaccinated animals and it remains independent of the animal becoming a carrier at a later stage. Moreover, viral determinants of conversion to carrier state could not be found [14]. At the same time, the host variability towards becoming a carrier and later causing a potential source of the outbreak cannot be ruled out under stress or alteration in health status due to negative energy balance as suggested by a study on transmission dynamics in endemic regions [43]. Further, the statistical analysis revealed that there was no significant difference ($P > 0.05$) in the blood parameters between DIVA-positive and DIVA-negative animals (Supplementary data, S2 and S3).

The present study provided a preliminary follow-up investigation to assess the status of NSP seroreactors to establish the circulation of the virus in the bovine population, if any, in the state of Haryana, India. While this study provides valuable insights, the sample size is relatively limited. A larger and more diverse dataset in terms of geography would enhance the applicability of the results. Probang sampling for virus detection was carried out on a subset of NSP seroreactors, as per ICAR-NIFMD guidelines. Nevertheless, conducting repeated or longitudinal sampling over a longer timeframe could yield more definitive evidence regarding the persistence and transmission dynamics of FMDV. Since probang sampling requires expertise, the probability of sampling error cannot be ruled out if conducted by inexperienced personnel at the field level. To mitigate this, all sampling in the present study was conducted by experienced professionals following standardized protocols, ensuring reliability and accuracy in sample collection. It has been previously shown that animals protected with vaccination are likely to become carriers upon exposure to infection and hence pose a threat to the control efforts in a given region [41]. The effectiveness of the ongoing vaccination programs could be assessed and potential disease-free zones could be identified. In a country where FMD is endemic, the establishment of disease-free zones may support the progressive control and eradication of the disease [44].

Conclusion

The presence of carriers and silent or neoteric animals in a vaccinated population under surveillance is a challenge for achieving sustainable FMD-free status. The present study was conducted in Haryana, a northern state of India, where FMDV NSP reactivity has been less than 10% consecutively for the past five years with no FMD outbreak in 2022. The study emphasized the importance of virus screening in OPF through molecular detection to identify carriers or neoterics following FMDV sero-surveillance in a vaccinated population of cattle and buffaloes. The role of other susceptible species (e.g., pigs, small ruminants, and wild animals) in FMDV maintenance and cross-transmission needs further exploration to understand the potential for virus circulation beyond cattle and buffalo populations. Such a framework should be implemented in a wider population and region to counter the threat of the re-emergence of FMD within the vaccinated population.

Abbreviations

DIVA	Differentiation between infected and vaccinated animals
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot-and-mouth disease
FMDCP	Foot-and-mouth disease Control Programme
FMDV	Foot-and-mouth disease virus
LHDCP	Livestock Health and Disease Control Programme
NADCP	National Animal Disease Control Programme
NSP	Non-structural Protein
OD	Optical density
OPF	Oropharyngeal fluid
PCP	Progressive Control Pathway
RT-mPCR	Reverse transcription-multiplex polymerase chain reaction
SP	Structural Protein
SPCE	Solid Phase Competitive ELISA
WOAH	World Organization for Animal Health

Supplementary Information

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Supplementary Material 1

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Author contributions

AP: Investigation, Methodology, Formal Analysis, Writing - Original Draft Preparation and Data Curation. SD: Conceptualization, Methodology, Formal Analysis, Writing - Review and Editing, Supervision, Project administration, Funding acquisition. AL: Investigation, Methodology, Review, and Editing. AK: Methodology and Formal Analysis. PS: Methodology and Formal Analysis. NR: Investigation, Methodology, and Editing. CSP: Statistical analysis. VY: Critical analysis and Editing. NKK: Review and Editing. RR: Conceptualization, Methodology, Resources, and Formal Analysis. JKM: Supervision, Writing-Review and Editing. All authors critically revised the document and agreed on its content.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Declarations

Ethical approval

The sample collection was carried out with the approval of the Institutional Animal Ethics Committee (IAEC) vide no. VCC/IAEC/2022/1679–1705 dated 17.05.2022. All the methods were performed as per the relevant government guidelines and regulations and with the approval of the competent authority of Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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