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Applied Microbiology and Biotechnology

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https://doi.org/10.1007/s00253-022-12151-2

Applied Microbiology and Biotechnology (2022) 106:6745–6757
https://doi.org/10.1007/s00253-022-12151-2

METHODS AND PROTOCOLS

A new blocking ELISA for detection of foot-and-mouth disease non-structural protein (NSP) antibodies in a broad host range

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Received: 22 November 2021 / Revised: 5 August 2022 / Accepted: 25 August 2022 / Published online: 12 September 2022

Abstract

Large-scale monitoring of foot-and-mouth disease (FMD) in livestock is imperative in an FMD control program. Detection of antibodies against non-structural proteins (NSP) of FMD virus (FMDV) is one of the best tools to estimate the prevalence of past infection; availability of such a well-validated test is therefore essential. Using a FMDV 3B protein-specific monoclonal antibody, we have developed a new NSP antibody blocking ELISA (10H9 bELISA) and validated it on large panels of sera from different susceptible species. The diagnostic sensitivity of the ELISA was 95% with a specificity of 98%, similar to the values found using a commercial kit (PrioCHECK FMD NS test). The 10H9 bELISA can be used in a broad range of FMD susceptible species making it a very useful tool in monitoring the foot-and-mouth disease control programs by detection of virus circulation in the vaccinated populations.

Key points

- *A new ELISA for detection of foot and mouth disease (FMD) antibodies.*
- *Diagnostic sensitivity of 95% and specificity of 98%.*
- *Tested with panels of validated sera from broad host range.*

Keywords Foot-and-mouth disease · Monoclonal antibody · Blocking ELISA · Non-structural protein antibodies

Introduction

Foot-and-mouth disease (FMD) is a livestock disease of economic importance affecting cloven-hoofed animals, such as cattle, buffaloes, pigs, goats, and sheep. Currently, the disease can be controlled through vaccination in the endemic countries while movement control combined with stamping out is often used in disease-free countries in case of an FMD outbreak. Detection of antibodies to non-structural protein (NSP) of FMD virus (FMDV) is most widely used as a support tool to monitor the disease situation in a herd or population. It is useful for monitoring the effectiveness of the control program where vaccination is followed. A test for detection of NSP antibodies is essential to establish freedom from FMDV NSP antibodies, a criterion used to regain the WOAH (World Organisation for Animal Health) disease status free of FMD. This is applicable in countries almost free of infection after prophylactic vaccination as well as in the aftermath of an FMD outbreak in a previously FMD-free country, when a vaccinate-to-live policy is

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practiced (Barnett et al., 2015). Hence, the availability of a robust diagnostic tool for the detection of antibodies against FMDV NSP is essential for application in such situations.

The presence of antibodies to NSPs as a result of FMDV infection forms the basis of using NSP tests as a useful tool for differentiating infected from vaccinated animals (DIVA) (Sørensen et al. 1998). Therefore, for the NSP test application to be relevant in the vaccinated population, it is imperative to use the FMD vaccines formulated from antigens that do not induce antibodies against FMDV NSP. While using these NSP-free FMD vaccines, the demonstration of antibodies, particularly to 3ABC/3AB, is considered as a reliable indicator of virus activity in both vaccinated and naïve animals (Berger et al. 1990). The WOAH has described an indirect ELISA using the NSP as a screening test, combined with blot assay as confirmatory test (Anonymous, 2021) for demonstrating freedom from infection. Several indirect ELISA-based tests currently used in India and elsewhere are species specific, which are not suitable to detect antibodies in different species of animals and non-domesticated animals including wild or captive animals. An indirect ELISA was developed using protein G-HRP conjugate (Hosamani et al. 2015); though useful in different species, its application in certain species is limited as protein G has a variable affinity for IgG from different animal species. In this study, a new blocking ELISA (10H9 bELISA) based on a 3B specific monoclonal antibody targeting a common sequence motif in three subunits (3B1/3B2/3B3) of 3B protein was developed. Assay validation (Jacobson, 1998) was performed using panels of sera available at Indian Veterinary Research Institute (IVRI), India and Wageningen Bioveterinary Research (WBVR), Netherlands. The performance of the 10H9 bELISA was also compared with a similar commercial assay using a repository of samples representing diverse FMD status and geographical regions.

Materials and methods

Samples

Samples at IVRI laboratory

To determine the assay specificity, known negative sera from livestock ($n=505$) comprising samples from different livestock species were used. This included sera from unvaccinated apparently healthy young calves ($n=340$) aged 6–8 months procured from isolated herds from mountainous regions of Karnataka, as well as unvaccinated calves and heifers from the experimental dairy unit of IVRI, pig sera ($n=62$), sheep sera ($n=94$), and goat sera ($n=9$) from herds that were not vaccinated and found to be negative in virus neutralization test (Anonymous, 2021) using the currently used FMDV vaccine strains serotype O (IND/R2/1975), A (IND/40/2000), and Asia 1 (IND/63/1972).

To determine the assay sensitivity, known positive sera ($n=480$) were tested. This included cattle sera ($n=226$) collected from clinically affected calves from day 10 to day 150 after experimental infection with any of three FMDV serotypes (O, A, and Asia 1), buffalo sera ($n=107$) from buffalo herds that had suffered an FMD outbreak, and confirmed to be FMDV positive (serotype O) in solid phase competition ELISA (SPCE), pig sera ($n=36$) collected from a commercial piggery farm 8 weeks after the recovery from an outbreak of FMD (FMDV serotype O) and sheep sera ($n=70$) from a herd that recovered from natural FMDV infection. The pig and sheep samples had high antibody titers against FMDV serotype O, when tested in liquid phase blocking ELISA (Hamblin et al. 1986) and VNT; these high titers were indicative of infection. Additionally, goat sera ($n=18$) collected 9 to 137 days following experimental infection with FMDV serotype O (IND/R2/75) and guinea pig sera ($n=23$) collected 3 weeks after experimental infection with guinea pig-adapted challenge virus (FMDV A/IND/40/2000 strain) (Dhanesh et al., 2020) were included.

Sera from 10 mithun (*Bos frontalis*) and 18 unvaccinated wild ruminants including 4 black bucks (*Antilope cervicapra*), 10 gaur (*Bos gaurus*), and 4 nilgais (*Boselaphus tragocamelus*) previously tested negative in SPCE (Paiba et al., 2004) were tested. Sera collected from yak (*Bos grunniens*) ($n=90$), affected with FMD (FMDV serotype O) were also screened. The status of these samples was confirmed in SPCE before applying in NSP ELISA.

A set of 17 samples from unvaccinated calves, experimentally infected with FMDV O/IND/R2/1975 and collected 7 days post-infection, and 2 samples collected at 43 months post-infection, were also screened. These sets of sera were meant to analyze the early and late NSP antibody response, respectively.

Samples at WBVR

Sera ($n=2381$) collected from various FMD experiments carried out by WBVR laboratory in different species of animals including cattle, sheep, and pigs were analyzed. As many sera were collected shortly after infection, sera collected less than 6 days after infection were considered negative. In total, 1146 sera (473 before exposure and 673 less than 6 days after exposure) were considered negative: 74 sera from non-vaccinated cattle (10 before exposure and 64 less than 6 days after exposure) and 40 sera from non-vaccinated pigs (22 before exposure and 18 less than 6 days after exposure) as well as 585 sera from vaccinated non-exposed cattle (195 before exposure and 390 less than 6 days after exposure), 253 sera from vaccinated non-exposed sheep (158 before exposure and 95 less than 6 days after exposure), and

194 sera from vaccinated and non-exposed pigs (88 before exposure and 106 less than 6 days after exposure). A total of 1090 sera from cattle ($n=594$), sheep ($n=300$), and pigs ($n=196$) that were vaccinated and subsequently exposed (collected 6 days or more after exposure) to FMDV, as well as 145 sera samples collected from non-vaccinated cattle ($n=108$), sheep ($n=3$), and pigs ($n=34$) that were exposed to FMDV, were analyzed.

A unique set of sera collected at day 769 post-infection of 16 cattle experimentally infected with FMDV A/TUR/14/98 (Moonen et al. 2004) were tested to study the longevity of the NSP antibody response. Of these 16 cattle, 14 were vaccinated (5 cattle vaccinated with a 2 ml, 4 with 0.5 ml, and 5 with 0.125 ml of vaccine). All cattle were infected with the virus homologous to the vaccine strain. In addition, the Pirbright NS validation panel (Parida et al. 2007) was available at WBVR.

Expression of 3AB protein using the baculovirus system and purification of the protein

Viral 3AB sequence (containing $3B_1$, $3B_2$, and $3B_3$) was derived from FMDV serotype O (IND/R2/1975 strain) by using reverse transcription PCR. Recombinant baculovirus was prepared as described earlier (Hosamani et al. 2015) by cloning 3AB sequence having 6 × His tag on the N-terminal, downstream of polyhedrin promoter sequence of *Autographa californica nucleopolyhedrovirus*. The baculovirus (designated as Ac-FMDV.3ABv) was used for infection of High-five insect cells (Tn5) at a multiplicity of infection of 3, for expression of the protein. Cells were collected on day 4 post-infection by centrifugation at low speed (192 × *g*) for 10 min. Cells were suspended in Tris-NaCl buffer (pH 7.8) containing Tris (10 mM), urea (6 M), and NaCl (300 mM) and subjected to sonication in the presence of protease inhibitor cocktail (Amresco, USA). The clarified lysate was used for purification of 3AB by using Ni–NTA resin (Thermo, USA) chromatography under denaturing conditions. Purified protein fractions were checked on a 12.5% SDS-PAGE gel for protein purity and size of the protein. The protein expression was confirmed by western blot and ELISA using a serum from convalescent animal (FMDV O/IND/R2/75 strain). Purified protein fractions were pooled, dialyzed, and concentrated for subsequent use in the immunization of mice or application in ELISA.

Monoclonal antibody and its characterization

Mouse monoclonal antibodies (mAbs) were produced using hybridoma technology (Yokoyama, 1995). Briefly, myeloma (Sp2/0) cells were fused with splenocytes from mice immunized with recombinant 3AB antigen. Hybridoma cells were cultured in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich, USA) containing 15% fetal bovine serum (FBS). Culture supernatants were screened by indirect ELISA against 3AB antigen. Following the subcloning of reactive hybridomas, mAbs were screened for their ability to compete with polyclonal antibodies present in the FMD convalescent serum for binding to 3AB antigen. We selected a mAb 10H9D8 (referred to as 10H9 hereafter) based on the fact that an NSP-positive serum was able to effectively block the binding of mAb 10H9 to the coated antigen. Isotype of the mAbs in culture supernatants was determined using goat anti-mouse isotype-specific antibody ELISA (ISO-2 kit, Sigma-Aldrich, USA), following the instructions of the manufacturer.

Immunofluorescence assay: The assay was used to detect the native viral antigen in BHK-21 cells (clone 13, ATCC) infected with FMDV. For this, the cells were grown in 96-well tissue culture plates at a density of 50,000 cells per well in 100 µl of Glasgow's minimal essential medium containing 10% FBS. When the monolayer was formed after 18–24 h, the cells were infected with either FMDV serotype Asia-1 (IND/63/72 strain), type O (IND/R2/75 strain), or type A (IND/40/00 strain) at a multiplicity of infection of 0.01. After 12–16 h of incubation, when cytopathic effect was apparent, wells were rinsed with PBS. The cells were fixed with a chilled fixative containing acetone–methanol (1:1) for 10 min at 20 °C. After two rinses with PBS, wells were blocked with PBS containing 1% FBS and incubated at 37 °C for 30 min. After three rinses, mAbs were added to the wells (1:400 dilution in PBS with 1% FBS) and incubated at 37 °C for 1 h. Following this, the wells were again rinsed and incubated with 1:500 dilution of goat anti-mouse IgG Alexa Fluor® 488 conjugate (Thermo Fisher Scientific) at 37 ºC for 1 h. The cells were washed as described above and examined under a fluorescence microscope (Nikon Eclipse Ti-S).

Epitope mapping: Peptides (synthesized at GL Biochem, China) were used in the peptide inhibition ELISA in order to detect the target epitope of mAb binding on the 3AB protein. Five peptides of FMDV 3A and 3B protein sequences (Table 1) that constitute major epitopes of NSP antibody response in animals were tested. Individual mAbs were pre-incubated with either peptides or PBS separately in a deep-well plate and the mixtures (mAb + peptide or mAb + PBS) were allowed to react with recombinant 3AB antigen (60 ng) coated in duplicate wells of ELISA plate (Polysorp, Nunc). Following washing, anti-mouse IgG antibody conjugated with HRPO (Dako, Denmark) was added and incubated for 30 min. After washing the wells, the substrate solution containing ortho phenylenediamine dihydrochloride and H_2O_2 was added. The plate was incubated in dark at 37 ºC for 15 min. Color development was stopped by adding 1 M H_2SO_4 (50 µl in each well). Absorbance was measured at 492-nm wavelength. The ability of the peptides

Table 1 Peptides used for checking the epitope specificity of the monoclonal antibodies

Peptide	Peptide sequence	Length	Protein (region)	Reference
P1	TDDKTLDEAEKNPL	14 mer	3A (101–114)	Fu et al. (2017) and Oem et al. (2007)
P2	GPYAGPLERQKPLK	14 mer	3B1 (1–14)	Chung et al. (2018)
P3	GPYAGPMERQKPLK	14 mer	3B2 (1–14)	Fu et al. (2014) and Oem et al. (2007)
P4	GPYEGPVKKPVALK	14 mer	3B3 (1–14)	Fu et al. (2014) and Oem et al. (2007)
P5	PMERQKPLKVKAKA	14 mer	3B2 (6–19)	Fu et al. (2014)
P6	PVALKVKAKNLIVTE	15 mer	3B3 (10–24)	Fu et al. (2014) and Oem et al. (2007)

to inhibit binding of the mAbs was checked by comparing the OD value difference between peptide + mAb and mAb + PBS wells. Percent reactivity of the mAb with antigen, in terms of the OD value of mAb + peptide, relative to mAb + PBS was calculated using the formula: $(OD_{mAb + peptide}/OD_{mAb + PBS}) \times 100$.

NSP blocking ELISA (10H9 bELISA) using peroxidase-conjugated mAb

For the preparation of the conjugated 10H9, mAb was produced by culturing hybridoma cells in serum-free medium (Thermo Fisher Scientific, USA). Culture supernatant was then used for purification of the antibody using protein G resin (GenScript, USA) affinity chromatography. Purified 10H9 mAb was chemically conjugated with horseradish peroxidase by periodate method using Peroxidase labelling kit (Sigma-Aldrich, USA) following the instructions of the manufacturer. Working dilutions of the conjugated mAb and antigen for the blocking ELISA were determined using checkerboard titration with known FMD-positive and -negative sera as feasibility study. Feasibility analysis also included the panel of sera from strong positive, weak positive, and negative FMD status from different species, namely, cattle, buffaloes, sheep, goats, and pigs.

Recombinant 3AB antigen (1.2 µg/ml) in 50 µl, diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was coated in ELISA plate (Polysorp, Nunc) by incubation for 60 min at 37 °C. After washing the plate with PBS, serum samples diluted (1:2) in blocking solution were added and incubated for 90 min at room temperature. Dilutions of sera were made in a dilution plate before dispensing into the antigen-coated wells. Following washing in Tris (25 mM) buffered saline containing Tween-20 (0.025%), conjugated mAb was added and incubated for 30 min at room temperature. The plate was washed 4 times with wash buffer and the bound antibody-HRPO conjugate was measured by addition of 50 µl TMB substrate solution. The plate was then incubated at room temperature for 15 min in dark to allow color development. At the end of incubation, the reaction was stopped by adding 50 µl of 1.5 M H_2SO_4. Absorbance was measured at 450 nm (Tecan infinite F500 or Thermo Scientific Multiskan EX). Based on the OD values obtained, percentage inhibition (PI) was calculated for each sample using the formula: $[1 - (OD_{TS}/OD_{NCS})] \times 100$, where OD_{TS} is the mean of OD of test serum + mAb and OD_{NCS} is the mean of OD of negative control serum + mAb.

The PrioCHECK FMDV NS test

The commercial test, PrioCHECK FMDV NS kit (Thermo Fisher Scientific, USA), was performed as per the manufacturer's instructions using the overnight protocol. After color development using TMB substrate, absorbance was measured at 450 nm and PI values calculated. Samples showing a PI value equal to or higher than 50% were considered "positive," and those below 50%, as "negative" as prescribed by the manufacturer.

Determining the cutoff value for 10H9 bELISA

A total of 505 known negative samples and 480 FMDV-positive samples were chosen for determining the cutoff. Based on the OD value obtained for this set of samples, the PI values were calculated for each sample. Diagnostic sensitivity and specificity of the assay at different cutoff values were assessed by MedCalc software (11.5.0) using Youden's index and the ROC curve was deduced based on the PI values. A maximum value of the Youden's index was used as a criterion for selecting the optimum cutoff point.

Analytical sensitivity

The analytical sensitivity of 10H9 bELISA and PrioCHECK FMD NS test was determined with three known positive sera tested in serial dilutions, to compare their detection limits. Samples included two convalescent sera obtained from calves infected with FMDV serotype A (IND/40/2000 strain) and a serum from a rabbit immunized with recombinant 3AB antigen.

Analytical specificity

The sera that were positive for antibodies to bluetongue (sheep, $n = 5$), PPR (sheep and goats, $n = 4$), BHV-1 (cattle, $n = 2$), and contagious ecthyma (goat, $n = 2$) available

at IVRI were analyzed. Similarly, samples positive for antibodies against bovine herpesvirus (BHV-1; $n=1$), bovine virus diarrhea virus (BVD; $n=1$), and vesicular stomatitis virus (VSV; $n=2$) available at WBVR were also tested in the 10H9 bELISA.

Test reproducibility

A prototype kit was prepared following optimization of the antigen concentration, antibody dilution, conjugate dilution, incubation times, and stabilization of antigen coated in the ELISA plate. The kit was provided to four different laboratories in three institutes along with a set of serum samples ($n=45$). The samples from different species with PI values in the range of 0 to 99% were included in the panel. The results of test performed in these labs (on different days) were compared for reproducibility using Cochran's Q test.

Statistical analysis

The Clopper-Pearson 95% confidence interval was calculated using the function "exactci" in the "PropCIs" library in R (R core team, 2013). The test reproducibility across different laboratories was evaluated using Cochran Q test (MedCalc 11.5.0) and the null hypothesis for the test was that there were no differences between the variables.

Results

Characterization of 3AB protein

The expression of recombinant 3AB was confirmed by SDS-PAGE analysis and western blot (Fig. 1). Prominent band of 34-kDa size was detected in western blotting on probing with convalescent serum from cattle. The purified band of 3AB also showed similar mass in SDS-PAGE analysis.

Characterization of mAb

The monoclonal antibody (mAb) 10H9 was selected from a panel of 28 mAbs raised against the 3AB protein. The mAb, 10H9, was used in the blocking ELISA as a detection antibody as it competed well with polyclonal antibody in the preliminary screening. The mAb was found to be specifically inhibited by the antibodies in the serum from FMD-positive animals, from binding to 3AB coated on the ELISA microplate. The isotype of the mAb was IgG2a. Strong reactivity of the mAb with the antigen was evident in ELISA even with a higher dilution of hybridoma culture supernatant (1:800),

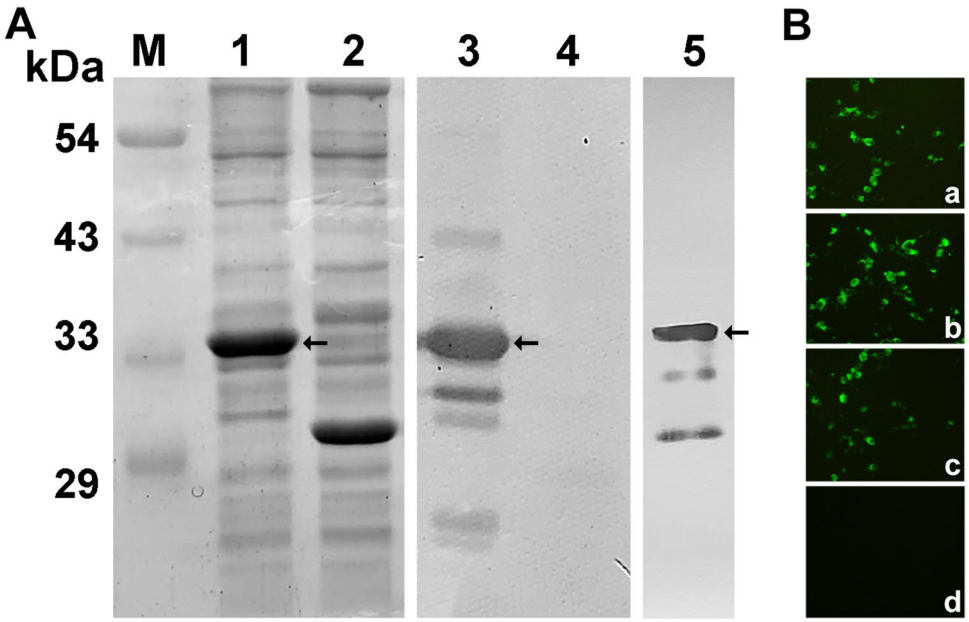

Fig. 1 Expression of 3AB protein and its antigenicity. **A** Lanes *M*, 1 and 2: SDS page analysis of protein, on staining with Coomassie brilliant blue R-250. Protein marker (lane 1), lysate from insect cells infected with recombinant baculovirus expressing either 3AB (indicated with an arrow, lane 1) or green fluorescent protein (GFP) as unrelated antigen (lane 2). Lanes 3–5: Western blot analysis. Reactivity of 3AB with FMD convalescent serum (lane 3), GFP showing no reactivity with the same serum. Reactivity of 3AB with the mAb 10H9 (lane 5). **B** Reactivity of mAb (10H9) with antigen expressed in BHK-21 cells. NSP antigen expressed in cells infected with either serotype O (**a**) or A (**b**) or Asia 1 (**c**) of FMDV showed reactivity with 10H9 mAb as evident from fluorescence, while no fluorescence detected in uninfected mock control (**d**)

in western blot (1:400 dilution), and immunofluorescence (1:250).

Western blotting and immunofluorescence assay

In western blot, mAb 10H9 reacted specifically with 3AB protein expressed in insect cells (Fig. 1 lane 5). BHK-21 monolayer cultures infected with different FMDV serotypes showed bright fluorescence in cell cytoplasm when incubated with mAb 10H9 and subsequently with goat anti-mouse Alexa Fluor® 488 conjugate. There was no fluorescence detected in the negative control (uninfected) BHK-21 cells (Fig. 1).

Mapping antigen-binding sites of the mAb 10H9 on the recombinant antigen 3AB

Three peptides (P2, P3, and P4; Table 1) covering amino acids 1–14 of 3B1, 3B2, and 3B3 (subunits of 3B) inhibited the binding of the mAb to the recombinant protein (Fig. 2).

Cutoff determination and assay validation

Using 480 positive sera and 505 negative sera tested, the optimal cutoff for having an acceptable Youden index was 49% with diagnostic sensitivity (Dsn) and diagnostic specificity (Dsp) of 95 and 99, respectively. A cutoff of ≥ 50% was hence chosen with the test Dsn of 95%, 95% CI (93, 97%) and a Dsp of 98%, 95% CI (96, 99%). For the PrioCHECK FMD NS test, the Dsn and Dsp values were 94% and 99%, respectively, for the given set of samples at the prescribed cutoff of 50%. The results of the 10H9 bELISA based on a total of 985 samples were highly correlating with the results obtained in the PrioCHECK FMD NS kit as evident from Table 2 and scatterplot (Fig. 3). The plot was based on a select panel of samples obtained from different species with known disease status with respect to FMD.

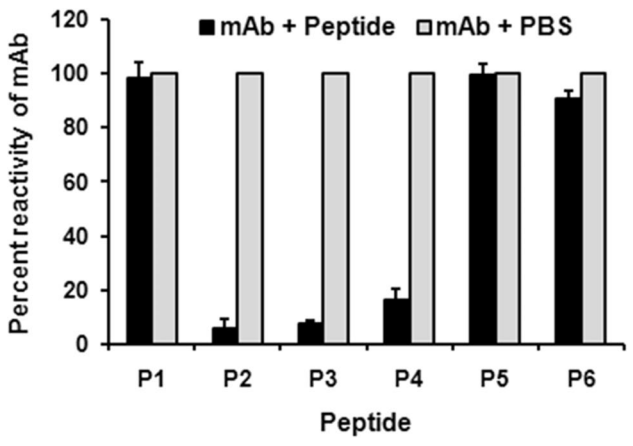

Fig. 2 Peptide blocking ELISA showing reactivity of the mAb in the presence or absence of peptide (Table 1) with the recombinant 3AB antigen

Antibody detection in different species

The performance of the test on serum samples, available at IVRI, from different species including cattle, buffaloes, sheep, goats, pigs, guinea pigs, yak, and various species of wild animals was analyzed (Table 2). The results in both the assays were highly consistent with an overall concordance of over 94% in different species. From cattle ($n = 115$) that had received trivalent (serotype O, A, and Asia 1) vaccine, 4/115 were positive in 10H9 bELISA and 2/115 in the PrioCHECK FMD NS test. Random samples of buffalo showed seropositivity of 16 and 19% in 10H9 bELISA and PrioCHECK FMD NS test, respectively. A limited number of samples collected from wild ruminants such as black bucks, gaur, mithun, and nilgai showed negative results with 100% concordance between both the assays.

Test reproducibility

The assay gave reproducible results with 100% agreement in the proportions across four labs in Cochran Q test, when tested with a panel of sera comprising samples from different species of animals (Supplementary Table S1).

Analytical sensitivity

The detection limit of the bELISA for three serum samples was compared to the PrioCHECK FMD NS kit and it was observed that the endpoint detection limits for both the assays are highly comparable (Fig. 4).

Analytical specificity

Sera positive to BHV-1, BVD, VSV, BTV, PPR, and contagious ecthyma did not show positivity in the assay.

Test validation

The scatterplot analysis of the PI values in both the 10H9 bELISA and the PrioCHECK kit performed at WBVR showed a high degree of similarity, with a small proportion of samples showing a discrepancy between the two assays (Fig. 5A). Among the discrepant samples, a total of 109 samples that are negative in PrioCHECK kit were positive in 10H9 bELISA. Of these, 69 were considered positive (6 or more days after infection) while the remaining samples were negative. Conversely 59 samples that were positive in the PrioCHECK test were negative in 10H9 bELISA of which 14 were negative samples and 40 samples originated from the infected cattle (6 or more days after infection).

Table 2 Comparison of the results between 10H9 bELISA and PrioCHECK test, obtained on panel of FMD sera available at IVRI laboratory

Species and sample details	Number tested	10H9 bELISA		PrioCHECK FMD NS ELISA	
		Positive 95% CI (range)	Negative (% < 95% CI >)	Positive (% < 95% CI >)	Negative (% < 95% CI >)
Cattle					
1. Not vaccinated and not infected	340	7 2.1 (0.8, 4.2)	333 97.9 (95.0, 99.2)	6 1.8 (0.65, 3.8)	334 98 (96.2, 99.4 >)
2. Vaccinated and not infected	115	4 3.5 (0.96, 8.7)	111 96.5 (91.3, 99.0)	2 1.7 (0.21, 6.1)	113 98.3 (93.9, 99.8)
3. Infected irrespective of vaccination status	226	209 92.5 (88.2, 95.5)	17 7.5 (4.44, 11.8)	205 90.7 (86.1, 94.1)	21 9.3 (5.8, 13.8)
Non-vaccinated and infected	167	160 95.8 (91.5, 98.3)	7 4.2 (1.7, 8.5)	160 95.8 (91.5, 98.3)	7 4.2 (1.7, 8.5)
Vaccinated and infected*	59	54 91.5 (81.3, 97.2)	5 8.5 (2.8, 18.7)	48 81.4 (69.1, 90.3)	11 18.6 (9.7, 31)
Buffalo					
1. Random	108	17 15.7 (9.5, 24)	91 84.3 (76, 90.5)	20 18.5 (11.7, 27.1)	88 81.5 (72.9, 88.3)
2. Infected	107	104 97.2 (92, 99.4)	3 2.8 (0.58, 8)	107 100 (96.6, 100)	0 0.00 (0, 3.39 >)
Pigs					
1. Non-vaccinated and not infected	62	0 0.00 (0, 5.78)	62 100 (94.2, 100)	0 0.0 (0, 5.78)	62 100 (94.2, 100)
2. Infected	36	33 91.7 (77.5, 98.3)	3 8.3 (1.8, 22.5)	34 94.4 (81.3,99.3)	2 5.6 (0.68,18.7)
Sheep					
1. Non-vaccinated and not infected	94	3 3.2 (0.7, 9.4)	91 97 (91, 99.3)	0 0.0 (0, 3.8)	94 100 (96.2, 100)
2. Non-vaccinated and infected	70	67 95.7 (88, 99.1)	3 4.3 (0.89, 12.0)	67 95.7 (88,99.1)	3 4.3 (0.89, 12.0)
Goat					
1. Non-vaccinated and not infected	9	0 0 (0, 33.6)	0 0 (66.4, 100)	0 0 (0, 33.6)	0 0 (66.4, 100)
2. Non-vaccinated and infected	18	18 100 (81.5, 100)	0 0.0 (0, 18.5)	18 100 (81.4, 100)	0 0.0 (0, 18.5)
Guinea pig					
1. Non-vaccinated and infected	23	21 (91.3 < 72, 98.9 >)	2 (8.7 < 1.1, 28.0 >)	19 (82.6 < 61.2, 95.1 >)	4 (17.4 < 5, 38.8 >)
Random screening					
1. Black bucks	4	0 0.00 (0, 60.2)	4 100 (39.8, 100)	0 0.0 (0, 60.2)	4 100 (39.8, 100)
2. Gaur	10	0 0.0 (0, 30.9)	10 100 (69.2, 100)	0 0.00 (0, 30.9)	10 100 (69.2, 100)
3. Mithun	10	0 0.0 (0, 30.9)	10 100 (69.2, 100)	0 0.0 (0, 30.9)	10 100 (69.2, 100)
4. Nilgai	4	0 0.0 (0, 60.2)	4 100 (39.8, 100)	0 0.0 (0, 60.2)	4 100 (39.8, 100)
5. Yak	90	43 48 (37.1, 58.6)	47 52 (41.4, 62.9)	43 48 (37.1, 58.6)	47 52 (41.4, 62.9)

*Sera from vaccine potency testing experiment, in which animals were vaccinated prior to infection

The frequency distribution of all the negative sera showed a normal distribution in both the assays (Fig. 5B). The samples "deemed negative" ($n = 1146$) are from the uninfected animals ($n = 473$) as well as samples collected less than 6 days post-infection ($n = 673$) and are irrespective of species or vaccination status (Table 3). The Dsp using these sera was 94% (1082/1146, 95% CI (93, 96%)) for the 10H9 bELISA and 96% (1103/1146, 95% CI (95, 97%)) for the

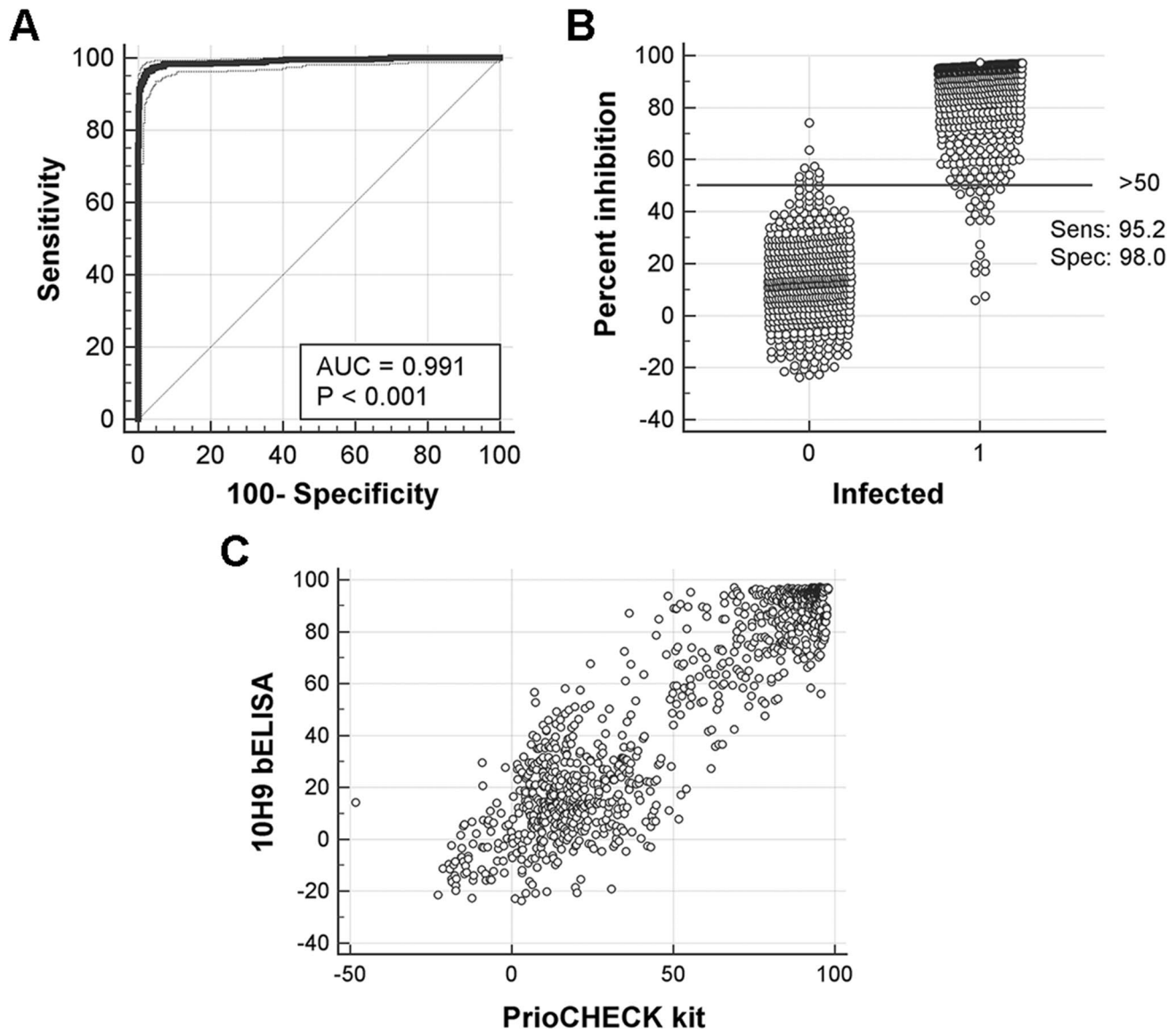

Fig. 3 Evaluation of a panel of positive and negative FMD sera using blocking ELISA. **A** Receiver operating characteristic (ROC) curve analysis of serum samples ($n=985$). **B** Dot plot analysis of negative (0) and positive (1) sera. Calculated Dsn and Dsp were 95% and 98%, respectively, when a cutoff was fixed at PI > 50 using known panel of positive ($n=480$) and negative ($n=505$) FMD sera. **C** Scatterplot analysis of results between the 10H9 bELISA and the PrioCHECK FMD NS test

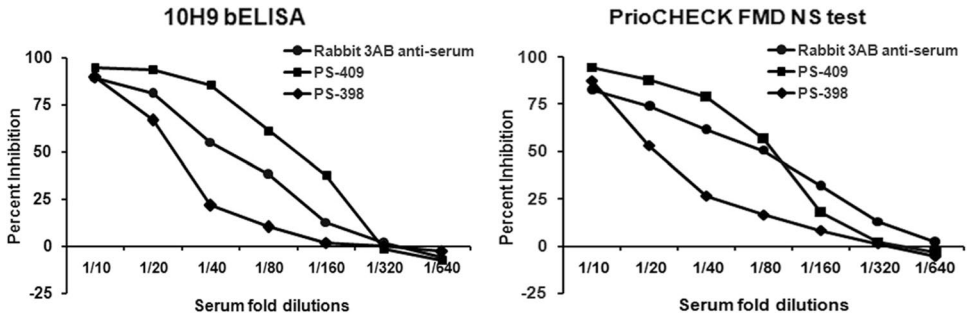

Fig. 4 Limit of detection of the assays. Samples ($n=3$) positive in NSP ELISA were serially diluted and tested in 10H9 bELISA and PrioCHECK kit in parallel to compare their end point detection limits at the indicated dilutions

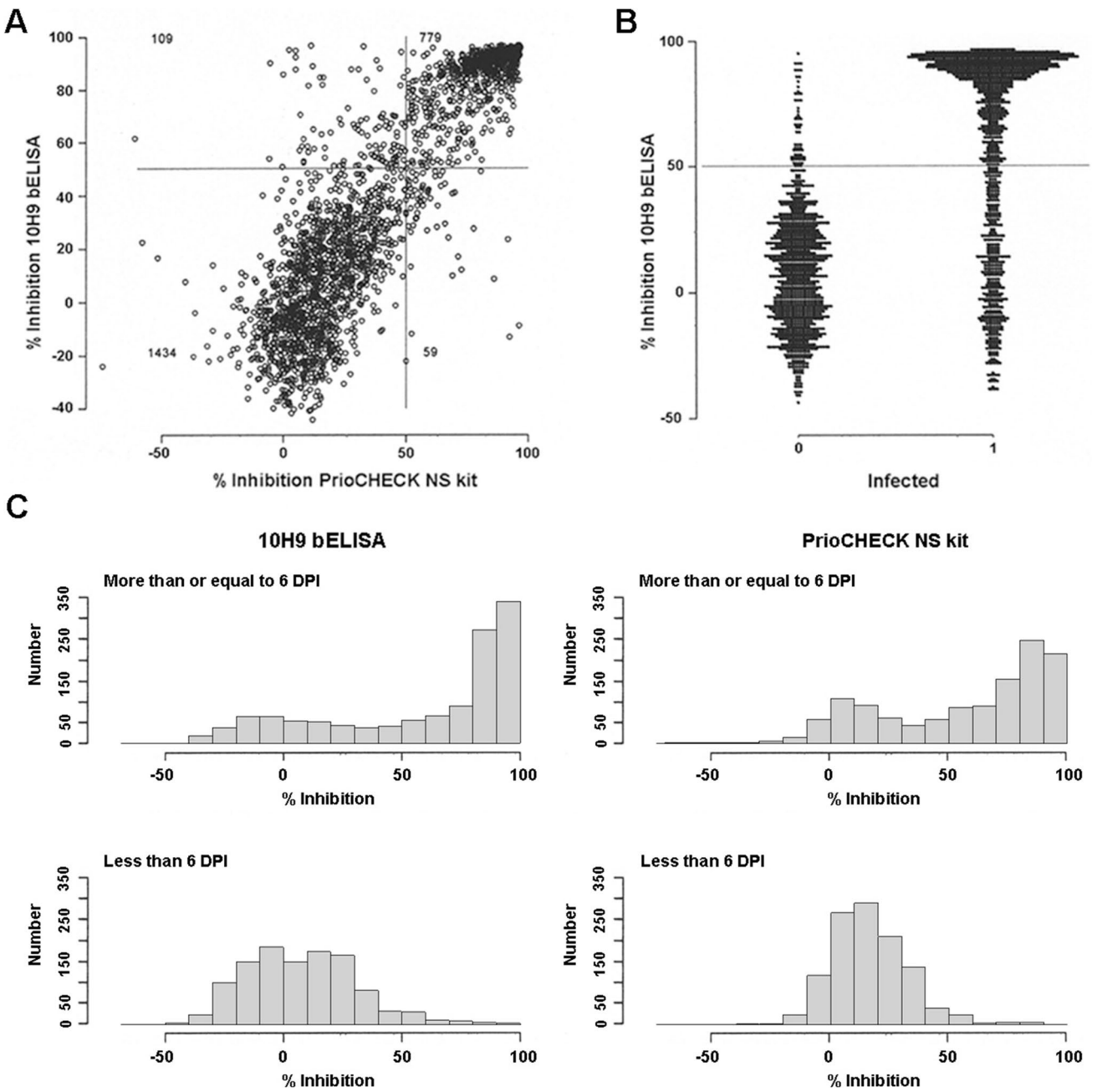

Fig. 5 Comparison of the results of the 10H9 bELISA and PrioCHECK kit. **A** Comparison of the results in both assays on WBVR serum panel of sera ($n=2381$) by scatterplot analysis. **B** Dot plot graph of the samples deemed negative for NSP antibodies, less than 6 days post-infection (or not infected), and samples deemed positive 6 days or more post-infection in 10H9 bELISA. **C** Frequency distribution of the samples deemed positive for NSP antibodies, 6 days or more post-infection (top graph), and samples deemed negative (bottom graph) in 10H9 bELISA (left) and the PrioCHECK test system (right)

PrioCHECK FMD NS kit. The Dsn for the 10H9 bELISA was 68% (824/1235, 95% CI (65,70%)), using sera collected 6 days or more post-infection, which was similar to the result in the PrioCHECK FMD NS kit (64% positive, 795/1235, 95% CI (62, 67%)). In Table 4, the results of the WBVR sera are specified for the route of infection. The Dsn of both ELISAs is higher in animals that are infected by injection.

Ability to detect early antibody response

Both the assays can detect NSP antibody response as early as 7 days post-infection when about 53% of the samples (9/17) were positive following infection under experimental conditions. Interestingly, only five of the nine positive reactive samples are commonly detected

Table 3 Comparison of the results between 10H9 bELISA and PrioCHECK test, using sera at WBVR

Sample details	Number tested	10H9 bELISA				PrioCHECK FMD NS			
		Positive	% positive	LC%	UC%	Positive	% positive	LC%	UC%
Naïve animals (including exposed < 6 dpi)									
Cattle	74	4	5.4	1.5	13.3	1	1.4	0	7.3
Pigs	40	0	0	0	8.8	0	0	0	8.8
Vaccinated and non-exposed (including exposed < 6 dpi)									
Cattle	585	51	8.7	6.6	11.3	40	6.8	4.9	9.2
Sheep	253	9	3.6	1.6	6.6	2	0.8	0.1	2.8
Pigs	194	0	0	0	1.9	0	0	0	1.9
Vaccinated and exposed									
Cattle	594	496	83.5	80.3	86.4	482	81.1	77.8	84.2
Sheep	300	142	47.3	41.6	53.2	120	40	34.4	45.8
Pigs	196	96	49	41.8	56.2	99	50.5	43.3	57.7
Not vaccinated and exposed									
Cattle	108	66	61.1	51.3	70.3	67	62	52.2	71.2
Sheep	3	3	100	29.2	100	3	100	29.2	100
Pigs	34	21	61.8	43.6	77.8	24	70.6	52.5	84.9

Table 4 Comparison of results on sera from animals exposed by different routes of infection

Sample details	Number tested	10H9 bELISA				PrioCHECK FMD NS			
		Positive	% Positive	LC%	UC%	Positive	% Positive	LC%	UC%
With or without vaccination, exposed by contact									
cattle	139	63	45.3	36.9	54	57	41	32.7	49.7
sheep	146	19	13	8	19.6	14	9.6	5.3	15.6
pigs	115	55	47.8	38.4	57.3	52	45.2	35.9	54.8
With or without vaccination, exposed by nasal route									
cattle	63	36	57.1	44	69.5	34	54	40.9	66.6
sheep	153	122	79.7	72.5	85.8	105	68.6	60.6	75.9
With or without vaccination, infected intradermally									
cattle	500	463	92.6	89.9	94.7	458	91.6	88.8	93.9
sheep	4	4	100	39.8	100	4	100	39.8	100
pigs	98	51	52	41.7	62.2	58	59.2	48.8	69

by both the assays while the other four samples were discordant.

The longevity of the NSP antibody in the convalescent animals

From the sera collected on day 769 post-infection from 16 cattle infected experimentally with A/TUR/14/98, 15 out of 16 samples were positive in the 10H9 bELISA, whereas all the 16 samples were positive in the PrioCHECK test (Fig. 6). The cow of the discrepant sample was however positive on day 638 post-infection in the 10H9 bELISA with a PI value of > 90%, whereas it was borderline positive on day 738 post-infection (PI value of 54 in PrioCHECK test and 31 in 10H9 bELISA). Two serum samples collected from cattle 43 months after experimental infection with FMDV serotype O/IND R2/75 were positive in both the assays.

Testing Pirbright panel of bovine sera

The Pirbright panel of bovine sera ($n = 35$) prepared for NSP assay validation were tested in both the assays and PI values plotted for each sample as shown in Fig. 7. Among all these known positive samples, 31 samples showed positivity in PrioCHECK kit as compared to 32 in 10H9 bELISA.

Discussion

In the present study, a new DIVA diagnostic ELISA has been developed and validated for sensitive and specific detection of NSP antibodies to FMDV. Purified recombinant protein (3AB) and the murine mAb 10H9 produced against this protein were employed in the assay. Insect cell-based baculovirus expression was chosen to produce protein, as it is easy to purify

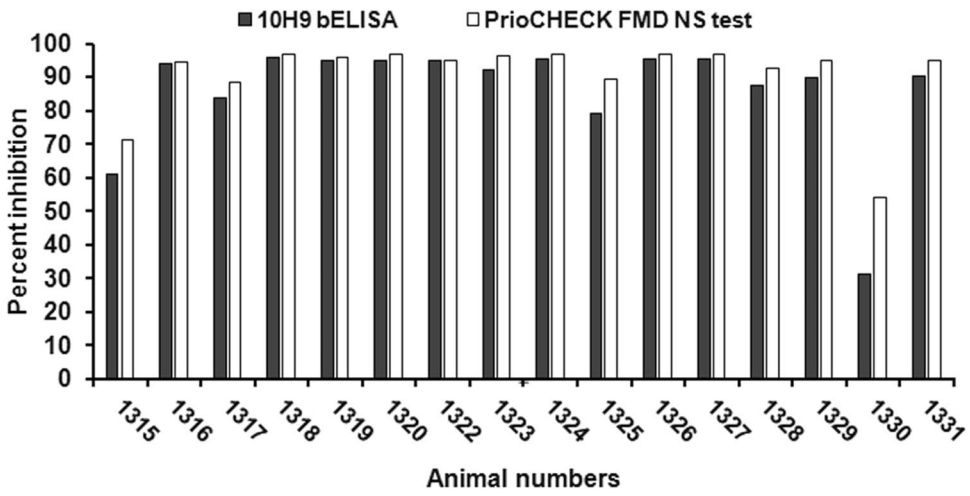

Fig. 6 Detectability of NSP antibodies 769 days post-experimental infection in cattle by 10H9 bELISA and PrioCHECK FMD NS kit

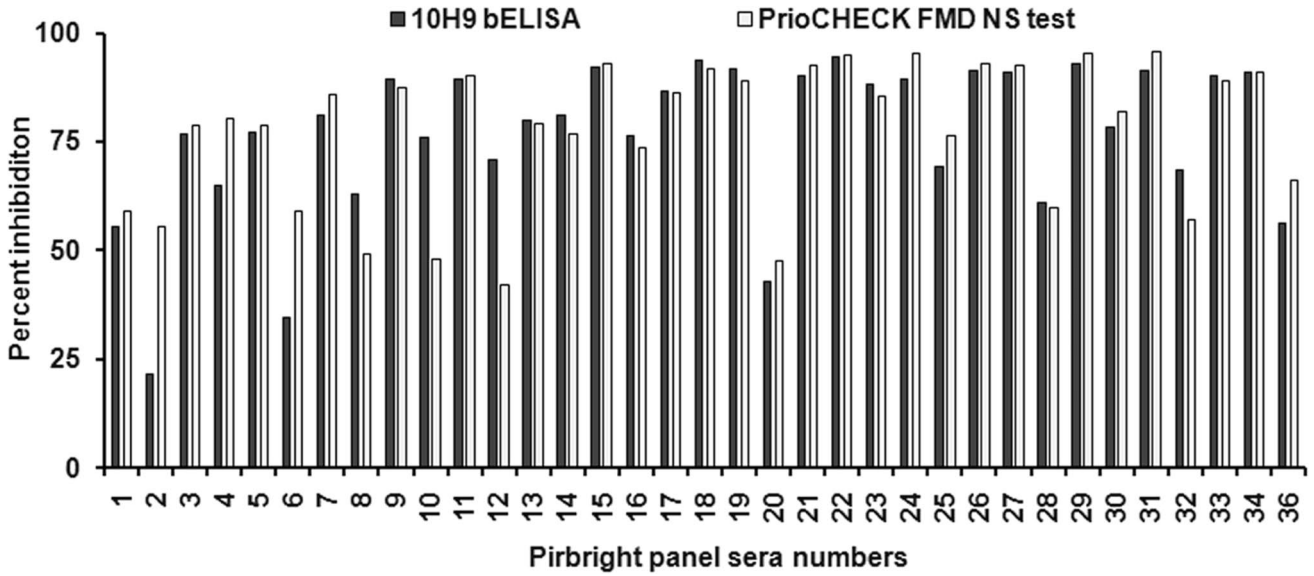

Fig. 7 Evaluation of Pirbright serum panel using 10H9 bELISA and PrioCHECK FMD NS kit

protein from insect cells as compared to *Escherichia coli*, for use either in mouse immunization or ELISA. The recombinant protein reacted in western blot with convalescent bovine sera as well as with mAb 10H9. The convalescent serum panel also blocked the reactivity of mAb 10H9 in blocking ELISA format. Using a peptide inhibition ELISA, the binding site of the mAb to 3AB was mapped; three 3B peptides GPYAGPLERQKPLK (3B1), GPYAGPMERQKPLK (3B2), and GPYEGPVKKPVALK (3B3) effectively inhibited the binding of mAb to the antigen. Since the sequences of these peptides are largely similar, it is apparent that the mAb is reactive with the common motif [GPYA(E)GP] present in these peptides. It is not surprising that the mAb we selected was directed against the 3B part of the protein, as previous reports also highlight that several of the mAbs employed in NSP Ab detection ELISAs were directed to 3B sequences (Sørensen et al. 2005, Fu et al. 2014; Chung et al. 2018; Yang et al. 2015) and 3A sequence (Fu et al. 2017).

The initial feasibility study showed that the 10H9 bELISA could be developed as a single test system to screen samples from wider host species susceptible to FMD. It was validated on different panels of sera in India and Netherlands by comparison with a similar commercial ELISA, PrioCHECK FMD NS kit. Based on known positive and negative sera tested, the 10H9 bELISA showed 95% sensitivity and 98% specificity, respectively. Known positive status of samples was based on presence of clinical signs and detection of serum antibodies to FMDV in the affected animals, and the infection status in all animals was however not checked by qPCR or virus isolation criterion. Graphical data analysis shows a good correlation with the PrioCHECK FMD NS kit, which is a well-validated test in different laboratories

across the world (Brocchi et al. 2006; Chung et al. 2018; Fukai et al. 2018). The sensitivity and specificity of the latter has been shown to be comparable to Panaftosa ELISA, a reference test described in the WOAH terrestrial manual, chapter 2.1.5 (Anonymous, 2021).

The 10H9 bELISA was shown to give consistent results when evaluated in 4 laboratories on a set of 45 samples of known FMD status. The assay showed no cross reactivity with the post-infection sera of various other viral diseases that produce similar clinical manifestation in cattle, sheep, goat, and pigs. The limit of detection was similar to the limit of detection in the PrioCHECK FMD NS kit (Fig. 4). Further comparison at WBVR Netherlands showed again a high correlation between the 10H9 bELISA and the PrioCHECK NS kit. The distribution pattern of the PI values differed from the distribution observed at IVRI. In the WBVR sera, there were more negative results in the sera collected 6 days or more after infection. But the negative results were mostly found in contact-exposed vaccinated animals in transmission studies (Table 4), and in those studies, absence of the NSP antibody response was considered absence of transmission and these animals were probably not infected (Eblé et al. 2004, 2006; Orsel et al. 2005, 2007a, 2007b, 2007c; Bravo de Rueda et al. 2014, 2015).

The 10H9 bELISA detected NSP antibodies in sera of infected animals of various species, both in the sera tested at IVRI as well as the cattle, pig, and sheep sera tested at WBVR. The ability to detect early NSP antibody responses and the longevity of the NSP antibody response were comparable in both the ELISAs. Antibodies were detectable in animals at 6–7 days after infection and up to more than 2 years after infection (Fig. 6). NSP antibodies were detectable for over 3 years though the antibody levels subsided gradually with the time (Elnekave et al. 2015). In this study, two Indian cattle sera that were collected 43 months post-experimental infection showed seropositivity in both the ELISAs, though the number of samples was small. Furthermore, the results of an international reference serum panel were comparable with published data (Parida et al. 2007) and results in both the tests (10H9 bELISA and the PrioCHECK FMD NS kit) were in alignment. Among the five commercial NSP Ab detection ELISAs evaluated by the latter group, the PrioCHECK FMD NS test (formerly called Ceditest) had exhibited the highest sensitivity and repeatability detecting 33/36 samples of the serum panel screened (Parida et al. 2007).

In conclusion, the novel assay described here proved to be a reliable diagnostic tool as it is validated using a reasonably large panel of sera, comprising samples from wide range of host species and against various FMDV serotypes. The test system with a shorter assay time has a high Dsn and Dsp. The assay would serve as a valuable tool for large-scale FMD serosurveillance and for monitoring the effectiveness of the disease control with or without vaccination.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-022-12151-2.

Acknowledgements The authors thank the Director, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, for facilitating this work. The authors acknowledge the directors of IAHVB, Bengaluru, ICAR-DFMD, and ICAR-NIVEDI for their kind cooperation in facilitating assay validation in different laboratories.

Author contribution M. H. conceived and designed the project. S. G., S. B., K.W., and M. H. carried out work including the assay optimization. S. H. B. and V. B. P. did the internal validation and edited the manuscript. A. B., D. P. B., and P. D. collected the reagents. B. P. S., R. K. S., A. S., and A. D. coordinated the assay validation in India and Netherlands and also edited the paper. A. D. and M. H. wrote the manuscript and did the analysis of the data. All authors read and approved the manuscript.

Funding The work was funded by the Consortia of Research Platforms (CRP) on Vaccines and Diagnostics Project (ICAR, New Delhi) and by the Ministry of Agriculture, Nature and Food Quality, project WOT-01–003-11, the Netherlands.

Data availability The associated data and the diagnostic kit are available on request.

Declarations

Ethics approval The animal experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of ICAR-IVRI, as well as the Animal Ethics Committee in the Netherlands for the experiments performed in the Netherlands.

Competing interests The authors declare no competing interests.

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