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Development of an ELISA to distinguish between foot-and-mouth disease virus infected and vaccinated animals utilising the viral non-structural protein 3ABC

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Abstract

Introduction. Foot-and-mouth disease (FMD) is a highly contagious and economically devastating viral disease of livestock and is endemic in much of Asia, including Pakistan. Vaccination is used to control disease outbreaks and sensitive diagnostic methods which can differentiate infected animals from vaccinated animals (DIVA) are essential for monitoring the effectiveness of disease control programmes. Tests based on the detection of the non-structural protein (NSP) 3ABC are reliable indicators of virus replication in infected and vaccinated populations.

Hypothesis/Gap statement. Diagnosis of FMD is expensive using commercial ELISA kits, yet is essential for controlling this economically-important disease.

Aim. The development of a low-cost diagnostic ELISA, using protein made in *Escherichia coli*.

Methodology. In this study, the viral precursor protein 3ABC (r3ABC) was expressed in *E. coli*, solubilised using detergent and purified using nickel affinity chromatography. The fusion protein contained an attenuating mutation in the protease and a SUMO tag. It was characterised by immunoblotting and immunoprecipitation, which revealed antigenicity against virus-specific polyclonal sera. Using r3ABC, an indirect ELISA was developed and evaluated using field sera from healthy/naïve, vaccinated and infected animals.

Results. The diagnostic sensitivity and specificity of the r3ABC in-house ELISA were 95.3 and 96.3% respectively. The ELISA was validated through comparison with the commercially available ID Screen FMD NSP competition kit. Results indicated good concordance rates on tested samples and high agreement between the two tests.

Conclusion. The ELISA described here can effectively differentiate between infected and vaccinated animals and represents an important low cost tool for sero-surveillance and control of FMD in endemic settings.

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease affecting cloven hoofed animals including cattle, buffalo, sheep, goats, and pigs, amongst many other species, and is a major constraint to the international trade of animals and animal products. The causative agent, foot-and-mouth disease virus (FMDV) has a single stranded, positive-sense RNA genome and belongs to the *Aphthovirus* genus of the *Picornaviridae* family [1]. The genome encodes a single polyprotein

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Keywords: foot-and-mouth disease; FMD; 3ABC; diagnosis; ELISA; uncleavable precursor.

Abbreviations: DDM, N-dodecyl-β-D-maltoside; DIVA, differentiating infected animals from vaccinated animals; DSn, diagnostic sensitivity; DSp, diagnostic specificity; ELISA, enzyme-linked immunosorbent assay; FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; IPs, immunoprecipitations; Ni–NTA, nickel–nitrilotriacetic acid; NSPs, non-structural proteins; OIE, Office International des Epizooties; PP, percentage positivity; ROC, receiver operating characteristic.

that is cleaved into four structural (capsid forming) proteins (VP1–4) and eleven non-structural proteins [L (Lab, Lb), 2A, 2B, 2C, 3A, 3B (1-3), 3C and 3D]. These proteins and a number of the precursors facilitate the viral life-cycle [2, 3]. The virus is highly variable and exists as seven serotypes, O, A, Asia1, C and South African territories (SAT 1–3), each of which includes many antigenically-different strains [4]. Asymptomatic FMDV infection is common and virus can be detected in oropharyngeal fluids for 28 days post-infection (dpi), which represents a potential source for the spread of the FMDV infection [5]. Infected animals produce antibodies against structural and non-structural proteins (NSPs), whilst animals immunised with the inactivated virus vaccine should elicit immune responses only against structural proteins [6]. Given that NSPs are only produced during FMDV replication, the detection of antibodies against these in sera of infected animals is a reliable indicator of historic or active infection [7]. In 2002, the Office International des Epizooties (OIE) updated the regulations for International Animal Health Code, permitting countries actively vaccinating animals during FMD outbreaks to regain disease-free status after a six month period, subject to differentiation between vaccinated and infected or convalescing animals [8].

Enzyme-linked immunosorbent assays (ELISAs) using immobilised NSPs have been used in FMD-free regions, such as Europe, South America and Southeast Asia, to demonstrate freedom from infection and in endemic countries, such as in Asia, some parts of South America and Africa, for disease surveillance [9, 10]. A range of ELISAs have been developed that utilise NSPs such as 2B [11], 2C [12], 3A [13, 14], 3B [15, 16], 3ABC [7, 17–23], 3AB [24, 25], 3D [26] or a combination of these proteins [27–29], generated as recombinant antigens in *E. coli* or in insect cells via baculovirus expression. Synthetic peptides mimicking 2B [30], 2C [31] and 3B [32] have also been produced to develop ELISA tests. Purified NSPs have been used as coating antigens for the development of competitive, indirect and blocking formats, used for differentiating infected animals from vaccinated animals (DIVA). Detection of antibodies against the 3ABC polyprotein is considered suitable for the identification of infection, as the protein is highly immunogenic [10, 23], does not interfere with vaccine formulations [28, 33] and induces long lasting antibodies [18]. Furthermore, the OIE recommends the use of 3ABC as the reference antigen in ELISA [34, 35]. A number of commercially-developed diagnostic ELISAs use 3ABC as a target antigen [36]. However, these assays are prohibitively expensive for developing countries, such as Pakistan, and present variable sensitivity and specificity, meaning they are unsuitable for large scale sero-surveillance of FMD [36, 37].

The 3ABC protein is an early product post-translation and is most likely to be an intermediate in the production of the 3C protease during the viral lifecycle. The 3ABC protein is also responsible for the cleavage of cellular proteins, such as histone H3, and it acts to inhibit host RNA transcription during FMDV infection [38, 39]. The proteolytic activity of 3C also causes several issues in the development of assays that use 3C-containing polyproteins. The expression of recombinant 3ABC in *E. coli* cells is reported to result in a truncated species similar to 3A [40] and in insect cells five different proteins have been detected, which is most likely to be due to the activity of 3C [41]. Protein 3C is a chymotrypsin-like protease, which contains the catalytic triad Cys163–His46–Asp/Glu84 in the active site. The protease is responsible for 10 of the 13 cleavage events which process the viral polyprotein into individual proteins [42]. However, Cys142Ser and Cys163Gly substitutions ablate protease activity, permitting cleavage-free expression of 3ABC in *E. coli* [40, 43] and insect cells [44] for development of DIVA tests. The proteolytic activity of 3C can also be attenuated through the substitution Cys163Ala [45–48]. However, recombinantly-expressed 3ABC harbouring the Cys163Ala inactivating mutation has not, to date, been used as an antigen in the development of DIVA tests.

In this study, we describe the expression of the 3ABC non-structural protein, containing the 3C protease Cys163Ala point mutation from FMDV strain O1K together with a His-SUMO-tag, in *E. coli* cells. This protein was characterised by Western blotting and immunoprecipitation. An indirect ELISA was then developed to evaluate infection status. Performance of the in-house ELISA was validated through comparison with the commercially available ID Screen FMD NSP competition kit, confirming that the in-house assay presents a cheaper and more accessible alternative to commercially available diagnostics to monitor FMDV infection and differentiate infection from vaccination.

METHODS

Construction of 6×His-SUMO 3ABC expression vector

The gene corresponding to the 3ABC polyprotein, containing the 3C mutation Cys163Ala was PCR amplified from FMDV serotype 01K mCherry replicon plasmid [49] using specific primers; Forward: 5′-CC**AGATCT**ATCTCAATTCCTTCTCAAAA ATCTG-3′ incorporating a *Bgl*II site (bold type) and reverse: 5′AT**GCGGCCGC**TCACTCGTGGTGTGGTTCG-3′ incorporating a *Not*1 site (bold type). Following restriction digestion, DNA products were inserted into the pET-28a-SUMO expression vector. Ligated products were transformed into DH5α *E. coli* competent cells. The insert was confirmed by colony PCR with gene specific primers and the correct orientation of insert was analysed by Sanger sequencing, performed by Genewiz (Takeley, UK). The resulting construct was termed pHis-SUMO-3ABC.

Expression and purification of r3ABC

BL21 DE3 *E. coli* competent cells were transformed with pHis-SUMO-3ABC and a single transformed colony was grown overnight at 37 °C in LB media supplemented with 50 µg ml^{-1} kanamycin with shaking. The overnight culture was diluted 1:100 into fresh

LB media supplemented with 50 µg ml^{-1} kanamycin and incubated at 37 °C until an OD_{600} of 0.6–0.8 was reached. The expression of recombinant 3ABC (r3ABC) was induced through addition of isopropyl-*β*-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated at 28 °C for 5 h. The culture was harvested by centrifugation at 4000 ***g*** for 15 min at 4 °C and the pellet was stored at 80 °C until further use.

The stored pellet was resuspended in 30 ml l^{-1} culture of lysis buffer (50 mM Tris, pH 7.6, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol, 0.1% *N*-dodecyl-*β*-D-maltoside (DDM) supplemented with 1 mg ml^{-1} lysozyme, 100 µg ml^{-1} DNase I and EDTA-free complete protease inhibitor cocktail (Roche). The suspension was incubated at 4 °C for 20 min with rocking prior to cell lysis by sonication (Soniprep 150, MSE Scientific Instruments) on ice with 15 cycles of 10 seconds separated by 30 second pauses. The cells were centrifuged at 18000 r.p.m. for 20 min at 4 °C using a Sorvall-34 rotor and supernatant containing r3ABC was loaded on a HisTrap column (1 ml), pre-equilibrated with five column volumes of equilibration buffer (50 mM Tris, pH 7.6, 300 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol, 0.1% DDM). Flow-through was collected and the column was sequentially washed with five column volumes of wash buffer (50 mM Tris, pH 7.6, 300 mM NaCl, 5% (v/v) glycerol, 0.1% DDM) containing 20 mM, 50 mM and 100 mM imidazole. Finally, the protein was eluted with elution buffer (50 mM Tris, pH 7.6, 300 mM NaCl, 5% (v/v) glycerol, and 0.1% DDM) containing 200, 300 and 500 mM imidazole. Fractions were separated by SDS-PAGE followed by Coomassie blue staining and Western blotting. The mass of protein in fractions was determined by Bradford assay in a 96-well plate format, with comparison to serially diluted bovine serum albumin of known concentration [50].

Immunoblotting and immunoprecipitation

For Western blot analysis, fractions were separated by SDS–PAGE prior to transfer to PVDF membrane using a TransBlot Turbo (Bio-Rad) for 30 min at 25 volts. Following blocking in 5% skimmed milk powder in TBS containing 0.1% Tween-20 (MTBST), the membrane was incubated with specific antibodies targeting the His-tag (1:1000, Bio-Rad) or FMDV 3A (1:500, a kind gift from Francisco Sobrino) [51] prepared in MTBST. Following three washes, membranes were incubated with species-specific HRP-conjugated antibodies (1:2000, Sigma) prepared in blocking solution for 1 h before final washes. Immunoreactive bands were detected using ECL chemiluminescence substrate (Promega) and photosensitive film which was developed using an XOgraph.

Immunoprecipitations (IPs) were performed using magnetic protein G Dynabeads (Thermo Fisher Scientific) as per the manufacturer's recommendations, with magnetic capture of beads performed before aspiration of solute at each stage. Beads were mixed with 1 µl of antibody (mouse anti-his or rabbit anti-3A) diluted in 20 µl wash buffer and incubated to allow complex formation for 30 min at room temperature. The beads–antibody complex was washed with wash buffer and supernatant was discarded following magnetic capture. The complexes were incubated with 10 µl r3ABC at room temperature for 2 h with gentle agitation. Beads were washed prior to addition of 2× Laemmli buffer. Samples were boiled at 95 °C for 5 min and briefly centrifuged to pellet beads prior to loading of samples. Western blotting was performed as described previously.

Collection of animal serum

A total of 149 serum samples were collected from cattle and buffalo with known disease/vaccination status and used in development of an indirect ELISA. Of these, 45 were collected from clinically healthy, unvaccinated animals with no FMD history within 10 years. Samples were confirmed negative for antibodies to FMDV serotypes O, A, and Asia1 using a solid-phase competitive ELISA kit (IZSLER, Italy, a kind gift from Dr Hermann Unger, University of Veterinary Medicine, Vienna). Samples were collected from 62 animals that had received one of three vaccines at documented times post-vaccination. Out of these, 16 samples were collected at 30 days post-monovalent vaccination (Merial), 18 samples at 27 days post-trivalent vaccination (ARRIAH) and 28 samples at 30 to 180 days post-trivalent vaccination (FMDRC). Serum samples from 42 infected animals with documented disease history were collected at different times post infection (5–60 dpi).

In addition, 121 serum samples (67 cattle, 54 buffalo) were provided by the District Veterinary Hospital, Faisalabad (Punjab, Pakistan). These samples were collected during disease outbreaks reported in December 2020 and January 2021 in which animals were infected with serotypes O or A and disease status confirmed by FMD serotyping ELISA (IZSLER). The samples were collected at 15 to 30 days post-outbreak to detect antibodies against 3ABC.

Indirect ELISA protocol development

The optimal concentrations of r3ABC and test serum were determined by checkerboard titration. Serum samples collected from an infected buffalo at 20 dpi (confirmed by RT-PCR) and from a young calf with no FMD history were used as positive and negative controls, respectively. 96-well ELISA plates (Costar) were coated with 100 µl per well of 5–0.5 µg ml^{-1} r3ABC, diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. The plates were washed four times with PBST [0.01 M phosphate buffered saline (PBS, Medicago (Uppsala Sweden) containing 0.05% Tween-20]. The plate was blocked with blocking buffer (2% BSA-V in PBST with 3% BL21 *E. coli* lysate [25]) and incubated at 37 °C for 1 h. After five washes with PBST, 100 µl of serum samples diluted 1:10, 1:20, 1:40 and 1:80 in blocking buffer were added and incubated at 37 °C for 1 h. Following five washes with PBST, the plate was incubated at 37 °C for 1 h with 100 µl per well of HRP conjugated rabbit anti-bovine antibody (Abcam) diluted 1:3000 in

blocking buffer. The colorimetric reaction was developed using 100 µl of 0.1 mg ml^{-1} 3,3,5,5-tetramethylbenzidine (TMB) with 0.01% hydrogen peroxide in phosphate/citrate buffer (pH 5.0), and the reaction was stopped after 15 min incubation through the addition of 50 µl of stop solution (1M H_2SO_4) in each well. The absorbance at 450 nm was determined using a microplate reader (Ledetect 96, Tec).

The OD value of the positive control (OD_{pos}) and the samples (OD_{sample}) were corrected by subtracting the OD value of the negative control (OD_{neg}). Results were expressed as percentage positivity (PP) using the formula:

$$PD = \frac{OD_{sample} - OD_{neg} \times 100}{OD_{pos} - OD_{neg}}.$$

Determination of ELISA threshold value

To determine the optimal threshold value of the ELISA, 45 serum samples collected from clinically healthy animals with no FMD history; 62 samples from uninfected, vaccinated animals; and 42 samples from non-vaccinated infected animals were tested. The optimal cut-off value was calculated through receiver operating characteristic (ROC) curve analysis using GraphPad Prism 9 software.

Assay reproducibility

Assay reproducibility was evaluated using five serum samples with various PP values and a negative control. Serum from each animal was tested in triplicate across several days using independently prepared ELISA plates, with mean OD, standard deviation and coefficient of variation calculated using raw absorbance values.

Comparison of 3ABC indirect ELISA with ID Screen FMD NSP competition kit

Diagnostic performance of the in-house ELISA was compared with FMDV 3ABC-Ab ELISA (ID Screen FMD NSP Competition, ID-VET), which is an accepted diagnostic test in use in Pakistan, by assaying 32, 40 and 20 sera from healthy, vaccinated and infected animals, respectively, and 74 field sera from the district veterinary hospital of unknown FMD status. The ID Screen FMD NSP kit is a competition-based ELISA for the detection of antibodies directed against *E. coli* expressed r3ABC and is therefore an appropriate diagnostic tool to determine infection status in all FMDV-susceptible species. The assay was performed according to the manufacturer's instructions.

Analytical sensitivity

Analytical sensitivity of the in-house ELISA was determined by using four positive sera collected from infected animals at different days post infection along with negative control serum. Sera from three infected and one uninfected animal were two-fold serially diluted, following an initial 1:10 dilution, and assayed using the in-house 3ABC ELISA alongside the ID Screen FMD NSP Competition kit as per the manufacturer's guidelines.

RESULTS

Expression, purification and characterisation of r3ABC

The expression of 3C containing the protease-inactivating single point mutation Cys163Ala in *E. coli* has been described previously [46, 48]. In order to develop an ELISA using FMDV 3ABC as an antigen, we introduced this attenuating 3C mutation into 3ABC as well as a N-terminal SUMO tag in an attempt to improve solubility. The construct was termed pHis-SUMO-3ABC and this was transformed into BL21 DE3 *E. coli* cells. Initial results were indicative of poor protein solubility following expression, with the majority of protein present in the insoluble fraction following cell lysis (data not shown). To increase solubility, buffers were supplemented with the detergent *N*-dodecyl-*β*-D-maltoside (DDM) (final concentration 0.1% v/v). r3ABC was eluted using buffer containing 200 mM imidazole, with a final yield of 1.5 mg protein per litre culture volume obtained (Fig. 1a). r3ABC expression was confirmed by Western blotting using anti-His and anti-3A specific antibodies, with detection observed at the expected molecular weight of r3ABC (Fig. 1b, c). IPs using immobilised anti-his and anti-3A antibodies were also performed to confirm antibody recognition of native r3ABC (Fig. 1d). Results confirmed that antibodies targeting 3A could react with native r3ABC. Following confirmation of successful r3ABC purification, the protein was taken forward and used in the development of an NSP-based ELISA for field surveillance of FMDV circulation in Pakistan.

Standardisation of the indirect ELISA

To determine the optimal combination of coating antigen (r3ABC) concentration and test serum dilution in an indirect ELISA a checkerboard assay was performed (Fig. 2). Results indicated that the optimal combination of r3ABC and sera dilution was 1 µg ml^{-1} and 1:20, respectively, with a signal to noise (positive to negative) ratio of 8.16.

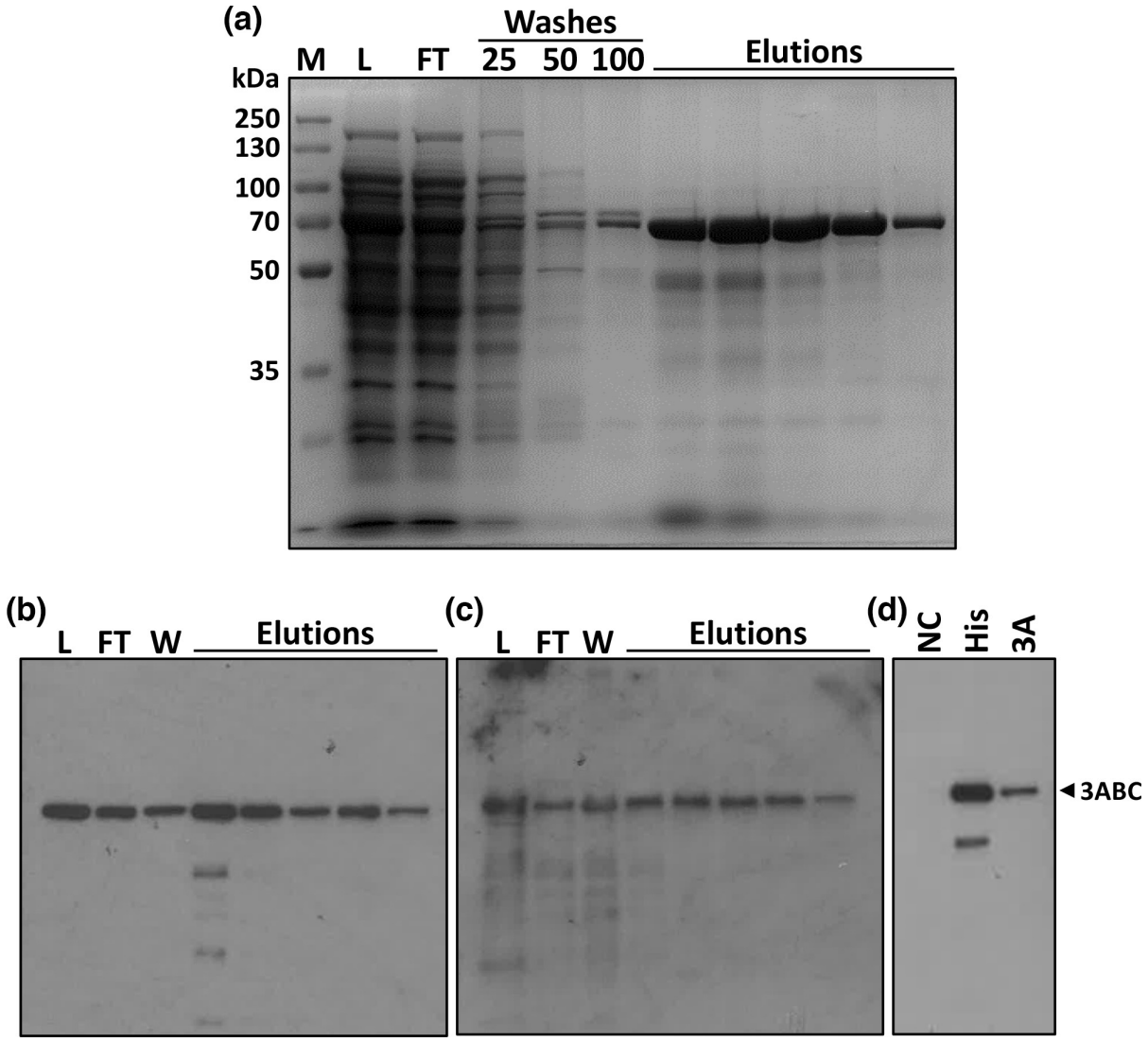

Fig. 1. Purification and characterisation of FMDV r3ABC. (a) Purification of FMDV r3ABC protein by nickel–nitrilotriacetic acid (Ni–NTA) chromatography prior to separation by SDS-PAGE and visualisation by Coomassie blue staining. M, pre-stained protein marker; L, load/cell lysate supernatant; FT, flow through, W, washes, with corresponding imidazole concentration (mM). Elutions represent 1 ml fractions collected sequentially using buffer containing 200 mM imidazole. (b) and (c) Western blot analysis of fractions using α-His (b) and α-FMDV 3A (c) antibodies to confirm successful expression and purification of r3ABC. Wash containing 100 mM imidazole was used to represent wash fractions (W). (d) Reactivity of non-denatured r3ABC was assessed by immunoprecipitation using α-His and α-FMDV 3A antibodies conjugated to magnetic Dynabeads prior to Western blotting using α-His antibodies. NC negative control containing no antibody-conjugated beads.

Determination of the threshold value, diagnostic sensitivity and diagnostic specificity of the in-house ELISA

The normalised PP values of 149 serum samples collected from animals with known FMD status (107 FMD-negative samples and 42 FMD-positive samples) determined by the in-house ELISA, were used to identify the most suitable threshold value by ROC curve analysis (Fig. 3a). The area under the ROC curve (AUC) of the in-house ELISA was determined as 0.9927 (95% CI=0.9837 to 1.00), indicating that the in-house ELISA was capable of distinguishing between positive and negative sample groups. The diagnostic sensitivity (DSn) and diagnostic specificity (DSp) were further determined by dot plot, using the optimal PP value of 27.80%, which was obtained from ROC curve analysis (Fig. 3b). Results indicated DSn of 95.3% and DSp of 96.3%, indicating that the in-house ELISA was suitable as a diagnostic test.

Serum samples of unknown status collected from disease outbreaks were tested using the in-house ELISA, which showed a 71% positivity rate for large ruminants, 76% and 65% for cattle and buffalo, respectively (Table 1).

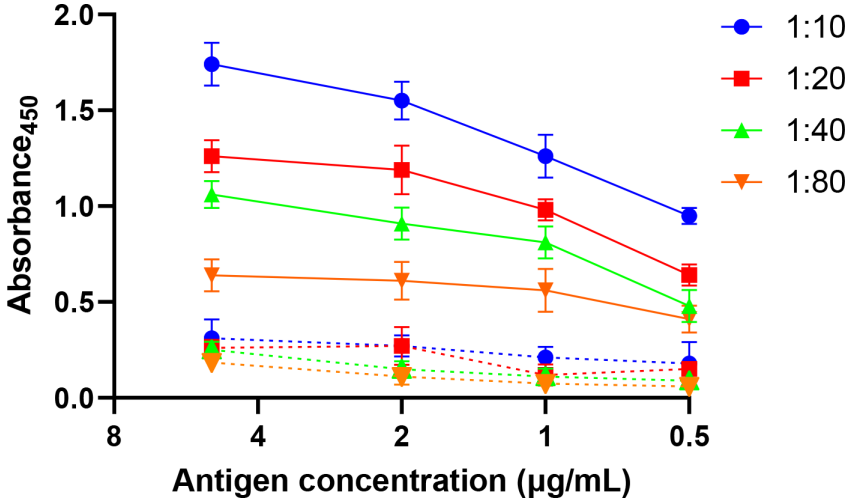

Fig. 2. Checkerboard titration to determine the optimal concentration of antigen and serum for use in ELISAs. r3ABC of varying concentrations was immobilised onto ELISA plates, prior to testing two-fold serially diluted FMDV positive (solid line) and negative (dashed line) sera to determine the optimal concentrations that yielded the highest signal:background ratio. *n*=2, symbols represent the mean and bars represent standard error of the mean.

Evaluation of diagnostic performance in comparison to a commercial kit

To validate the performance of the in-house ELISA, 166 serum samples including samples from from 32 healthy, 40 vaccinated and 20 infected animals and 74 random field samples were tested in comparison to the commercially available ID Screen FMD NSP competition kit. Both assays showed good concordance (95.2%) across all categories (Table 2). The rate of agreement between the two tests was calculated using kappa coefficient values, with a mean of 0.87 (95% confidence interval=0.77–0.98) across all the given categories showing good concordance.

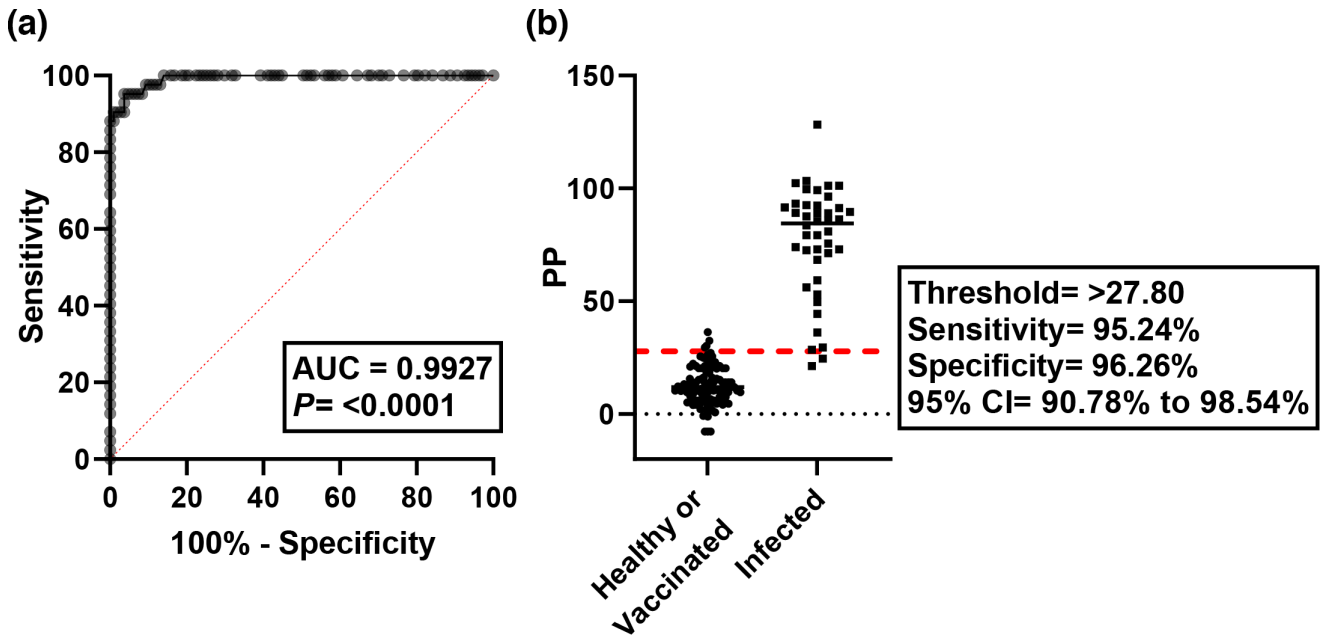

Fig. 3. Diagnostic performance of the in-house ELISA using sera from healthy, vaccinated and infected animals. (a) ROC curve analysis of the in-house r3ABC ELISA using healthy (*n*=45), vaccinated (*n*=62) and infected (*n*=42) sera. Healthy and vaccinated samples were treated as uninfected controls. (b) Dot-plot analysis to determine the optimal threshold value, based on sensitivity, specificity and 95% confidence intervals (CI).

Table 1. Prevalence of 3ABC NSP antibodies in samples collected from disease outbreaks. Samples from cattle and buffalo, provided by the District Veterinary Hospital, Faisalabad, were tested using the in-house ELISA to determine the presence of antibodies specific for r3ABC

Species	Total number of samples	Positive	Negative	Positivity rate (%)
Cattle	67	51	16	76
Buffalo	54	35	19	65
Total	121	86	35	71

Evaluation of assay reproducibility

Assay reproducibility was determined by comparing the raw absorbance values obtained from positive sera with different PP values, along with negative sera to calculate intra-assay and inter-assay variations across three different days. Intra-assay coefficient of variation (C.V.) was determined to be less than 10%, while inter-assay C.V. was calculated to be between 5.79 and 18.08% (Table 3). C.V. values were less than 20% and no sample result changed from positive to negative or vice versa, which indicated that the assay was reproducible.

Comparison of analytical sensitivity with a commercial kit

Analytical sensitivity of the in-house ELISA, in comparison to the ID Screen FMD competition kit, was determined using three FMDV-positive sera taken at different times post infection and an FMDV negative control that were serially diluted. The previously determined optimal sera dilution of 1:20 resulted in all three positive and the negative sera being correctly identified using both assays (Fig. 4a, b).

In order to determine assay sensitivity, samples were two-fold serially diluted to 1:5120 prior to testing. Results obtained for the three positive sera showed similar sensitivities for both tested assays, with values close to the respective thresholds observed where one test was positive and the other negative at a given dilution. For both assays there was a striking difference between the negative sera in comparison with positive sera in low dilutions (1:10 – 1:80). Sera taken at 30 dpi was determined to be positive by the in-house ELISA when diluted 1:640, whilst the commercial kit still identified the sample as positive when diluted 1:1280. Sera taken 50 dpi showed a similar result, with both assays determining the sample to be positive when diluted to 1:160. Sera taken at 80 dpi indicated an increased sensitivity of the in-house assay, with a positive result determined when diluted 1:160, whilst the commercial kit had crossed the threshold to return a negative result at this dilution. Taken together, these data indicate that the ELISA developed here performed in a manner that was similar to a commercially available alternative.

DISCUSSION

In this study, we describe the development of an indirect ELISA that detects antibodies from animal sera against recombinant FMDV 3ABC polyprotein. The performance of the ELISA developed here was evaluated using a range of sera collected from uninfected, infected and vaccinated animals and performance was further validated through comparison with the commercially available ID Screen FMD NSP competition kit.

In Pakistan FMD is endemic, with FMDV serotypes O, A and Asia1 the most prevalent serotypes in circulation, leading to huge economic losses [52, 53]. Pakistan has a vast livestock population including 41.2 million buffaloes, 49.6 million cattle, 78.2 million goats and 30.9 million sheep, which are susceptible to FMDV infection [54]. Many commercial dairy farmers vaccinate their animals through the use of imported trivalent vaccines such as Aftovax (Merial) or locally manufactured monovalent vaccines. Lack of diagnostic facilities, inappropriate control of vaccine markets and lack of control of animal movements are major barriers

Table 2. Concordance of the in-house r3ABC ELISA and ID-Screen FMD NSP kit. Samples of animals of known disease status and unknown disease status (field) were tested using the in-house r3ABC ELISA and ID-Screen FMD NSP kit to determine concordance

		r3ABC ELISA		ID-Screen FMD NSP Kit		
Status	Samples	Positive	Negative	Positive	Negative	Concordance (%)
Healthy	32	1	31	2	30	96.8
Vaccinated	40	3	37	4	36	92.5
Infected	20	17	3	15	5	90.0
Field	74	52	22	51	23	94.5
Total	166	73	93	72	94	95.2

Table 3. Determination of assay reproducibility through testing of six known samples over three separate days. Samples were assayed using the in-house ELISA on three separate days to determine reproducibility. S.D. = standard deviation, C.V. = coefficient of variation

Status	Repeat 1			Repeat 2			Repeat 3			Inter-run reproducibility		
	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.
Healthy	0.12	0.01	8.33	0.16	0.02	9.35	0.15	0.01	6.67	0.14	0.09	11.91
Vaccinated	0.33	0.02	4.90	0.23	0.02	9.18	0.23	0.02	8.77	0.26	0.05	18.08
Infected	0.58	0.03	4.71	0.51	0.02	3.09	0.44	0.03	6.18	0.51	0.08	17.07
Infected	1.05	0.03	2.92	0.84	0.03	3.58	0.75	0.03	3.80	0.88	0.15	16.94
Infected	0.66	0.05	6.94	0.61	0.03	4.98	0.59	0.03	4.43	0.62	0.04	5.79
Infected	0.81	0.07	8.07	0.64	0.03	3.95	0.76	0.04	5.50	0.73	0.02	15.37

in controlling FMD [55]. Determination of FMDV sero-prevalence using NSP-based assays has previously been performed in large [56] as well as small ruminants [57, 58] using commercially available kits. However, such kits are not economically viable for use on a large scale in countries such as Pakistan. Post-vaccination surveillance in the country is also scarce, further adding to the demand for an inexpensive assay so that monitoring of disease status in vaccinated populations can be performed.

FMDV polyproteins such as 3ABC are ideal candidates for use in assays to differentiate between vaccinated and healthy animals, as NSPs are only expressed during infection. The proteolytic activity of 3C can, however, lead to undesirable processing of 3ABC into the derivative proteins 3A, 3B1, 3B2, 3B3 and 3C. In order to maintain the unprocessed and highly antigenic 3ABC, we therefore utilised a single point mutation within 3C, namely Cys163Ala, which successfully ablated proteolytic activity in a manner similar to that observed with the mutations Cys163Gly and Cys142Ser, reported previously [40, 42, 43]. Previous studies have used baculovirus expression systems to express such proteins, however, such methods require cell culture and generally produce lower protein yield in comparison to bacterial systems and are therefore costly [21, 22]. Another factor in the development of assays using FMDV polyproteins such as 3ABC is that such proteins tend to display poor solubility during purification, which is likely to be due to the hydrophobic transmembrane domain of 3A [13]. Previous methods to ensure that r3ABC remained soluble utilised high concentrations of urea [21] or low concentrations of the ionic detergent *N*-laurylsarcosine followed by steps to refold the polyprotein into its native structure [17, 22]. To address this issue, an N-terminal SUMO-tag was introduced and the mild non-ionic detergent DDM was supplemented into buffers throughout purification, removing the need for additional steps post-purification. The majority of recombinant protein was recovered in soluble fractions during Ni–NTA chromatography, with yields of 1.5–1.8 mg protein per litre of culture. Further optimisation of expression and purification to boost protein yield per litre

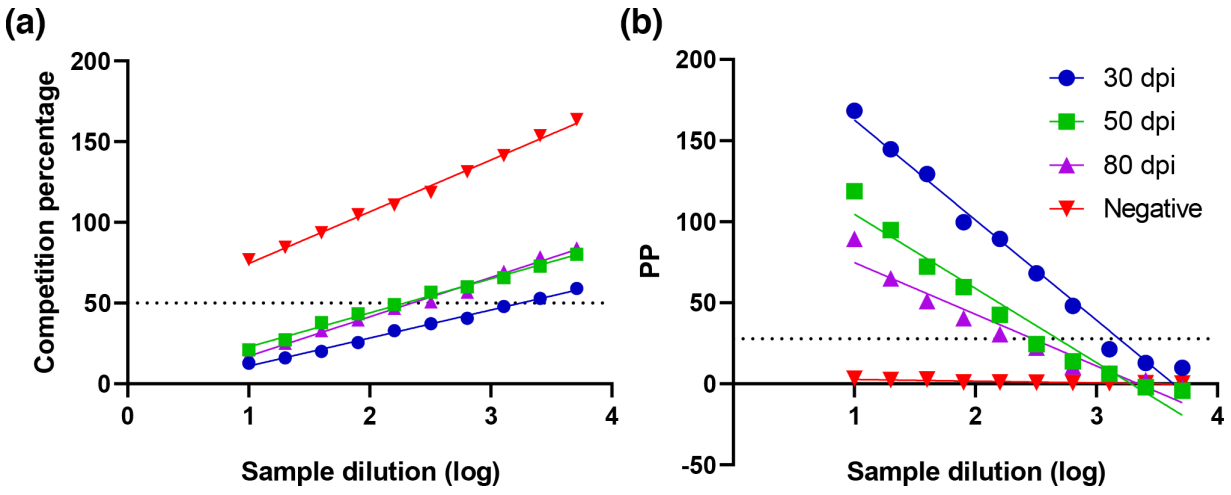

Fig. 4. Comparison of the ID Screen FMD NSP competition kit (a) with the in-house r3ABC ELISA (b) using animal sera. Sera from animals 30, 50 and 80 days post-infection (dpi) were serially diluted and tested to determine assay sensitivity of the in-house ELISA in comparison to a commercially available kit. Sera from a healthy, uninfected animal was used as a negative control. Dashed lines represent the threshold value for each assay of 50 and 27.80% for the ID Screen FMD NSP competition kit and in-house ELISA, respectively. *n*=1 for each sample.

of culture will be performed in future studies in order to improve the efficiency of recovery. As part of this optimisation, further experimentation to characterise protein stability and potential issues, such as aggregation, will be undertaken.

Using r3ABC, an indirect ELISA was developed to capture antibodies present in sera collected from cattle. An indirect ELISA format was selected over a competition-based assay due to the probability of increased sensitivity resulting from the binding of multiple epitopes present in sera, as compared with a competition-based ELISA, which would target only one or two epitopes [21]. The Pan American Center for Foot-and-Mouth Disease (PANAFTOSA) ELISA test, which is used as reference test for virus surveillance in regions of South America, such as Argentina, is also an indirect assay [25]. Results indicated that the in-house ELISA was able to detect FMDV-specific antibodies in infected animals regardless of their vaccination status. Optimisation revealed that the optimal concentration of antigen was 1 $\mu g\,ml^{-1}$ with a 1 in 20 dilution of test sera, which is in agreement with findings described in previous reports [17, 21]. The optimal threshold value of 27.80% was determined through ROC curve analysis, with diagnostic sensitivity and specificity of 95.24 and 96.26%, respectively. It should be noted that an indirect ELISA utilising *E. coli* expressed 3ABC has been reported previously. This included different 3C mutations to those included here and the insolubility of 3ABC necessitated urea-mediated unfolding and refolding of the protein, which is both time consuming and costly in terms of protein yield [7, 28]. This assay displayed 98.05 and 93.2% agreement with the Ceditest FMDV-NS and UBI NSP ELISA kits, respectively, when testing sera collected from PR China [17]. An indirect ELISA utilising 3ABC expressed in insect cells has also been developed in India, where antibodies were detected in sera of different animals using recombinant protein G conjugate [21]. This ELISA presented diagnostic sensitivity and specificity of 95.8 and 97.45%, respectively, and showed strong agreement with the PrioCHECK NSP kit. These figures provide supporting evidence that the ELISA using r3ABC described here produces results that are similar to those from other available assays.

Our assay detected one false positive sample in the naïve cohort of 32 samples and three out of 40 vaccinated animals, which in both cases was lower than the number identified using the ID Screen FMD NSP competition kit (two out of 32 naïve animals, four out of 40 vaccinated animals), which is also a competitive ELISA (Table 2) and is an accepted diagnostic assay in use in Pakistan. The in-house assay also performed better when detecting infected animals, with 17 of 20 cases correctly identified, compared with 15 of 20 positive results using the ID Screen FMD NSP competition kit. Assay reproducibility was also determined by evaluating control serum samples over three consecutive days, with results indicating that variance across assays was within acceptable limits (Table 3). The detection of false positives was increased for both the in-house ELISA and the ID Screen FMD NSP competition kit in animals that had been vaccinated when compared with naïve animals. These false positive results may be associated with insufficient specificity of the assay or to the presence of 3ABC during some vaccine preparations, which should have been depleted during the manufacturing process [10, 59]. It has previously been reported that animals receiving repeated local vaccination may elicit immune responses to 3ABC, which probably contributes to this observation [60]. NSP contamination in inactivated vaccines is therefore a barrier which may hinder the accuracy of NSP detecting assays and indicates the need to evaluate the vaccination history of an animal prior to use of such assays and interpretation of the reliability of the results. Our findings indicated that the ID Screen FMD NSP competition kit was more sensitive than our in-house ELISA (Fig. 4). It may therefore be the case that increased detection of NSPs in vaccinated animals using this commercial kit is due to increased sensitivity of the assay. This is further supported by a decrease in relative specificity in sera collected from animals vaccinated with the trivalent vaccine produced locally in Pakistan. Moreover, relative sensitivity may be affected by different geographical location. It has previously been reported that the commercial ID Screen FMD NSP competition kit gave lower sensitivity (76.9%) but 100% specificity upon comparison with sera identified after virus neutralization test (VNT) and 3B ELISA [59]. Analytical specificity is also an important parameter for newly developed assays, which is determined by using sera from animals infected with clinically similar diseases. This aspect could not be tested in this study due to a lack of available samples, but will be an important step in the future validation of this assay. In diseased herds of cattle and buffalo, the in-house ELISA determined an average positivity rate of 71% (Table 1), which showed good concordance rates with a commercially available test when comparing a smaller group of samples (Table 2). The detection of antibodies against 3ABC is a useful indicator of virus activity within diseased herds, which is essential in determining appropriate control measures within countries such as Pakistan.

FMD diagnosis in Pakistan is typically performed using molecular techniques such as RT-PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP). Although these are more sensitive than NSP ELISAs in detecting infection, they require sophisticated instruments and trained laboratory staff [53, 61]. The potential use of such tests in routine screening for disease is further challenged by transport and cold-chain storage in rural settings. Inexpensive diagnostic approaches that can be deployed in rural areas are therefore more logistically viable. It should also be noted that the costs of commercially available NSP ELISA kits are also prohibitive for widespread use. To bypass such economic challenges, there has been a need to develop an inexpensive assay to be used at large scale in FMD surveillance. Through the production of an assay in-house, which is scalable through using bacterially-expressed protein, there is the potential to produce a not-for-profit alternative that will drastically reduce the costs associated with such diagnostics.

The method described here is standardised for large ruminants (buffaloes and cattle), with a limited number of samples collected from several geographical locations. To further evaluate the suitability of the in-house ELISA developed here, a

wider range of animal species infected with FMDV will be tested, with hope that such studies will expand the potential use of this assay to several more species. Each FMDV serotype includes a number of subtypes with subtle differences in protein sequence that could influence assay sensitivity and specificity. Samples from more distant geographical locations will therefore be tested to determine whether this assay is suitable for use on a wider scale where there may be increased genetic variability. Antibodies against 3ABC can be detected by ELISA two years after initial infection, which is also considered as a marker of import and export serology [17, 23]. Testing of sera from three animals with historical FMDV infection using the in-house assay determined that two out of three cattle were positive, indicating that this ELISA was capable of detecting antibodies against 3ABC long after initial infection. Whilst this shows initial promise, further screening will be essential to improve confidence that historical infection can be routinely detected.

In conclusion, we describe the expression and purification of His-SUMO-tagged 3ABC containing the single point mutation Cys163Ala for use in an ELISA locally within Pakistan for the first time, to our knowledge. The assay described here, which could successfully differentiate between infected, healthy and vaccinated animals in a manner comparable to commercially available kits, provides promise of a robust and financially accessible diagnostic test for detection and control of FMD within Pakistan.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Blood samples from animals were taken by veterinarians as part of routine surveillance, in accordance with standard national guidelines in Pakistan. Their use here was approved by the local ethics committee at the Pakistan Institute of Engineering and Applied Sciences.

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