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Short Paper

A developed recombinant antigen and monoclonal antibody based solid phase competitive ELISA for detection of foot and mouth disease virus serotype O specific antibodies

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Abstract

Background: Foot and mouth disease (FMD) is a highly contagious and economically burdensome viral disease of cloven-hoofed animals. Out of the seven FMD virus (FMDV) serotypes, type O is one of the most prevalent serotype in several parts of the world. **Aims:** This study aimed to develop a recombinant capsid polypeptide (rP1) and monoclonal antibody (MAb)-based solid phase competitive ELISA (rP1-MAb-SPCE) to detect anti-FMDV/O antibodies in serum samples. **Methods:** For the development of rP1-MAb-SPCE the concentrations of rP1, MAb and dilution of serum were optimized. Percent inhibition (PI) cut-off was determined based on ROC curve analysis of cattle sera (n=53) of known FMD vaccination status with reference to the gold standard virus neutralization test (VNT). The assay performance was further validated in comparison to the conventional polyclonal antibody (PAb)-based SPCE using FMDV-positive and negative serum samples (n=612) obtained from multiple species (cattle, goat, pig, fallow deer, antelope, giraffe and African buffalo). **Results:** At the selected cut-off value of 30.77 PI, the rP1-MAb-SPCE exhibited 86.21% diagnostic sensitivity and 95.83% diagnostic specificity. A significant correlation (r=0.84; P<0.0001) was observed between the PI values of sera tested by rP1-MAb-SPCE and PAb-based SPCE and also the limits of detection of both of the assays were comparable. Repeatability tests demonstrated that the coefficients of variation within and between runs were <10% and <14%, respectively. **Conclusion:** The developed rP1-MAb-SPCE has the potential to facilitate the detection of antibodies against FMDV serotype O while mitigating biosecurity concerns. However, further validation involving a larger serum cohort is essential to confirm the assay's robustness.

Key words: Foot and mouth disease virus, Monoclonal antibody, Recombinant protein, Serotype O, Solid phase competitive ELISA

Introduction

Foot and mouth disease (FMD) is a highly infectious and rapidly spreading viral disease of cloven-footed animals including cattle, buffalo, sheep, goat and pig. The disease is caused by FMD virus (FMDV) which is a member of the genus *Aphthovirus* within the *Picornaviridae* family (Belsham, 1993). The economic impact of FMD on livestock industry is enormous due to significant decrease of animal productivity and international trade restrictions on animals and animal products. Additionally FMD causes significant morbidity and stress in affected animals (Grubman and Baxt, 2004; Mallick *et al.*, 2021). FMD is endemic in most Southeast

Asian countries including India. Prophylactic bi-annual vaccination with trivalent (O, A, C, Asia 1) FMDV vaccine is practiced as a strategy to prevent FMD in India (Subramaniam *et al.*, 2022; Ranjan *et al.*, 2025). Serological tests for FMD diagnosis include detection of antibodies against viral nonstructural (non SPs) or structural proteins (SPs). The detection of antibodies against the SPs can be used to monitor seroconversion in both infected and vaccinated animals (Robiolo *et al.*, 2010; Asfor *et al.*, 2020). This is important to confirm presence or absence of FMDV infection, to certify the FMD-status of animals prior to import or export, and to determine the efficacy of FMD vaccination (OIE, 2008). Serological tests which are internationally accepted for

detection of FMDV-specific SP antibodies include virus neutralization test (VNT), liquid-phase blocking ELISA (LPBE) and solid-phase competitive ELISA (SPCE) (Mackay *et al.*, 2001). Virus neutralization test is considered as the gold standard for detection and quantification of antibodies against FMDV SPs (OIE, 2008; Ranjan *et al.*, 2026). However, it is costly, labor intensive, time consuming and requires a bio-containment facility. These constraints make the VNT difficult to apply in the field-level laboratories (Sala *et al.*, 2023; Cao *et al.*, 2025). In contrast, ELISA techniques are quicker to perform and are not dependent on cell culture systems or the use of live viruses. While LPBE requires overnight incubation of test serum with viral inactivated antigen, SPCE involves simultaneous addition of the polyclonal antibody (PAb)/monoclonal antibody (MAb) and test sera, leading to competition for a specific epitope on FMDV antigen (Chenard *et al.*, 2003; Paiba *et al.*, 2004). Moreover, SPCE has demonstrated higher specificity for detecting antibodies against FMDV than LPBE (Şevik and Öztürk, 2013; Afroz *et al.*, 2024). Standard polyclonal antisera prepared via hyperimmunization of laboratory animals have limited supply and show batch to batch variations. Conversely, MAbs once generated do not require repeated production in animals. They are homogenous in nature and unlimited in supply (Ko *et al.*, 2012). Foot and mouth disease virus antigens which are used for diagnostic purposes are usually derived from cell-culture-grown inactivated virus capsids. Alternatively, recombinant antigens produced via prokaryotic protein expression system are safer, more efficient and cost-effective than viral inactivated antigens (Biswal *et al.*, 2015). Notably, a MAb and FMD VLP/P1-based blocking ELISA was developed earlier by Lee *et al.* (2022) and unfortunately was tested only on few (23) vaccinated swine sera for the assay validation. Also, a MAb-based blocking ELISA that uses rabbit anti-FMDV/O polyclonal antibody and inactivated FMDV antigen along with MAb for the detection of FMDV/O-specific antibody is developed by Kashem *et al.* (2024). In the present study, we aimed to develop a SPCE for FMDV/O antibody detection using in-house recombinant FMDV/O capsid polyprotein P1 and anti-FMDV/O MAb.

Materials and Methods

Animal ethical statement

Ethical approval from Institutional Animal Ethics Committee (IAEC) was not required for this study as no live animals were handled, housed, or experimented upon. The study utilized pre-existing archival samples in the investigation.

Preparation of recombinant capsid polyprotein (rP1) of FMDV serotype O

The preparation of rP1 antigen was carried out as mentioned previously (Biswal *et al.*, 2015). Briefly, the overnight culture of BL21(DE3) *E. coli* cells harboring

the pETite N-His SUMO-P1 construct was inoculated into Luria-Bertani broth medium at 1:10 dilutions with antibiotic Kanamycin. The *E. coli* cells were grown at 37°C until the optical density (OD) at 600 nm reached to 0.7-0.8. Protein expression was induced with 1 mM of Isopropyl β-d-1-thiogalactopyranoside at 28°C for 4 h. Bacterial cells were harvested by centrifugation at 4000 ×g for 20 min and then re-suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). The bacterial suspension was subjected to three freeze-thaw cycles. The clarified supernatant was subjected to nickel-nitrilotriacetic acid metal affinity chromatography for purification of the protein. Finally, the bound rP1 protein was eluted in the elution buffer containing 250 mM imidazole and its purity was analyzed on 12% SDS-PAGE. Immunoreactivity of the rP1 protein was assessed by indirect-ELISA (Biswal *et al.*, 2015).

Anti-FMDV/O monoclonal antibody

A high-affinity, FMDV/O-specific MAb#5B6 previously developed by our team was used in the present study (Mallick *et al.*, 2023). The ability of MAb#5B6 to compete with polyclonal antibodies in FMDV/O vaccinated/infected cattle sera was examined in a preliminary study. The MAb demonstrated strong inhibition of the serum antibodies to bind to FMDV/O inactivated antigen as well as rP1 in separate ELISAs. Hence, it was chosen as the competitor antibody of the test serum in the new rP1-MAb-SPCE.

Serum samples

Sera (n=612) from naïve, FMDV-infected and vaccinated animals belonging to multiple species (Table 1) available at ICAR-National institute on Foot and Mouth Disease (ICAR-NIFMD) serum repository were tested by rP1-MAb-SPCE and the results were compared with in-house PAb-based SPCE (ICAR-DFMD, Annual report 2022). Naïve sera collected from unvaccinated and uninfected animals were included in the study. FMDV-infected cattle sera were obtained from confirmed cases of FMDV/O field outbreaks collected at different time points of FMD outbreak ranging from acute viral infection phase to one month post outbreak. FMDV vaccinated sera were collected at 0 and 28 days post-vaccination from cattle vaccinated with inactivated trivalent (O, A and Asia 1) FMD vaccine.

Table 1: Characteristics of serum samples used in the study

Type of serum samples	Species	Number of serum samples
Pre-vaccinated	Cattle	80
Infected vaccinated	Cattle	259
	Cattle	120
Naïve	Pig	30
Naïve	Goat	24
Naïve	Antelope	34
Naïve	Fallow deer	20
Naïve	Giraffe	30
Naïve	African buffalo	15
		Total=612

Development of rP1-MAb-SPCE

The optimal concentrations of rP1 and MAb were determined by checkerboard titration method. The serum dilution factor for the test was established based on known combinations of FMDV-positive and negative serum dilutions, which resulted in the greatest differential in percent inhibition (PI) between the positive and negative samples. These optimized concentrations of rP1, MAb and serum dilution were used for further development of the assay. Briefly, Maxisorp 96-well microplates (Nunc, Denmark) were coated with rP1 protein diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times (3 min for each wash) with PBS containing 0.1% Tween-20 (PBST). After washing, 50 µL of test/control serum samples diluted to 1:5 (final working dilution of 1:10) in blocking buffer (PBST, 3% skim milk) were added to the plates in duplicate. Immediately, MAb#5B6 diluted to 1:4 (final working dilution of 1:8) in the blocking buffer was added to each well except the background control wells and the plate was incubated at 37°C for 1 h. The microplate underwent a washing procedure. Subsequently, an anti-mouse-horseradish peroxidase (HRP) conjugate (Sigma Aldrich), diluted to a ratio of 1:3000 in blocking buffer, was added at a volume of 50 µL per well and incubated at a temperature of 37°C for 1 h. After final washing, 50 µL of substrate solution containing orthophenylenediamine dihydrochloride/hydrogen peroxide (Sigma Aldrich) was added. The plate was allowed to stand at 37°C for 15 min for colour development. The reaction was then stopped using 1 M H₂SO₄. The OD values were read at 492 nm using an ELISA plate reader (Thermo Scientific, Switzerland). OD₄₉₂ values of all samples were expressed as PI of reaction relative to the mean OD₄₉₂ value of the MAb control. The PI of the test serum was calculated using the following formula:

$$PI = 100 - \left(\frac{\text{mean OD of test serum sample}}{\text{mean OD of MAb control}} \right) \times 100$$

Virus neutralization test (VNT)

Virus neutralization test was performed as described in the manual of diagnostic tests and vaccines for terrestrial animals, WOAHA (World Organisation for Animal Health, 2018) using BHK-21 cells. A representative set of cattle serum samples (n=53) with known vaccination status were tested for virus-neutralizing antibodies against FMDV vaccine strains O/IND/R2/75. Serum samples with a titer of <1/16 and ≥1/45 were considered as negative and positive, respectively. Serum samples with the titer of 1/16-1/45 were not included in the present study.

PAb-based SPCE

PAb-based SPCE was carried out as per the method previously established (ICAR-DFMD, Annual report 2022). Briefly, Maxisorp 96-well plates (Nunc, Denmark) were coated with 50 µL/well of appropriately

diluted rabbit anti-FMDV/O polyclonal antibody and left overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween-20 (PBST), 3 min each wash. Then 50 µL of FMDV/O antigen diluted in PBS was added to each well of the ELISA plate except the wells assigned as background controls. Test serum diluted to 1:45 in blocking buffer (3% lactalbumin hydrolysate, 5% healthy new born calf serum, 5% healthy rabbit serum in PBST) was added. Immediately, appropriately diluted guinea pig anti-FMDV/O antibody was added to each well. The plate was incubated at 37°C for 1 h. After washing, 50 µL of HRP-conjugated rabbit anti-guinea pig immunoglobulin diluted in blocking buffer was added. The plates were incubated for 1 h at 37°C. The subsequent phases of the assay followed the methodology outlined in the section pertaining to the development of rP1-MAb-SPCE. The PI values for the serum samples under investigation were derived from the median absorbance values obtained from the antigen control wells.

Determination of the PI cut-off

The determination of the PI cut-off was conducted using 53 serum samples from cattle with known vaccination status. This analysis employed receiver operating characteristic (ROC) curves and dot plot diagrams utilizing MedCalc software. The diagnostic sensitivity was defined by the proportion of serum samples that tested positive with rP1-MAb-SPCE among those confirmed positive via VNT. Conversely, the diagnostic specificity was established based on the count of serum samples that tested negative with rP1-MAb-SPCE relative to the total number of samples that were negative in the VNT.

Repeatability test

Three FMDV vaccinated bovine serum samples of high (>128), medium (64-128) and low (<45) antibody titers were taken as FMDV-strongly positive, moderately positive and negative control sera and tested in quadruplicate in twenty plates to evaluate the repeatability of the rP1-MAb-SPCE. To determine the precision within a run and between the runs, intra-assay and inter-assay coefficient of variations (CV) % were calculated.

Analytical sensitivity

Analytical sensitivity of the assay was determined by testing FMDV-infected serum samples found positive in VNT (n=3). The sera were serially diluted and tested in rP1-MAb-SPCE vs. PAb-based SPCE in parallel to compare their end point detection limits at the indicated dilutions.

Analytical specificity

To determine the analytical specificity of rP1-MAb-SPCE, confirmed FMDV-O (n=13), -A (n=6) and -Asia 1-positive (n=2) serum samples of cattle sera from clinical cases of FMD field outbreaks were used.

Statistical analysis

Correlation between PI values obtained by rP1-MAb-SPCE and PAb-based SPCE for the same set of sera (n=612) were analyzed by Pearson correlation coefficient using Graphpad prism software version 6 (Graph-Pad Software, Inc., San Diego, CA).

Results

The optimal concentrations of rP1, MAb and serum dilution

For the development of rP1-MAb-SPCE, the optimal concentrations of rP1 protein were fixed at 1.25 µg/50 µL/well and MAb dilution at 1:8 (Fig. 1A). Serum dilution of 1:10 was selected as the maximum difference in the PI values of FMDV-positive and negative sera that were observed at this dilution (Fig. 1B). The suitable dilution ratio of anti-mouse-HRP conjugate was determined as 1:3000. These optimized parameters were applied for further validation of the rP1-MAb-SPCE.

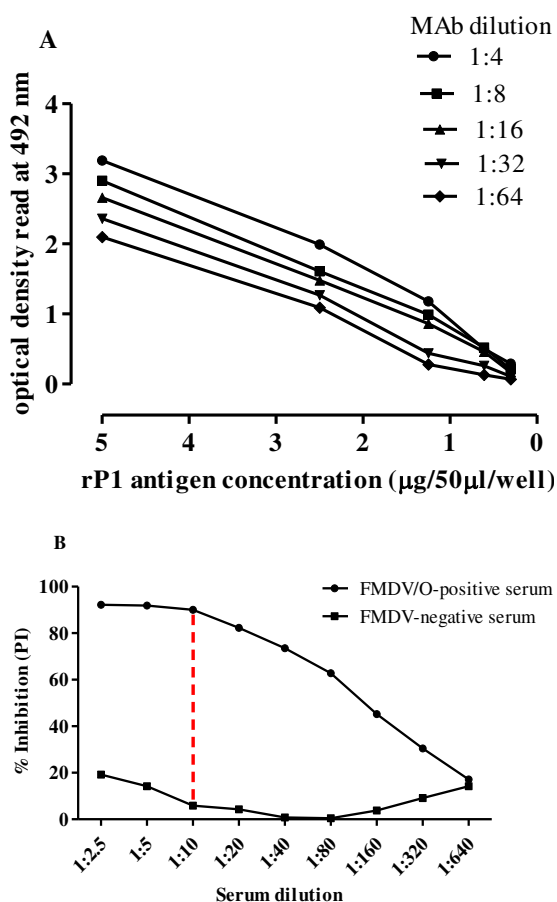


Fig. 1: Determination of concentration of coating antigen rP1, MAb and dilution of test serum. (A) Checkerboard titration to determine the optimum coating antigen (rP1) concentration and MAb dilution, and (B) Test serum dilution of 1:10 showed maximum difference in the PI of FMDV/O-positive and negative serum

The PI cut-off determined by ROC analysis

ROC analysis of the PIs of all the samples is depicted

in Table 2. An area under curve (AUC) of 0.889 is predictive of accuracy of the developed assay. The 95% confidence intervals for diagnostic sensitivity was 68.3-96.1% and this value for specificity was 78.9-99.9%. At a cut-off point of 30.77 PI, the developed SPCE had diagnostic sensitivity and specificity of 86.21% and 95.83%, respectively. Among the 29 test positive serum samples (based on VNT), 25 samples were found to be true positive. While, among the 24 test negative sera (based on VNT), 23 samples were considered to be true negative (Figs. 2A and B). Test positive predictive value was 96.20% and test negative predictive value was 85.20% (Table 2).

Table 2: ROC analysis of rP1-MAb-SPCE for anti-FMDV/O antibody detection

Parameters	Overall ROC analysis
AUC	0.889
AUC at 95% CI	0.773 to 0.959
Standard error	0.0539
P-value for null hypothesis AUC=0.5	<0.0001
Optimal test cutoff	>30.77
Positive likelihood ratio	20.69
Negative likelihood ratio	0.14
Sensitivity (%)	86.21
Specificity (%)	95.83
Test positive predictive value (%)	96.20
Test negative predictive value (%)	85.20

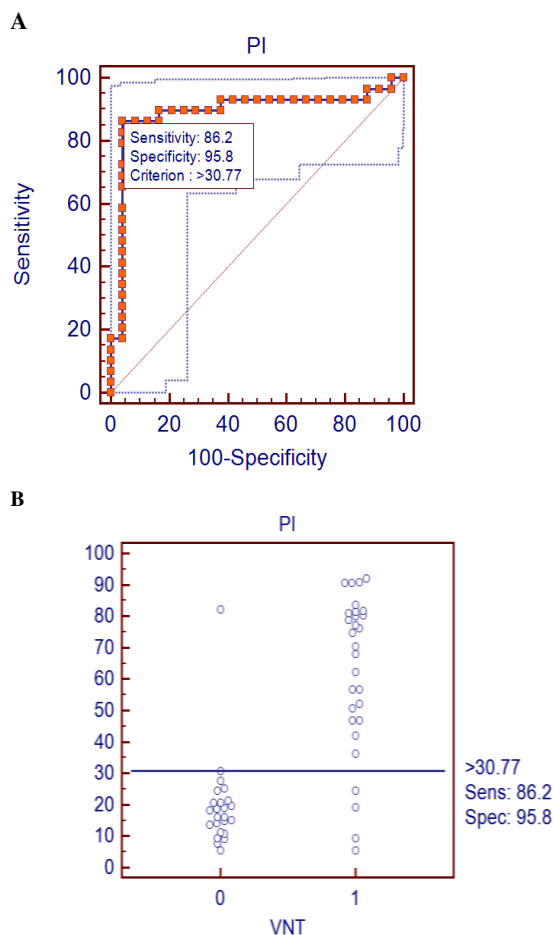


Fig. 2: (A) Receiver operating characteristic (ROC) curve

analysis of serum samples ($n=53$), and (B) Dot plot analysis of negative (0) and positive (1) sera. Diagnostic sensitivity and specificity of the assay was 86.2% and 95.8%, respectively at the cut-off PI of 30.77

Analytical sensitivity

The analytical sensitivity of the rP1-MAb-SPCE was determined from serially diluted FMDV-strongly positive serum samples (S-1, S-2 and S-3). PIs of the serum samples obtained by rP1-MAb-SPCE were compared to the PI values of PAb-based SPCE. It was observed that the endpoint detection limits for both of the assays were highly comparable (Figs. 3A and B).

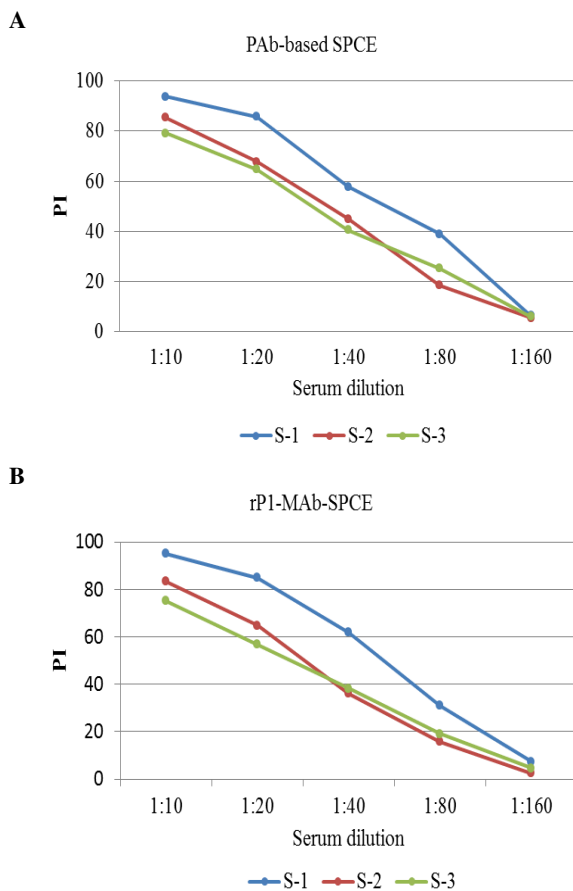


Fig. 3: Determination of limit of detection of PAb-based SPCE (A), and rP1-MAb-SPCE (B). Serially diluted FMDV-positive sera ($n=3$) were tested in both the assays to compare their endpoint detection limits which was determined at 1:40 dilution

Analytical specificity

The rP1-MAb-SPCE showed significant inhibition of homologous FMDV/O-positive sera whereas the PI values of heterologous FMDV/A and Asia 1-positive

sera were well below the cut-off value of 30.77% (Fig. 4).

Repeatability test

The intra and inter assay CVs were both $<5\%$ for the strongly positive and moderately positive control serum samples while for the negative control serum the values were $<10\%$ and $<14\%$ respectively (Table 3).

Performance of rP1-SPCE as compared to PAb-based SPCE

The PI values obtained by rP1-MAb-SPCE demonstrated a statistically significant correlation with the PI values of PAb-based SPCE ($r=0.84$; $P<0.0001$) for a set of sera ($n=612$) originating from multiple species (Fig. 5).

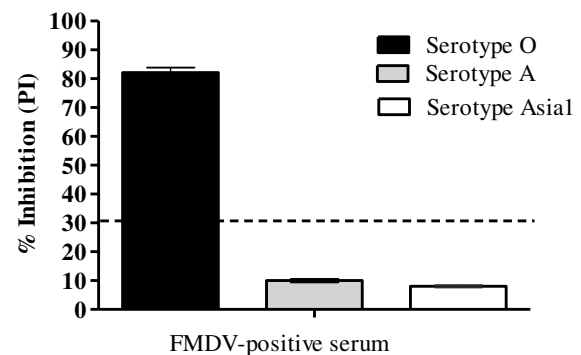


Fig. 4: Analytical specificity of rP1-MAb-SPCE. The horizontal line indicates the cut-off PI of the assay. The assay detected FMDV/O antibody specifically without showing cross-reactivity against FMDV-A and Asia1 serotypes

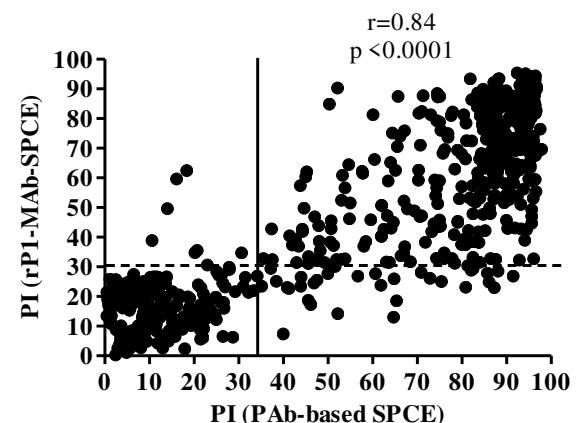


Fig. 5: Correlation between rP1-MAb-SPCE and PAb-based SPCE. The horizontal line indicates the cut-off PI of the assays. The PI values of 612 tested serum samples demonstrated good correlation as evident from the scatterplot analysis

Table 3: Repeatability of rP1-MAb-SPCE

Type of serum samples	Percent inhibition (PI)					
	Intra-assay			Inter-assay		
	Mean	SD	CV%	Mean	SD	CV%
Strongly positive	88.53	0.92	1.03	89.26	1.19	1.33
Moderately positive	68.95	1.12	1.62	69.26	2.64	3.81
Negative	6.89	0.68	9.86	6.78	0.92	13.56

Discussion

The aim of the study was to develop a SPCE for the detection of FMDV/O-specific antibody in serum samples using recombinant capsid polyprotein rP1 and MAb as diagnostic reagents. During the development of rP1-MAb-SPCE several key operational steps were optimized. The concentration of rP1 and dilution of the MAb showing an OD value ranging between 1.0-1.5 by checkerboard titration method was chosen for further development of the assay. Serum dilution of 1:10 was selected as the maximum difference in PI of FMDV-positive and negative serum was observed at this dilution. We determined the PI cut-off at 30.77% by using ROC curve analysis and dot plot diagram. The diagnostic sensitivity and specificity of the assay was found to be 86.21% and 95.83%, respectively compared to the gold standard VNT. The developed rP1-MAb-SPCE had an appropriate area under ROC curve, test predictive values and likelihood ratios at the optimal cut-off point. The results indicated that the new assay is suitable as a first line test to monitor regular FMD vaccine-induced sero-conversion. The samples scoring positive could be tested further by VNT for confirmation. This approach would reduce the time and labour invested in the gold standard test. Earlier, Kang *et al.* (2018) reported that the FMD vaccination coverage determined by SPCE were relatively similar to those determined by VNT at 4-12 weeks after vaccination which is supported by our findings. Further, our results were comparable to previously published reports of SPCE for FMDV type O antibody detection (Biswal *et al.*, 2015; Ran *et al.*, 2019). However, these tests relied on virus like particle (VLP) or recombinant capsid protein in combination with PAb as diagnostic reagents. Lee *et al.* (2022) developed a VLP/recombinant protein and MAb-based blocking ELISA for FMDV/O antibody detection the test was validated with only vaccinated swine sera whereas our assay exhibited broader species validation. In our study we tested sera from multiple species (n=612) and compared the results with conventional PAb-based SPCE. The positive/negative proportions of the sera per species as observed in rP1-MAb-SPCE were as follows: cattle-350/29, pig-0/30, goat-8/16, antelope-0/34, fallow deer-0/20, giraffe-0/30 and African buffalo-0/15. Overall, an excellent agreement ($\kappa=0.87$) was observed between the two tests (95% CI, 0.83-0.91). The results also corroborated with a recently developed MAb-based blocking ELISA for anti-FMDV O antibody detection (Kashem *et al.*, 2024). Furthermore, the limit of detection of rP1-MAb-SPCE was similar to that of PAb-based SPCE as observed from serially diluted FMDV-strong positive cattle serum samples. The assessment of the analytical specificity of rP1-MAb-SPCE demonstrated that Monoclonal Antibody #5B6 exhibited a significantly stronger capability to compete with sera positive for FMDV/O. Conversely, it displayed minimal inhibition of the reactions with sera positive for FMDV/A and Asia 1, thereby indicating that the assay is specifically tailored for FMDV/O. Repeatability tests

showed high reproducibility of the assay. Coefficient of variation values were both below 5% for FMDV-strongly and moderately positive sera indicating consistency of the new test. Despite the fact that the intra-assay and inter-assay CVs for the negative control serum sample were somewhat elevated, they remained within the acceptable thresholds of less than 10% and less than 15%, respectively. In a nutshell, the rP1-MAb-SPCE could be useful for FMDV/O antibody detection in all FMDV susceptible species without biosecurity concern. Additional testing using large numbers of serum samples will be needed to confirm the preliminary validation.

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Conflict of interest

The authors declare no competing interest.

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Use of generative AI and AI-assisted technologies

No use of generative AI or AI-assisted technologies was used in the preparation, writing, or editing of this research manuscript.

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