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Research paper

Improvement of a recombinant antibody-based serological assay for foot-and-mouth disease virus

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ABSTRACT

Differentiating foot-and-mouth disease virus (FMDV) antibodies generated during a natural infection from those due to vaccination (DIVA) is crucial for proving freedom from disease after an outbreak and allowing resumption of trade in livestock products. The World Organisation for Animal Health (OIE) recommends that FMDV vaccines are composed of inactivated virus that has been purified to remove non-structural viral proteins. Such purified vaccines primarily induce antibodies to viral structural proteins, whereas replicating virus stimulates host antibodies specific for both structural and non-structural proteins. The current preferred FMDV DIVA test is a competitive ELISA (C-ELISA) designed to detect antibodies to the non-structural protein 3ABC. Previously, we described the development of an FMDV DIVA test based entirely on recombinant proteins (the recombinant detecting antibody and the 3ABC coating antigen) produced in *Escherichia coli*. In this study, we have determined the precise binding site of the recombinant detecting antibody to a conserved sequence within the 3B region of the 3ABC protein, replaced the original E-tag of the detecting antibody with two in-house tags and engineered a direct antibody–reporting enzyme (alkaline phosphatase) fusion protein. These modifications have further improved the DIVA test, providing great potential for large scale production and uptake due to its simplicity, reproducibility and low cost.

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1. Introduction

Foot-and-mouth disease (FMD) remains the greatest threat to livestock industries worldwide. For a country to be considered free from the FMD virus (FMDV), different diagnostic approaches are required according to whether vaccine has been used and if so whether it was used in response to an outbreak of disease or as part of routine prophylaxis. The ability to differentiate between these different immune states has been an area of much research. The current preferred test to differentiate infected from vaccinated (DIVA) animals is a competition ELISA (C-ELISA) designed to detect antibodies to the non-structural protein 3ABC as an indicator of infection (Sorensen et al., 2005; Clavijo et al., 2004; Foord et al., 2007).

Most approved FMDV vaccines are composed of mainly viral structural proteins and hence primarily induce antibodies to these proteins, whereas replicating virus stimulates host antibodies against both structural and non-structural proteins. Although non-structural proteins can in theory contaminate the vaccine preparation, it is expected that FMDV vaccines produced by most current commercial manufacturers will not induce significant antibody responses to non-structural proteins. Provided these DIVA tests are used under controlled

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Abbreviations: AP, alkaline phosphatase; C-ELISA, competition enzyme-linked immunosorbent assay; CRAb, chicken recombinant antibody; DIVA, differentiation of infected from vaccinated animals; FMDV, foot-and-mouth disease virus; GST, glutathione *S*-transferase; IBDV, infectious bursal disease virus; IPTG, isopropyl-beta-D-thiogalactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SARS, Severe Acute Respiratory Syndrome; scFv, single chain variable fragment.

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circumstances and knowledge of vaccine quality understood, they can be of tremendous benefit for FMDV diagnosis and surveillance.

Previous FMDV DIVA tests relied on polyclonal or hybridoma-derived monoclonal antibody reagents which are expensive, difficult to produce and maintain. To improve the DIVA C-ELISA, we produced the two critical reagents, the detecting antibody and coating antigen, in *E. coli*, making them safe and more economical to produce without the requirement for infectious virus or animals (Foord et al., 2007). However, the recombinant antibody-based test still relies on two additional commercial antibodies (the epitope-tag-specific antibody and an enzyme conjugated antibody), which makes it expensive and difficult for quality assurance due to the requirement for multiple antibodies.

In the current study, we have further improved this recombinant protein-based DIVA test by: 1) determination of the precise binding site and binding affinity of the detecting antibodies which can facilitate fine tuning of assay sensitivity and specificity if required; 2) replacement of the commercial E-tag with two in-house epitope tags allowing further reduction of production costs; 3) development of an antibody–reporter fusion protein for a simple assay requiring fewer steps.

2. Materials and methods

2.1. Antigen and antibodies used in this study

The non-structural protein 3ABC previously described by Foord et al. (2007) was used as the coating antigen for the C-ELISA. Production of the two detecting antibodies CRAb-FM26 and FM27 with different epitope tags is described in Section 2.3. Monoclonal antibody F26G8, specific to the SARS coronavirus spike protein (Berry et al., 2004) was kindly provided by Dr. J. Berry and mAb 4–9D4 against the E2 protein of classical swine fever virus was produced in-house (Yu et al., 1996).

The following panels of experimental sera were used in the evaluation of the C-ELISA. The sera from FMDV-infected and FMDV-vaccinated cattle representing O, A, Asia-1 and C serotypes and the associated pre-treatment sera were kindly provided by Dr. Alan R. Samuel, IAH, Pirbright, UK. Infected pig sera were generated against serotype A24 and were kindly provided by Dr. J. Lubroth, Plum Island Animal Disease Center, New York, USA. Naïve pig sera were obtained in-house at AAHL. The vaccinated pig sera were serotype O and were kindly provided by Dr. Dong Manh Hoa, Regional Animal Health Center Ho Chi Minh City, Vietnam. Two sera from sheep infected with O-UKG were kindly provided by Dr. Bob Armstrong, IAH, Pirbright, UK. Serum from a sheep that had been experimentally infected sequentially with multiple serotypes; O1-Tunisia, A5, A10, O1-BFS and C1-Detmold was kindly provided by Dr. Aldo Dekker, Central Institute for Animal Disease Control, Lelystad, The Netherlands. Naïve sheep sera and sera from sheep vaccinated with serotypes O, A and Asia-1 were kindly provided by Dr. Jef Hammond, AAHL.

2.2. Expression of fusion proteins for epitope mapping

Primers were designed to amplify the three fragments of the FMDV 3B coding region denoted 3B-1, 3B-2 and 3B-3 (Table 1) and engineered to include Asc I and Not I unique restriction sites. Small gene fragments were amplified by PCR (94 °C 5 min, 30 cycles [94 °C 45 s; 55 °C 45 s; 72 °C 1 min], 72 °C 10 min, 4 °C) using the original pGD3-3B plasmid (Foord et al., 2007) as the template. Purified PCR products were digested with the restriction enzymes Asc I and Not I and cloned in-frame with the GST-tag in the pGD3 vector (Wang et al., 1996). Protein expression was performed as previously described for 3ABC with *E. coli* MC1061 cells (Foord et al., 2007).

2.3. Production of scFVs with different epitope tags

PCR fragments containing coding regions for the SARS (Yu et al., 2008) and YYEP (Yu et al., 1996) epitopes were created by PCR assembly of overlapping primers (see Table 1) under the following conditions: 94 °C 2 min, 35 cycles [94 °C 30 s; 45 °C 30 s; 72 °C 30 s], 72 °C 7 min, 4 °C. PCR products were purified and digested with the restriction enzymes Not I and Eco RI to facilitate cloning in-frame with the antibody gene sequence in the modified pCANTAB-link vector (Sapats et al., 2003).

2.4. Epitope mapping using phage display random peptide library

The Ph.D.-12 Phage Display Peptide Library (New England BioLabs) was utilized for epitope mapping of the FM26 and FM27 CRAbs, following the manufacturer's instructions. Briefly, 10 µl (~1.5×10^{11} phage particles) of phage peptide library was added to 1 µl of FM26 or FM27 scFv solution and incubated at room temperature for 15 min. For affinity binding of FM26 or FM27 to protein G, 1 µl of anti-E-tag monoclonal antibody (Pharmacia Biotech) was added to the Phage/scFv solution and incubation continued for a further 1 h. Protein G-Dynabeads (Invitrogen) were prepared by washing four times in PBST and blocking in 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA for 1 h. The phage/scFv/monoclonal antibody complex was added to the blocked Dynabeads and incubated for 1 h at room temperature. The bead complex was washed ten times in PBST and bound phage eluted using 1 ml 0.2 M Glycine–HCl (pH 2.2), 1 mg/ml BSA, followed by neutralization with 1 M Tris–HCl (pH 9.1). Phage titrations were performed to give inputs of 1 to 2×10^{11} plaque forming units for the following three rounds of binding and recovery. Single plaques from the third round of bio-panning were isolated, ssDNA purified and the 12-mer peptide sequence determined by DNA sequencing.

2.5. Affinity determination using biosensor

2.5.1. Cloning, expression and purification of CRAb scFvs

Large scale protein expression of CRAb-FM26 and FM27 was performed using a pGC *E. coli* expression vector (Coia et al., 1997). Specifically, the pCANTAB-FM26 and FM27 clones were digested with Nco I and Not I and the resulting scFv gene fragments cloned into a likewise digested pGC vector that contained C-terminal FLAG and His tags (Robert et al., 2009). The resulting pGC-FM26/27-FlagHis constructs were expressed according to previously described methodology (Dolezal et al., 2000). Proteins were isolated from the periplasmic space (Skerra and Pluckthun, 1988). The subsequent supernatant

Table 1
Primers used in this study.

Application	Name	Sequence (5′–3′)
Epitope tagging	SARS-F	AAGACGACGGCGGCCGCACTCACACCAGCTTG
	SARS-R	CCCGGGAATTCATTAGCGCCAAGCTGGTGTGAG
	YYEP-F	AAGACGACGGCGGCCGCATTGAAGAACAAGTACTATGAGCCCAGGGAC
	YYEP-R	CCCGGGAATTCATTAGCGGTAGCTGTCCCTGGGCTCATAGTACTT
Sequencing	H-chain-F	ATGTCTCTATGGCGCGCCGTGACGTTGGACG
	L-chain-F	ATGTCTCTATGTCGACAGCGCTGACTCAGCCGT
	AP-F	CCAGAATCGTTGGGTGATCT
Expressing	3B1-F	ATTAGGCGCGCCAGGACCCTACGCCGGACCA
	3B1-R	GTTAGCGGCCGCTACTCCTGCTGTGGGAGCTTG
	3B2-F	ATTAGGCGCGCCAGGGCCTTACGCTGGTCCG
	3B2-R	GTTAGCGGCCGCTATTCCTTCACGACCGGGGCT
	3B3-F	ATTAGGCGCGCCAGGACCTTACGAGGGACCG
	3B3-R	GTTAGCGGCCGCTACTCAGTGACAATCAGGTTC

was filtered through a 0.45 μm filter and the FM26 and FM27 scFv proteins purified by a two-step automated procedure using an AKTAxpress purification system (GE Healthcare). In brief, the scFv proteins were first purified using a Ni^{2+}–NTA HisTrap FF (1 ml) affinity column followed by size exclusion chromatography on a HiLoad 16/60 Superdex 200 column equilibrated in PBS. Purified proteins were quantified by their absorbance at 280 nm using the theoretical extinction coefficient calculated from the sequence (Gill and von Hippel, 1989).

2.5.2. Immobilization of scFv proteins for surface plasmon resonance study

A Biacore T100 biosensor instrument (Papalia et al., 2006) was used to measure the kinetic binding interactions of 3ABC antigen with immobilized FM26 and FM27. The scFv proteins were immobilized at 25 °C in running buffer (HBS-EP + buffer [10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20, pH 7.4]) in separate flow-cells on a CM5 (S series) sensor chip using standard amine-coupling (Johnsson et al., 1991). The scFv proteins were diluted to 30 μg/ml in 10 mM sodium acetate pH 4.5 and injected for 7 min (10 μl/min) over the activated surface. Final immobilized protein levels achieved were 318 RU (1 RU = 1 pg protein/mm^2) for FM26 in flow-cell 2 and 304 RU for FM27 in flow-cell 3. For all subsequent binding experiments flow-cell 1 was used as a "mock surface" for referencing purposes.

2.5.3. SPR binding analysis

The 3ABC antigen in 0.1% SDS was subjected to size exclusion chromatography on a Superdex 200 HR 10/30 column (GE Healthcare) in PBS (pH 7.4) calibrated with Bio-Rad Gel Filtration Standard proteins. Protein fractions corresponding to monomeric species of 3ABC were collected and concentrated to 0.4 mg/ml (7.5 μM). Binding experiments were performed in triplicate in HBS-EP + running buffer at 25 °C with a series of antigen concentrations, diluted two-fold from 320 nM to 10 nM, serially injected at a constant flow rate of 30 μl/min over the four flow-cells within the Biacore T100 instrument. Antigen association and dissociation phases were each monitored for 180 s and 300 s, respectively. At least one buffer blank injection was included for the purpose of double-referencing (Myszka, 1999). The FM26 and FM27 scFv protein surfaces were regenerated with a 15 s injection of 90 mM phosphoric acid at 30 μl/min. Scrubber software (version 2.0c; BioLogic Software, Campbell, Australia) was utilized to process all Biacore T100 generated sensorgrams and to determine association rate (k_a) and dissociation rate (k_d) constants by globally fitting to the processed data sets to a 1:1 Langmuir binding model. The equilibrium dissociation constant (K_D) was calculated from the quotient of k_d/k_a.

2.6. Production of scFv-alkaline phosphatase fusion proteins

Plasmids containing a gene encoding a His tag and an *E. coli* AP gene, designated pDAP2 and pDAP2/S, were sourced from Dr. Randolf Kerschbaumer (Kerschbaumer et al., 1996, 1997). Both pDAP2 and pDAP2/S were compatible with the pCANTAB-series of vectors, designed for the simple construction and production of CRAbs. pDAP2, pDAP2/S and the plasmid encoding CRAb-FM27 were digested with the restriction enzymes Sfi I and Not I, to enable the antibody-encoding gene to be cloned in-frame with both the His tag and the AP gene. All constructs were transformed into electrocompetent MC1061 *E. coli* cells. Positive clones were confirmed by PCR (94 °C 5 min, 30 cycles [94 °C 15 s; 55 °C 15 s; 72 °C 30 s], 72 °C 5 min, 4 °C) using the chicken heavy-chain primer and a primer specific for the AP gene in the vector (Table 1, sequencing primers) and direct sequencing of the PCR product.

The resulting plasmids, pDAP2-27 and pDAP2/S-27, were then transformed into electrocompetent *E. coli* TG1 cells for protein expression. A single transformant was inoculated into LB broth containing 100 μg/ml of Ampicillin and 1% glucose and incubated at 30 °C overnight with shaking. The overnight culture was diluted 1:200 in M9ZB/Ampicillin broth (Kerschbaumer et al., 1996) and grown at 30 °C with shaking, until the optical density of the culture was 0.5 as measured using an LKB Biochrom Ultrospec 4050 ($\lambda = 600$ nm). Cells were harvested by centrifugation at 4500 × g for 5 min at 25 °C, the supernatant was discarded and the bacterial pellet resuspended in fresh M9ZB-GY/Ampicillin media (Kerschbaumer et al., 1996), and further incubated at 16 °C for 72 h to achieve maximal expression of CRAb proteins. The cultures were centrifuged at 11,000 × g for 10 min at 4 °C, the pellet was discarded and the supernatant was stored at 4 °C until further analysis.

2.7. Western blot analysis

Equal amounts of each 3B protein, approximately 1 μg/lane, were loaded onto a 12% SDS-PAGE and electrophoresed.

The proteins were transferred to a nitrocellulose membrane, blocked and probed with either goat anti-GST-HRP (1:5000); CRAb-FM26-E-tag (1:40), CRAb-FM27-E-tag (1:500) or the control CRAb-20-T-tag (1:100) (Sapats et al., 2003). Each membrane was washed three times for 5 min in TBST and all bound antibodies, with the exception of the anti-GST-HRP control, had the addition of a secondary mouse anti-E-tag antibody (1:2000), followed by goat anti-mouse-HRP (1:1000). The bound antibodies were detected using a HRP substrate (50 ml PBST containing 1 tablet [30 mg] 4-chloro-1-napthol [Sigma] dissolved in 10 ml methanol and 30 µl H_2O_2 30% [w/v] just before use). Color development was stopped after 10 min by washing the membrane in water.

2.8. C-ELISA

CRAbs with different epitope tags were compared in a C-ELISA format. The C-ELISA was performed as previously described in Foord et al. (2007) with the exception of 2% sterile filtered BSA (Sigma, USA) in PBST used as blocking buffer. The 2% BSA/PBST was used in place of the skim milk powder/PBST block because it had been shown in previous studies to interfere with binding of the YYEP-mAb (M. Yu, unpublished results). The optimal working concentration of each antibody was determined by serial titration across the coating antigen 3ABC (62 ng/well) and were as follows; CRAb-FM26-E-tag (1:40 final 1:80), CRAb-FM26-SARS (1:5 final 1:10), CRAb-FM26-YYEP (1:5 final 1:10); CRAb-FM27-E-tag (1:400 final 1:800), CRAb-FM27-SARS (1:80 final 1:160), CRAb-FM27-YYEP (1:80 final 1:160). Each CRAb was detected using either the E-tag-specific antibody (Pharmacia; 1:1000), the SARS-specific antibody F26G8 (1:500) or the YYEP-specific antibody 4–9D4 (1:100). All of these tag-specific mAbs were detected using goat anti-mouse-HRP (Jackson ImmunoResearch Laboratories Inc.; 1:1000).

2.9. Detection of alkaline phosphatase activity

For rapid and simple determination of enzyme activity of the scFv–AP fusion proteins, to a microtitre plate well was added 90 µl of water, 10 µl of CRAb-FM27-AP supernatant and 50 µl of AP substrate (50 mM Tris pH 10 containing 150 mM NaCl, 2 mM $MgCl_2$ and 1 mg/ml p-nitrophenyl phosphate [Sigma]). The plate was placed at room temperature in the dark for 1 h and the enzyme activity was determined by measuring the change in optical density of the solution at a wavelength of 405 nm on an automated ELISA plate reader (Thermo Multiskan Ascent, Finland).

2.10. One-step C-ELISA

The C-ELISA was performed essentially as outlined above with the following modifications: the incubation of test serum with the recombinant CRAb-FM27-AP/S produced in 2.6 above (1:30: final 1:60) was performed at room temperature for 30 min and then developed by incubation with AP substrate for 1 h at room temperature in the dark as described above.

3. Results

3.1. Differentiation of the three repetitive sequences in the 3B region for scFv binding

The FMDV 3B region contains three repetitive sequences as shown in Fig. 1A. To determine the exact binding site(s) utilized by the CRAbs, each 3B peptide was cloned, expressed and tested for reactivity with FM26 and FM27 and the non-related CRAb-20 (specific to IBDV) in a Western blot. The results shown in Fig. 1B indicated that both FM26 and FM27 bound the full-length 3B, the 3B-1 and 3B-2 peptides, but not the 3B-3 peptide. The non-related CRAb-20 exhibited no reactivity to any of these fusion proteins (data not shown). The relative mobility and quantity of each fusion protein were determined using the goat anti-GST HRP antibody. Amino acid sequence alignment of the three repeated regions within the 3B protein indicates a high degree of similarity between 3B-1 and 3B-2. In comparison, the 3B-3 region is less similar. This observation is consistent with the Western blot results obtained (Fig. 1B). Taken together, the data suggests that there are two independent binding sites in the 3ABC protein, which are recognised by both FM26 and FM27.

3.2. Fine mapping of key binding residues using phage display peptide library

After three rounds of affinity selection as detailed in the Materials and methods, specific reactivity of the selected phage clones with the CRAbs was confirmed by ELISA (data not shown). Of those showing a positive ELISA reactivity, twelve clones were randomly selected and sequenced to determine their peptide insert sequences. The results obtained (Fig. 2) revealed several important findings: a) a consensus sequence motif was identified from the peptide sequences selected by both CRAbs; b) the motifs recognised by both CRAbs share a high level of similarity all contained a Φ-DPLE(D) sequence (Φ = aromatic amino acid residue); c) the Φ–PLE(D) motif was found in the 3B-1 and 3B-2 peptides; d) there is a subtle difference between the two motifs recognised by both CRAbs where the P residue is more conserved in the motif recognised by FM26 than that of FM27; and e) the LE(D) residues are highly conserved in both motifs, but missing from the 3B-3 peptide, which may explain the failure of both CRAbs to react with this peptide.

3.3. Kinetic analysis of binding between 3ABC and scFv using biosensor

Kinetic parameters were determined for 3ABC antigen binding to immobilized CRAb-FM26 and FM27 (Table 2). Differences were detected mainly in the association rate constant (k_a) whereby 3ABC associated approximately 3-fold faster with FM27 than with FM26. Dissociation rate constants (k_d) were comparable for both CRAbs although FM27 dissociated approximately 1.3-fold slower. It is therefore the association rate that contributes most to the overall difference in affinity (K_D) whereby FM27 exhibits a four times higher affinity for 3ABC in comparison to FM26.

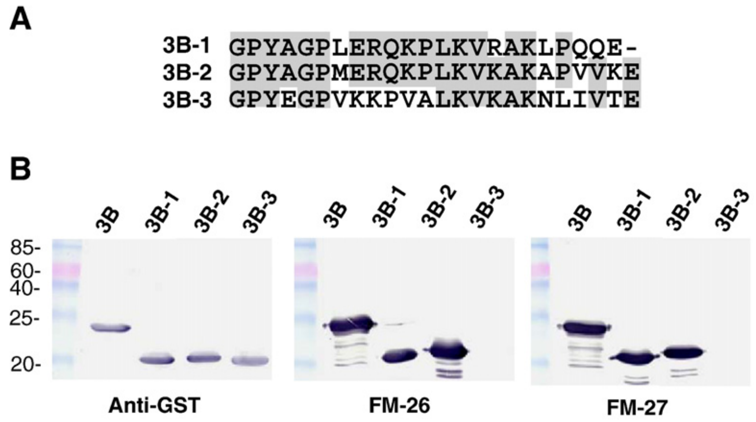
A
3B-1 GPYAGPLERQKPLKVRAKLPQQE-
3B-2 GPYAGPMERQKPLKVKAKAPVVKE
3B-3 GPYEGPVKKPVALKVKAKNLIVTE
B
3B
3B-1
3B-2
3B-3
85-
60-
40-
25-
20-
Anti-GST
FM-26
FM-27

Fig. 1. Mapping of antibody binding sites in the 3B region. (A) Alignment of aa sequences of the three repetitive sequences in the 3B region, named 3B-1, 3B-2 and 3B-3, respectively, from N- to C-terminus. (B) Western blot analysis of the four GST-fusion proteins, probed by different antibodies shown at the bottom of each panel.

3.4. Replacement of the detection tag and its effect on assay performance

To enhance the diagnostic application of the CRAbs, the commercial E-tag was replaced with in-house epitope tags to decrease associated costs with secondary reagents. A C-ELISA was used to compare the relative performance of each CRAb with different epitope tags. Results obtained with paired sera of pre-treatment and FMDV-infected cattle, sheep and pigs indicated that the performance of the CRAbs with in-house tags was comparable to those with the E-tag (Fig. 3). Performance of the YYEP tag was shown to be slightly better than the original E-tag.

3.5. Development of a one-step assay using scFv–AP fusion

To further simplify the assay, an antibody–reporter fusion protein using AP as the reporting enzyme was generated. The plasmids pDAP2 and pDAP2/S contain the wild type AP gene and a mutant gene (AP/S), respectively (Kerschbaumer et al., 1997). The AP/S has been shown to enhance the specific activity of the AP up to 35-fold (Kerschbaumer et al., 1997). Because the AP gene is derived from *E. coli*, FM27-YYEP was included as a control alongside the FM27-AP and FM27-AP/S constructs for all studies. Western blot analysis demonstrated the presence of the CRAb–AP fusion protein with the predicted molecular mass of approximately 72 kDa (Fig. 4A). The functionality of the fusion protein was demonstrated by directly measuring AP activity in the crude supernatant as shown in Fig. 4B. Background AP activity was negligible as indicated by the control FM27-YYEP. As expected, the FM27-AP/S clone exhibited higher enzyme activity than the FM27-AP clone. Furthermore, the specific reactivity of the CRAb component in the fusion was confirmed by ELISA, which indicated that the CRAb–AP proteins reacted with 3ABC, but not with a non-related SARS virus antigen containing the same His tag (data not shown).

Since the AP/S fusion showed higher specific activity, subsequent studies were conducted using FM27-AP/S only. After optimization of the one-step C-ELISA using different dilutions of the FM27-AP/S protein, a DIVA test was performed with a panel of sera representing naïve, FMDV-infected and FMDV-vaccinated cattle, sheep and pigs (Fig. 5). The results demonstrated that the FM27-AP/S protein retained the ability to function in the C-ELISA format and differentiate between naïve, FMDV-infected and FMDV-vaccinated animals despite the introduction of an enzyme molecule fused at its C-terminus.

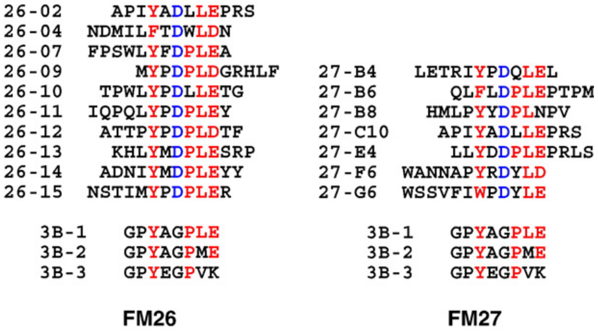
26-02 APIYADLLEPRS
26-04 NDMILFTDWLDN
26-07 FPSWLYFDPLEA
26-09 MYPDPLDGRHLF
26-10 TPWLYPDLLETG
26-11 IQPQLYPDPLEY
26-12 ATTPYPDPLDTF
26-13 KHLYMDPLESRP
26-14 ADNIYMDPLEYY
26-15 NSTIMYPDPLER
27-B4 LETRIYPDQLEL
27-B6 QLFLDPLEPTPM
27-B8 HMLPYYDPLNPV
27-C10 APIYADLLEPRS
27-E4 LLYDDPLEPRLS
27-F6 WANNAPYRDYLD
27-G6 WSSVFIWPDYLE
3B-1 GPYAGPLE
3B-2 GPYAGPME
3B-3 GPYEGPVK
3B-1 GPYAGPLE
3B-2 GPYAGPME
3B-3 GPYEGPVK
FM26
FM27

Fig. 2. Mapping of epitopes using phage display random peptide library. Random 12-mer aa sequences selected by FM26 (on the left) or FM27 (on the right) are aligned for identification of consensus motifs, which are in turn aligned with the predicted matching residues in the 3B repetitive sequences shown underneath. Residues in red indicate conserved random peptide sequences present in the 3B region whereas residues in blue are those conserved in the peptide sequences, but absent in the 3B region.

Table 2
Apparent kinetic rate constants and equilibrium binding constants for the interaction of recombinant 3ABC with immobilized FM26 and FM27 scFv proteins (± standard deviation; $n = 3$).

scFv	$k_a \times 10^4 (M^{-1} s^{-1})$	$k_d \times 10^{-4} (s^{-1})$	K_D (nM)
CRAb-FM26	1.0 ± 0.2	10.2 ± 0.5	107 ± 21
CRAb-FM27	3.2 ± 0.6	7.8 ± 0.5	25 ± 7

Of the cattle sera, the six naïve and six FMDV-vaccinated sera demonstrated less than 35% inhibition. The six sera from FMDV-infected cattle, representing O1-Manisa, C-Oberbayern, Asia-1 India and three different strains of serotype A (A15, A22 Iraq and A24 Cruzeiro), all had an inhibition of greater than 93% with the exception of C-Oberbayern with 84% inhibition. The six sera from naïve and FMDV-vaccinated pigs (serotype O) showed an inhibition of less than 20% and 39% respectively, with the sera from FMDV-infected pigs (serotype A) demonstrating a range of inhibition from 54% to 96%. For the sheep sera tested, the six naïve and six FMDV-vaccinated sera showed an inhibition of less than 20% and 31% respectively and the sera from FMDV-infected sheep showed inhibition of 76, 81 and 84%.

4. Discussion

Recombinant antibodies are being used as an alternative to mAbs for a range of research applications. Chicken recombinant antibodies are not only simple to produce but they are also less cross-reactive when used in combination with mammalian antibodies in immunoassays. Our previous FMDV DIVA C-ELISA (Foord et al., 2007) utilized a commercial E-tag which can only be detected using the commercial anti-E-tag antibodies (the E-tag mAb is no longer commercially available). In this current study, we determined the epitope sequences recognised by the two closely related CRAbs and the binding affinities by biosensor. To further improve their application, the CRAbs were modified to include non-commercial tags and an antibody–enzyme fusion protein.

Using two independent methods, i.e., expression of truncated peptide sequences and selection of mimotopes from phage display random peptide libraries, the key binding sites/residues of CRAb-FM26 and FM27 were determined. It was interesting to note that both CRAbs have two binding sites within the 3B region, designated 3B-1 and 3B-2 in this study. Although both CRAbs bound the same site, the data obtained from the phage library suggests a subtle difference in the key residual contacts between the two antibodies. In the Φ-DPLE (D) motif, CRAb-FM26 has a more stringent requirement for the Y residue at the Φ position and the presence of the P residue than that of CRAb-FM27. This difference in binding between FM26 and FM27 was further demonstrated by absolute affinity measurements using a biosensor. This was most notable with the association rate constant (k_a), which showed an approximate 3-fold difference between the antibodies, with FM27 having a faster on-rate. Since the variable heavy (V_H) chain complementary determining regions of the two antibody molecules is identical (Foord et al., 2007), one could conclude that the observed difference in binding affinity and mimotope specificity is entirely dependent on the variable light (V_L) chain. These data would suggest that further V_L-randomization may be used as a strategy to improve the assay sensitivity in the future.

To improve the viability of the recombinant antibody as a potential commercial reagent, alternative epitope tags were explored. For this application we chose two epitope tags derived from the SARS coronavirus spike protein and the classical swine fever virus E2 protein, which have been developed in our group (M. Yu, unpublished results). Although both tags maintained the functionality of the CRAbs, the YYEP tag was more effective in the C-ELISA than the SARS-tag. For this reason, the in-house produced YYEP tag will be used in all future studies. A potential future improvement will be a direct conjugation of HRP with the 4–9D4 antibody for detection of the YYEP epitope tag.

Although the introduction of the YYEP epitope tag had improved the DIVA C-ELISA, the assay strategy remained reliant upon a mAb and a conjugate for detection. We moved to a direct antibody–enzyme fusion system to simplify the assay to a single antibody in a one-step assay format. To do this, we employed the AP-fusion vectors developed by Kershbaumer's group (Kerschbaumer et al., 1996, 1997), that contained both the wild type and a mutant AP gene. The mutant gene denoted AP/S contained a serine residue (S) at position 101 instead of the wildtype aspartate residue (D), which has the potential to increase the specific AP activity by 35 fold (Kerschbaumer et al., 1997). The CRAb–AP fusion proteins generated in this study

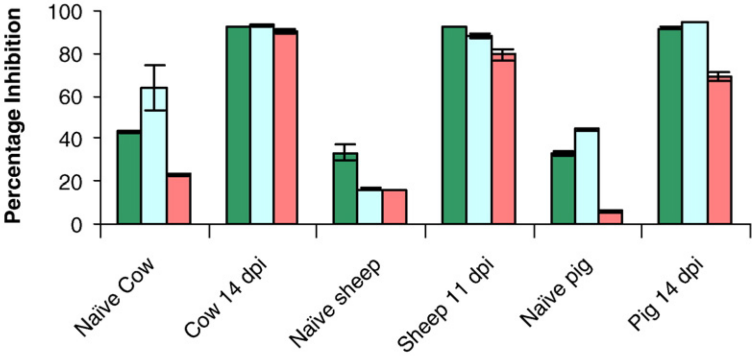

Fig. 3. Performance comparison of three different epitope tags in C-ELISA. Results shown are C-ELISA readings conducted under identical conditions using FM27 with the E-tag (green), the YYEP-tag (blue) and the SARS-tag (red). Error bars represent the standard deviation of the mean.

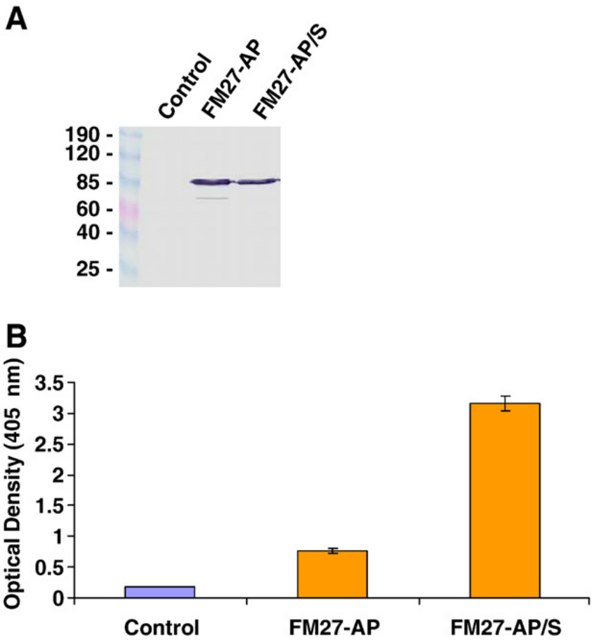

Fig. 4. Characterization of the antibody-alkaline phosphatase (AP) fusion proteins. (A) Western blot analysis using anti-His antibody, to confirm protein size and full-length expression. (B) AP activities displayed by each of the fusion proteins. Negative control: FM27 alone with no AP-fusion. Error bars represent the standard deviation of the mean.

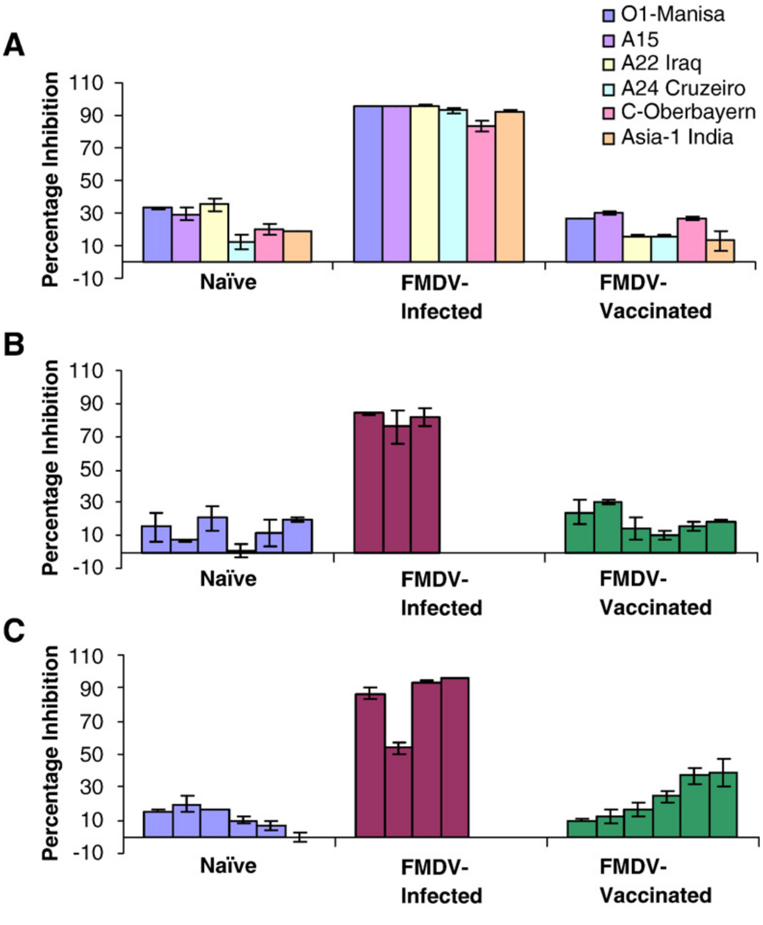

Fig. 5. Testing of FM27-AP/S fusion protein in a one-step C-ELISA using cattle (A), sheep (B) and pig (C) sera. Error bars represent the standard deviation of the mean.

maintained both the AP activity and the antibody specificity of the two fusion partner proteins. However, the FM27-AP/S was only 3 fold more reactive than the FM27-AP. Nevertheless, the FM27-AP/S represents a very promising reagent for wider application of the one-step FMDV DIVA test.

In conclusion, the current study has made significant progress in our understanding of the two FMDV-specific CRAbs produced in our previous study. The introduction of the antibody–enzyme fusion protein into the DIVA test has realized the potential of a one-antibody one-step assay format which will greatly facilitate its production and application in both developed and developing countries. The advantages of the current test platform over other existing tests include simple operation, less assay time, low production cost and high reproducibility.

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