Journal of Virological Methods 188 (2013) 161–167

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of an inactivated 3C^{pro}-3ABC (mu3ABC) ELISA to differentiate cattle infected with foot and mouth disease virus from vaccinated cattle

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Article history:
Received 18 May 2012
Received in revised form
12 December 2012
Accepted 17 December 2012
Available online 7 January 2013

Keywords:
FMDV
3ABC-based ELISA
Non-structural protein
DIVA

ABSTRACT

Foot and mouth disease, a highly contagious disease of cloven-hoofed animals, is still endemic in Asia, Africa, and a few countries in South America. Subclinical and persistent infections usually occur in vaccinated cattle exposed to FMDV. Successful control and eradication measures need a diagnostic assay that can distinguish between immune responses to infection and vaccination. The non-structural 3ABC ELISA is the most reliable differential diagnostic assay. However, expression of the native 3ABC gene in insect cells yielded truncated versions of the proteins; thus, a monoclonal antibody to capture digested proteins is needed to develop the assay. The purpose of this study was to develop a simple indirect 3ABC ELISA using complete 3ABC protein. The full-length mutated 3ABC protein with inactive 3C^{pro} (mu3ABC) gene was constructed. The histidine-tagged mu3ABC protein was produced in insect cells for easy purification and measuring. This permits simple assay design and reproducible assay development. mu3ABC ELISA had diagnostic specificity and sensitivity of 96.6% and 84%, respectively, compared to Ceditest® FMDV-NS. Agreement of both assays was excellent with κ value of 0.823 ($p < 0.05$). The mu3ABC ELISA could distinguish infected from vaccinated animals. These factors are necessary for the successful development of an in-house NSP-based ELISA. Availability of a reliable assay with acceptable costs would facilitate successful disease control and the establishment of disease-free zones. Expansion of such zones may ultimately decrease the risk of introducing FMDV into disease-free countries, thus accelerating global FMD control.

1. Introduction

Foot and mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, such as cattle, sheep, pigs, and goats. The causative agent, Foot and mouth disease virus (FMDV), is a member of the genus *Aphthovirus* in the family *Picornaviridae* (Loeffler and Frosch, 1897). FMDV is a non-enveloped virus with icosahedral symmetry. The diameter of the virion is about 30 nm; it contains a single-stranded positive-sense genomic RNA of 8500 nucleotides. The genome is transcribed as a single open reading frame (ORF), which is translated into a single polyprotein. This polyprotein is cleaved by viral proteases, generating several individual structural proteins (VP1, VP2, VP3, and VP4) and non-structural proteins (NSPs; 2A, 2B, 2C, 3A, 3B, 3C^{pro}, and 3D^{pol}) (Domingo et al., 2002; Mason et al., 2003). The 3C^{pro} is an important viral protease enzyme, which processes at least 10 positions on the primary polyprotein of FMDV (Curry et al., 2007). In infected cells, the non-structural polyprotein 3ABCD of FMDV is cleaved into six single proteins: 3A, 3B1, 3B2, 3B3, 3C, and 3D.

These viruses are highly variable and are divided into seven serotypes: O, A, C, Asia1, South Africa Territories (SAT) 1, SAT2, and SAT3 (Gleeson, 2002). They may persist in the oropharyngeal area of infected cattle for at least 28 days (Parida et al., 2005). Vaccinated animals can be infected with FMDV, causing subclinical or persistent infection. These animals may serve as carriers, although there has been no clear evidence of their role as a source of FMDV infection (Sørensen et al., 1998; Kitching, 2005; Lee et al., 2006; Paton et al., 2006). A diagnostic test with the ability to differentiate between infected and vaccinated animals (DIVA) will enhance the effectiveness of control strategies.

The most effective and high-throughput diagnostic assay that can differentiate infection from vaccination is the NSPs-based ELISA

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http://dx.doi.org/10.1016/j.jviromet.2012.12.016

(Robiolo et al., 2006; Yadin et al., 2007). The recombinant NSPs expressed in *E. coli* (Suryanarayana et al., 1999) or insect cells (Meyer et al., 1997) were produced and used to develop differential diagnostic tests. Antibodies to the NSPs were produced only in the infected cells, and appeared in infected animals starting from 1-week to more than 1-year post-infection (Oem et al., 2007; Mohapatra et al., 2011). More antibodies to 3ABC protein presented in the animals than other NSPs (Clavijo et al., 2006) and for a longer period than antibodies against 3C alone (Lubroth et al., 1998). In an experimental comparison of various NSP-based ELISA, 3ABC ELISA demonstrated the best diagnostic performance (Mackay et al., 1998; Sørensen et al., 1998; De Diego et al., 1997; Robiolo et al., 2006). A number of 3ABC-based ELISAs are commercially available, but remain fairly expensive. Recently, other NSP-based ELISAs have been developed, including one competitive ELISA that uses single-chain Fab fragments produced by phage-display technology (Foord et al., 2007), another *E. coli*-derived 3ABC ELISA (Lu et al., 2007), a 2C3AB ELISA (Lu et al., 2010), a $3AB_3$ ELISA (Mohapatra et al., 2011), an rAB1 ELISA (Jaworski et al., 2011), and an ELISA produced from 3AB protein with one deletion at the amino terminus (He et al., 2010). These assays are all claimed to be more sensitive and/or specific than other assays.

The expression of 3ABC gene in *E.coli* yielded a truncated 3ABC version with a molecular weight similar to the 3A protein (Sariya et al., 2011). Furthermore, expression of the native 3ABC gene within insect cells generated five different proteins, with molecular weights of 51, 38, 34, 27, and 18 kDa (Kweon et al., 2003). These evidences indicated that the polyprotein 3ABC was cleaved into each individual protein, probably by $3C^{pro}$ activity (Curry et al., 2007). The 3ABC proteins utilized in the aforementioned 3ABC-based ELISA were produced from the wild-type 3ABC gene. These native 3ABC proteins presented in previous studies might be generated as processed protein, not the full length version. On the other hand, the NSPs used in 2C3AB (Lu et al., 2010), $3AB_3$ (Mohapatra et al., 2011) and rAB1 ELISAs (Jaworski et al., 2011) did not included $3C^{pro}$ in their constructs to avoid the digestion of the NSPs into each individual protein. $3C^{pro}$, a chymotrypsin-like cysteine protease, is a major viral protease that cleaves ten of thirteen cleavage sites within the viral polyprotein (Mason et al., 2003), and has a Cys163-His46-Asp/Glu84 catalytic triad in the active site (Birtley and Curry, 2005; Grubman et al., 1995). However, substitution of Cys142 to Ser and Cys163 to Gly resulted in a completely inactive $3C^{pro}$ enzyme (Sariya et al., 2011).

The purpose of this study was to develop an NSP-based ELISA from $3C^{pro}$ inactivated 3ABC protein to differentiate infected from vaccinated animals. The 3ABC gene of an FMDV serotype O was mutated by site-directed mutagenesis, which altered the two key amino acids – Cys163Gly and Cys142Ser – within the catalytic triad of the $3C^{pro}$. The 55 kDa full-length mutated 3ABC (mu3ABC) was cloned into the Baculovirus expression system and expressed in insect cells. The mu3ABC protein with inactive $3C^{pro}$ was used as antigen for the development of a modified indirect ELISA, mu3ABC ELISA, to differentiate FMDV-infected from-vaccinated cattle.

2. Materials and methods

2.1. Molecular cloning and construction of the mutated 3ABC (mu3ABC) gene

3ABC gene of FMDV serotype O (O/Thailand/02) containing inactivated $3C^{pro}$ protease was constructed as described previously (Sariya et al., 2011). Two point mutations were introduced into the $3C^{pro}$ active site at the nucleotide positions 1103 and 1166 of the 3ABC gene. As a result, Cys (TGC) 142 and 163 were changed to Ser (AGC) and Gly (GGC), respectively. Briefly, the mutation at nucleotide position 1103 was introduced using a fusion PCR technique with two pairs of primers [3ABC_F (5′ AGTCGGATC CCC AATTCCTTCCCA AAA ATC T 3′)/C142S_R (TCCGTC CAT GCTCACCACAAT G and C142S_F (CAT TGTGGT GAG CAT GGACGG A)/3ABC_R (AGCTAAGCT TAG TGGTGTGGTTCGGGGTCA A)]. The mutation at nucleotide position 1166 was generated similarly by using two different internal primers, C163G_F (TGG CTA CGGCGGGGGAGC)/C163G_R (GCT CCC CCGCCG TAG CCA). The purified 3ABC DNA containing Ser142 and Gly163 was cloned into pGEM-T Easy (Promega) and the nucleotide sequences were verified by sequence analysis. The selected clone with correct nucleotide sequences was designated pmu3ABC.

2.2. Expression of viral genes

The Bac-to-Bac (baculovirus) expression system (Invitrogen) was used for the expression of the mu3ABC gene, as described elsewhere (Nawagitgul et al., 2000). Briefly, the mu3ABC gene was subcloned into pFastBac (Invitrogen) at *BamHI* and *HindIII* restriction sites, and transformed into *E.coli* DH10Bac (Invitrogen). Within *E.coli* DH10Bac, the mu3ABC gene was transposed into the bacmid. The colonies of *E.coli* containing the recombinant bacmid were collected by blue-white selection. The recombinant bacmid DNA was isolated, purified, and transfected into Sf 9 cells (Invitrogen). Baculovirus (AcMNPV) carrying the FMDV mu3ABC gene referred to as AcMNPV.mu3ABC was collected from the transfected cells and supernatant. The expression of mu3ABC gene of FMDV in Sf9 cells was confirmed by indirect immunoperoxidase assay (IPMA) (Lekcharoensuk et al., 2010) using monoclonal antibody against the histidine-tag (anti-6xHis antibody; Millipore). The soluble protein and dissolved-inclusion body were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The presence of tagged-6xHis fused recombinant protein was further confirmed by Western blot using anti-6xHis antibody (Millipore). In addition, the specificity of the expressed protein was determined by Western blot, using a serum containing antibody to nonstructural protein (NSP) of FMDV.

2.3. Production and purification of non-structural polyprotein mu3ABC

Wild type baculovirus (AcMNPV.wt) and recombinant baculovirus carrying the mu3ABC gene of FMDV (AcMNPV.mu3ABC) were prepared as previously described (Nawagitgul et al., 2000). Briefly, AcMNPV.mu3ABC was inoculated onto Hi 5 insect cells (Invitrogen) at a multiplicity of infection (MOI) of eight. After incubation at 27 °C for 48 h, the infected cells were collected, washed with cold PBS pH7.2, and partially digested in a lysis buffer containing 25 mM sodium phosphate, 150 mM NaCl and 1% NP40. The lysates were sonicated on ice for a total of 3 min and then spun at 10,000 rpm at 4 °C for 20 min. The pellet was collected and further digested using the denaturing solubilization buffer (50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.0). The cell lysates were diluted at a ratio of 1:2 in the denaturing solubilization, containing 0.5% 2-mercaptoethanol, before being loaded into the Protino® Ni-IDA Resin column (Macherey Nagel). The resin was washed three times with five bed volumes of denaturing solubilization buffer, containing 20 and 50 mM imidazole, consecutively. Then, the mu3ABC protein was eluted with 8 ml of denaturing solubilization buffer containing 250 mM imidazole. The purified mu3ABC was dialyzed in 0.01 M phosphate buffer saline, pH 7.4. The concentrated purified AcMNPV.mu3ABC protein was measured using a BCA protein assay kit (Pierce). The proteins were stored at −80 °C until used.

2.4. Serum samples

One thousand, one hundred and five (1105) serum samples were obtained from different sources. Twenty-eight samples were from an FMD-free herd. Eighty-one samples were collected from an animal experiment, in which calves were vaccinated with an inactivated FMD vaccine and then challenged at three weeks' post-vaccination. Of 81 sera from the experimental animals, 22 samples were sera from calves prior to vaccination; 21 and 22 serum samples were from calves at one-and three-weeks' post-vaccination, respectively; the other 16 samples were obtained from calves after being challenged with FMDV for one week. The remaining 996 serum samples were obtained from cases submitted to the Regional Reference Laboratory for Foot and Mouth Disease in Southeast Asia.

2.5. mu3ABC ELISA

A modified ELISA method, utilizing antigen prepared from the recombinant mu3ABC protein, was performed as described previously (Nawagitgul et al., 2002) with slightly modification. Briefly, purified mu3ABC protein was used as positive antigen. In addition, recombinant baculovirus expressing non-FMDV-derived protein was produced similarly to the mu3ABC protein and used as negative antigen. Both positive and negative antigens were adjusted to a final concentration of 5 μg/ml in carbonate-bicarbonate coating buffer, pH 9.5. High binding EIA polystyrene microtiter plates (Costar®) were coated with positive and negative antigens by adding 100 μl of diluted antigen into each well of alternate columns. The coated plate was incubated at 4 °C for 24 h and stored at −20 °C until used.

To perform the mu3ABC ELISA on the cattle sera, the plate was equilibrated at room temperature, then washed five times with 30 s incubation using PBST washing buffer (0.1 M PBS, pH 7.2 and 0.1% Tween 20). One hundred microliters of serum diluted 1–20 in PBST were incubated with positive and negative antigens at 37 °C for 30 min. Excess antibodies were removed by washing five times with PBST buffer. One hundred microliters of an optimum dilution of peroxidase-labeled anti-bovine IgG (Sigma) in 0.5% skimmed milk (Difco) were incubated with bound antibodies at 37 °C for 30 min. After washing with PBST five times, the enzyme was incubated with 100 μl of freshly prepared and warmed (37 °C) 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate (KPL) at 37 °C for 15 min. The enzyme-substrate reaction was stopped by adding an equal amount of 1% SDS into each well. Positive and negative control sera were included in each ELISA plate to verify assay validity.

The optical density of the reaction in each well was measured at 405 nm using a multimode plate reader (Beckmann). The corrected optical density (COD) of each sample and control sera was calculated by subtraction of the mean optical density (OD) value of the well containing negative antigen from that of the parallel well containing positive antigen. The COD of the positive and negative control sera must be greater and lower than the cutoff of the test, respectively.

2.6. Evaluation of assay repeatability

Evaluation of assay repeatability within and between runs was performed as described elsewhere (Jacobson, 1998). Briefly, 24 field serum samples representing negative, weak positive and strong positive sera (provided by Regional Reference Laboratory for Foot and Mouth Disease in Southeast Asia) were selected for the repeatability test. For intra-assay (within plate) repeatability, three replicates of each serum sample were assigned in the same plate. For inter-assay (between run) repeatability, three replicates of each sample were run in different plates on different occasions. mu3ABC ELISA was performed using conditions as described earlier in the mu3ABC ELISA section. Mean COD, standard deviation (SD) and coefficient of variation (CV) of three replicates of each test were calculated.

2.7. Selection of negative/positive cutoff

To set a negative/positive cutoff value for the assay, 1105 field bovine sera were classified into positive and negative sera using a competitive 3ABC ELISA (Ceditest® FMDV-NS). Similar sets of sera were subsequently tested by the mu3ABC ELISA. The COD obtained from the mu3ABC ELISA was compared with the results of the competitive 3ABC ELISA. A negative/positive threshold for each assay was determined using a modified receiver-operator characteristic analysis, as previously described (Nawagitgul et al., 2002). A cutoff point was determined so that diagnostic sensitivity and specificity were maximized, while the numbers of false-negative and false-positive results were minimized. Data were calculated using a Microsoft Excel® spreadsheet.

2.8. Evaluation of assay performance

Diagnostic accuracy was determined by calculating the diagnostic sensitivity (Dsn) and diagnostic specificity (Dsp) of the test (Jacobson, 1998). The competitive 3ABC ELISA (Ceditest®FMDV-NS) was used as the gold standard or reference method to classify samples as positive or negative. The Dsn of the ELISA is the proportion of serum samples that give positive results in both competitive 3ABC and mu3ABC ELISA (Dsn = TP/(TP + FN) where TP is true positive and FN is false negative. Dsp is the proportion of serum samples that give negative results by both assays (Dsp = TN/(TN + FP) where TN is true negative and FP is false positive. Positive and negative predictive values as well as accuracy of the test were also calculated from the formulas: TP/(TP + FP) × 100, TN/(TN + FN) × 100 and (TP + TN)/total tested serum × 100, respectively. Kappa value (κ) was calculated using WIN Episcope 2.0 to determine the proportion of agreement between mu3ABC ELISA and the reference assay. A kappa of 0 indicates no agreement, while a kappa of 1 indicates perfect agreement. Also, the 95% confident interval (95% CI) of Dsn and others were calculated using WIN Episcope 2.0 by the formula: (percentage ± 1.96) × standard error of percentage (SE).

2.9. Analytical sensitivity

Six serum samples represented strong, moderate, and weak-positive sera as well as negative sera, were selected to determine the analytical sensitivity of the mu3ABC ELISA. Fetal bovine serum (FBS, Invitrogen) was also included as real negative serum control. Briefly, 100 μl of PBST was dispensed into each well of two mu3ABC ELISA plates. One hundred microliters of each serum sample and FBS were added to the first row of positive and negative antigen wells. The sera were mixed by pipetting up and down. Subsequently, 100 μl of the diluted sera were transferred to the second row of the plates and so on to make two-fold-serial dilution of the sera from 1:5 to 1:10,240. The mu3ABC ELISA was performed as described earlier. A similar set of sera was also used to determine analytical sensitivity of Ceditest®FMDV-NS ELISA.

3. Results

3.1. Cloning and expression of mu3ABC in insect cells

The full-length 3ABC ORF containing Cys142Ser and Cys163Gly was successfully constructed and transposed into the baculovirus DNA, as determined by sequence analysis. Transfection of the recombinant baculovirus DNA containing the mutated 3ABC gene into Sf 9 cells generated the recombinant baculovirus referred to

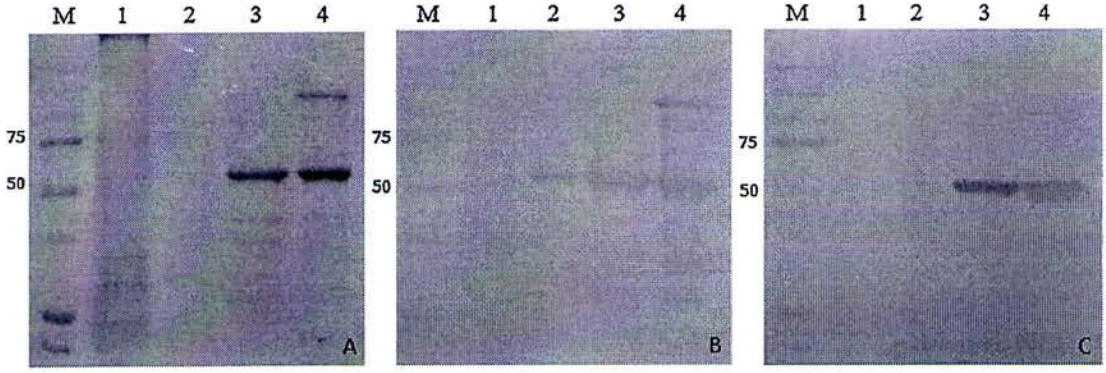

Fig. 1. SDS-PAGE (A) and Western blot (B and C) demonstrating characteristics and specificity of the mu3ABC protein. Proteins were separated in 10% SDS-PAGE, transferred onto nitrocellulose membranes, and reacted with an FMDV-convalescent serum (B) or monoclonal antibody to histidine-tag (C). Lanes M and 1 are molecular weight markers and lysates from AcMNPV.wt-infected Sf 9 cells, respectively. Lanes 2 and 3 are lysates from AcMNPV.mu3ABC-infected Sf 9 cells in mild and denaturing lysis buffer, respectively. Lane 4 is the mu3ABC protein after purification by affinity chromatography.

as AcMNPV.mu3ABC in the supernatant over the transfected cells. Further inoculation of the transfection supernatant onto the Sf 9 cells and overlaying the inoculated cells with agar yielded clear visible plaque surrounded by the pink color of the living cells (Supplemental Fig. A). In addition, Sf 9 cells inoculated with similar supernatant produced a protein that was recognized by a monoclonal antibody to the histidine-tag (6XHis) when examined by IPMA (Supplemental Fig. B). This indicates that the recombinant virus expressed the histidine-tag fusion protein within the Sf 9 cells. The expressed protein was further analyzed using SDS-PAGE and Western blot techniques. The result revealed that a majority of protein was produced as insoluble form (Fig. 1), and was then purified under denaturing conditions. The recombinant mu3ABC fused with histidine-tag had a molecular weight of 55 kDa. It reacted specifically to antibody to histidine tag and a convalescent serum from an FMDV-infected cow (Fig. 1). Therefore, the recombinant mu3ABC was ready to be processed and used in developing an ELISA.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2012.12.016.

3.2. mu3ABC ELISA development and evaluation of assay repeatability

The purified mu3ABC protein showed specific reactivity to the serum samples from the FMDV-infected animals. The mu3ABC ELISA could differentiate FMDV-positive and -negative sera, as well as strong from weak positive sera. The precision of the mu3ABC ELISA was further determined, and results showed that the assay was reproducible when performed on different occasions and in different positions. The intra-assay coefficient of variation (CV) of the 24 serum samples in mu3ABC ELISA ranged from 1 to 14% (mean 8%). The inter-assay CV for positive serum samples in mu3ABC ELISA was between 1 and 28% (mean 12%). These CVs are in an acceptable range (<30%), indicating low variability among each replicate of similar samples.

3.3. Cutoff determination

The results of mu3ABC ELISA on negative and positive sera are summarized in Supplemental Table. The COD of samples varied from a minimum of −0.6950 to a maximum of 0.4933 for negative sera, and from a minimum of −0.1519 to a maximum of 1.0250 for positive sera. The negative/positive cutoff value of 0.14 gave the fewest false results compared with the competitive 3ABC ELISA; of 1105 samples, 50 were false-negative and 21 were false-positive (Table 1). This yielded diagnostic sensitivity (Dsn) and specificity (Dsp) of 82.3 and 97.4%, respectively. By decreasing the negative/positive cutoff value to 0.125, Dsn increased to 84.0% (95% CI = 81.9–86.2) while the Dsp slightly decreased to 96.6% (95% CI = 95.5–97.7). Consequently, total false results increased to 73 samples. When the cutoff was moved to 0.15, the Dsp of the assay increased slightly to 97.8%, but the Dsn decreased to 79.4%. The cutoff value 0.125, which resulted in acceptable Dsn and Dsp, was therefore selected. Samples were considered negative when the COD was equal to or less than 0.125, and positive when the COD was greater than 0.125.

Table 1
Performance of mu3ABC ELISA at different cutoff points compared with the reference assay.

mu3ABC ELISA cutoff	Sum of false	Diagnostic sensitivity	Diagnostic specificity	Accuracy
0.100	86	87.9%	93.7%	92.5%
0.125	73	84.0%	96.6%	93.4%
0.130	73	83.0%	97.0%	93.5%
0.140	71	82.3%	97.4%	93.6%
0.150	76	79.4%	97.8%	93.0%

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2012.12.016.

3.4. Performance of mu3ABC

Table 2 shows the number of serum samples that tested positive and negative using the mu3ABC ELISA, compared with Ceditest® FMDV-NS. At cutoff 0.125, the accuracy of mu3ABC was 93.4% (95% CI = 91.9–94.9) compared with the reference assay. Agreement of both assays (κ) was 0.823 ($p < 0.05$). At this cutoff, mu3ABC

Table 2
Number of sera classified as positive and negative by mu3ABC ELISA compared with the reference assay.

			Reference assay (Ceditest® FMDV-NS)		
			Positive	Negative	Total
mu3ABC ELISA	COD > 0.125	Positive	237	28	265
	COD ≤ 0.125	Negative	45	795	840
	Total		282	823	1105

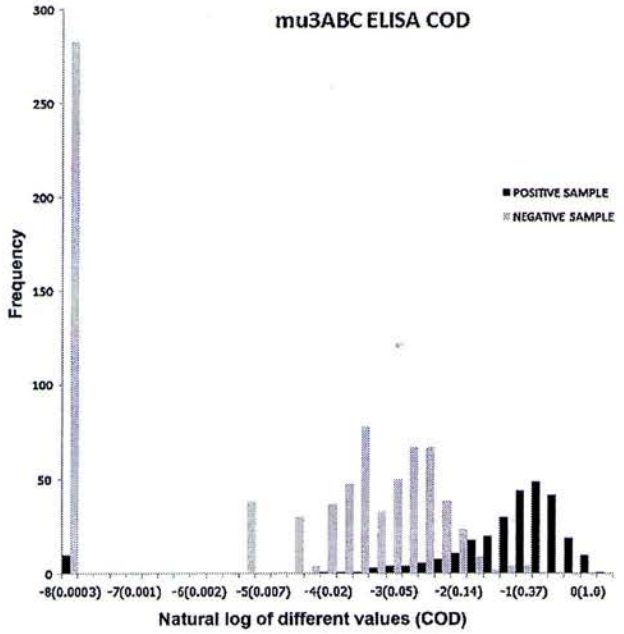

Fig. 2. A frequency plot of 823 negative and 282 positive serum samples as classified by the reference assay, by a natural log of COD results of the mu3ABC ELISAs. The plot shows the distribution of negative and positive bovine sera. The numbers in parenthesis are the COD of the mu3ABC ELISA.

ELISA had positive and negative predictive values of 89.4% (95% CI = 87.6–91.2) and 94.6% (95% CI = 93.3–96.0), respectively. When the numbers of positive and negative serum samples, as classified by the competitive ELISA, were plotted against the natural log of COD obtained from mu3ABC ELISA, two overlapping populations appeared, as shown in Fig. 2. This overlap area represents the equivocal zone where a sample has an equal probability of being positive or negative.

Of 1105 cattle sera, 760 samples were previously examined for the presence of antibodies to the NSPs using an ELISA developed by Regional Reference Laboratory (RRL) for FMD in Southeast Asia using reference reagents provided by World Reference Laboratory for FMD. All 760 sera were classified into 18 positive and 742 negative sera by Ceditest® FMDV-NS. At cutoff 0.125, mu3ABC ELISA could detect 16 positive samples, while RRL ELISA was unable to detect any positive samples. Since the Ceditest® FMDV-NS was used as the gold standard assay, assuming its Dsp and Dsn equaled 100%, the mu3ABC ELISA was more sensitive than RRL ELISA.

3.5. Analytical sensitivity of mu3ABC ELISA

The results of the analytical sensitivities of mu3ABC ELISA and Ceditest® FMDV-NS are shown in Fig. 3. At the optimum dilution of the test sera, 1:20 for mu3ABC ELISA and 1:5 for Ceditest® FMDV-NS, both assays could consistently differentiate between strong (sera 3 and 5), moderate (sera 4 and 6), and weak (serum 2) positive sera and negative sera (serum 1 and FBS). In addition, at the specific cutoff of each assay, the highest dilutions of the positive sera that mu3ABC ELISA and Ceditest® FMDV-NS could detect as positive were almost equivalent.

3.6. Differentiation between infection and vaccination by mu3ABC ELISA

To determine the ability of mu3ABC ELISA to differentiate sera from vaccinated and infected cattle, 81 serum samples from an experimental animal vaccination were tested by both mu3ABC ELISA and Ceditest® FMDV-NS. These sera were collected from calves prior to vaccination, after vaccination for 1 and 3 weeks, and 1 week post challenge by FMDV. The results showed that all serum samples were classified as negative by mu3ABC ELISA and Ceditest® FMDV-NS. In addition, mu3ABC ELISA was used to detect the 3ABC antibodies from cattle in FMD-affected herds. Eight samples were convalescent sera from FMDV-infected cattle collected one month after the outbreak of FMD in Northern Thailand. All serum samples gave positive results with COD range 1.2989–1.8011 (mean 1.5630; median 1.4969). The second group of nine samples was collected from a cattle herd in Western Thailand during an FMDoutbreak, one month after the onset of the outbreak. Five samples were taken from cattle that had previously showed clinical signs. As expected, these sera were found positive by mu3ABC ELISA with COD range 0.5979–1.7056 (mean 1.2262; median 1.2700). The other four samples were from vaccinated cattle exposed to FMDV, but without clinical signs. Two of these samples were positive with COD of 0.2321 and 0.2382, while the other two samples were negative by both mu3ABC ELISA and Ceditest® FMDV-NS. The third group of 49 samples was collected from a herd in Western Thailand, two months after the outbreak. Forty-eight serum samples were classified positive by mu3ABC ELISA, with COD range 0.1452–2.1131 (mean 1.1005; median1.557). The results confirmed that the mu3ABC ELISA could distinguish naturally infected animals from vaccinated ones.

4. Discussion

This is the first report of an NSP-based ELISA produced from the 3ABC gene with two point mutations, Cys142Ser and Cys163Gly,

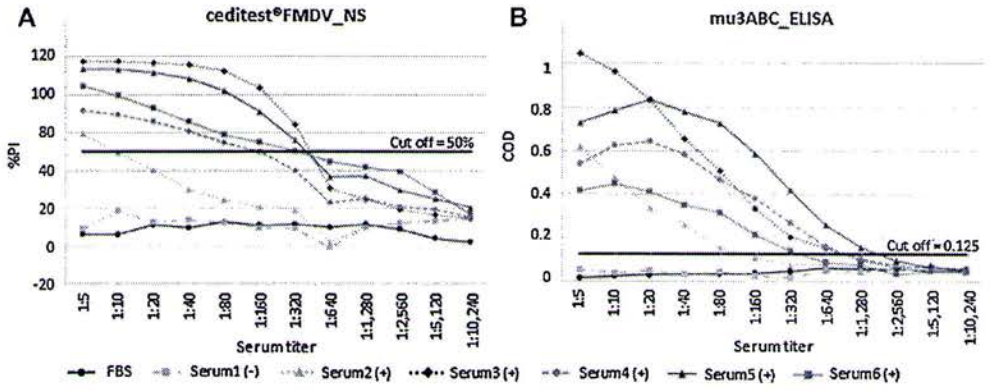

Fig. 3. Analytical sensitivity of Ceditest® FMDV-NS (A) and mu3ABC ELISA (B) performed on six serum samples. Serum 1 represents negative samples, serum 3 and serum 5 are strongly positive, and serum 4 and serum 6 are moderately positive. FBS was included as a real negative serum.

in the $3C^{pro}$. The full-length mu3ABC with inactivated 3C protease was expressed in insect cells. The mu3ABC protein produced in this study can be separated from the insect cellular components by binding property of the histidine-tag and nickel beads. Purified mu3ABC protein can be measured accurately using a spectrophotometer. Thus, a precise amount of antigen can be used in each batch, leading to high reproducibility of the assay, similar to the 3C deficient NSP ELISA, since no 3C protease interferes with the integrity of the protein (He et al., 2010; Jaworski et al., 2011; Mohapatra et al., 2011). In addition, the mu3ABC protein retains antigenic properties similar to the native 3ABC protein, and can be utilized directly as antigen for diagnostic purposes.

The mu3ABC ELISA was developed as an indirect ELISA, and cutoff as well as assay performance were determined by using Ceditest® FMDV-NS, as the gold standard. When real negative sera from disease-free herds are scarce, a gold standard or reference assay is utilized to classify sera as negative or positive. Ceditest® FMDV-NS was selected as the reference assay because it is one of the most sensitive and specific differential diagnostic assays, and its performance is comparable to the OIE index test, NCPanaftosa (Brocchi et al., 2006; Chen et al., 2007). Relative diagnostic sensitivity of mu3ABC ELISA is 84% compared to the Ceditest® FMDV-NS. However, relative to real animal status, the gold standard may also contain a few equivocal results that may be transferred to the new assay. Comparison of six commercial NSP-based ELISA–NCPanaftosa, IZS-Brescia, Ceditest® FMDV-NS, Svanovir, Chekit, and UBI – using cattle sera collected for post-outbreak surveillance, revealed 22.7% and 39.5% discordant results when these tests were used to detect anti-NSP from sera from Israel and Zimbabwe, respectively (Brocchi et al., 2006). This discrepancy reflects the ambiguous results inherent in each assay.

Comparison of mu3ABC ELISA with other NSP-based ELISAs in the literature is quite difficult, since the sources of the sera used in each study were different. Even within the same study, the sensitivity and specificity of one assay may be different if various sources of sera are used. The relative specificity of the assay decreased when the tested sera were from animals vaccinated with NSP contaminant vaccine (Chen et al., 2007). On the other hand, the relative sensitivity of the assay was different when the tested sera were from dissimilar geographical areas (Brocchi et al., 2006) or from different farms (Inoue et al., 2006). Brocchi et al. (2006) compared performance of various NSP ELISAs using cattle sera from post-outbreak surveillance in Zimbabwe, and demonstrated that IZS-Brescia had the highest relative sensitivity (100%). This was followed by NCPanaftosa (99.6%), Ceditest (99.6%), UBI (88.8%), Svanovir (83.6%) and Chekit (81.5%), respectively. In another study, a comparison was performed using cattle sera from different FMDV-affected farms in Zimbabwe (Inoue et al., 2006). The results showed that the 2B peptide ELISA developed in the study was the most sensitive, as the detection rate was 79.2%. Ceditest and UBI could detect 75% and 61% of the samples, respectively, while the detection rate of both Bommeli and Svanova was 56%.

Recently, various NSP-based ELISAs have been developed and compared with commercial test kits. The 3ABC-I-ELISA used antigen produced from recombinant 3ABC protein expressed in *E.coli* (Lu et al., 2007). By using negative sera from naive and vaccinated cattle, and positive sera from FMDV-infected cattle from China, the comparative performance of 3ABC-I-ELISA, Ceditest and UBI were 97.5, 100, and 96.7%, for Dsp and 100, 99.2, and 81.8%, for Dsn, respectively. The high specificity and sensitivity of this assay might be because the established cutoff defined the equivocal results as suspect, neither positive nor negative. Furthermore, r3AB ELISA was produced from recombinant 3AB protein, in which N terminal of the 3A was deleted to get rid of cysteine at amino acid position 65 (He et al., 2010). Relative diagnostic specificity of r3AB ELISA, Ceditest and UBI performed on sera from Chinese cattle was 97.3, 96.7, and 95.1%, respectively. In addition, $r3AB_3$ ELISA developed by using cattle sera from India had analytical sensitivity comparable to Svanovir, while relative Dsn and Dsp were 97.7% and 94% for $r3AB_3$ ELISA and 88% and 95.5% for Svanovir, respectively (Mohapatra et al., 2011). In another 3AB-based ELISA, r3AB1 ELISA, the cutoff was determined from results of normalized true negative sera plus 3 standard deviations (Jaworski et al., 2011). r3AB1 ELISA had a Dsp and Dsn of 84% (95% CI = 79–88) and 98.6% (95% CI = 97–100), respectively, and had higher analytical sensitivity than NCPanaftosa. The comparative performance of r3AB1 ELISA and NCPanaftosa on positive and negative sera from Argentina were 84 and 80% for Dsn, and 98.6 and 95% for Dsp, respectively. As above-mentioned, comparing the relative performance of different assays from a variety of studies can be problematic. Most of these studies established the assay cutoff from the true positive and negative sera, while the mu3ABC ELISA utilized positive and negative sera classified by the gold standard. Nevertheless, the Dsn (84%) and Dsp (96.4%) of mu3ABC ELISA was acceptable for a screening assay, especially at herd level.

In conclusion, the mu3ABC ELISA developed in this study is a relatively simple and reliable diagnostic test. It can differentiate infected from vaccinated cattle. The mu3ABC protein is easily purified using affinity chromatography, which has potential for large scale purification. The indirect ELISA format of the mu3ABC ELISA is simple but reproducible. All reagents and materials used in the assay are commercially available. The advantage over the commercial test kits is that the cost per sample is much lower. These factors are necessary for successful development of an in-house NSP-based ELISA, especially in countries where budgets are limited. The availability of a reliable assay with acceptable costs would facilitate successful disease control and the establishment of new disease-free zones.

Acknowledgements

This research was supported by the National Council Research Institute (NCRI), Thailand. The authors express their gratitude to Prof. Dr. Worawidh Wajjawalku and Ms. Wilairat Chumsing at the Faculty of Veterinary Medicine, Kasetsart University, for providing the FMDV isolates. We thank Drs. Somchai Sajjapitak and Adisorn Yawongsa at the Faculty of Veterinary Medicine, Kasetsart University and Dr. Therdsak Yano at the Faculty of Veterinary Science, Chiangmai University for providing the FMDV convalescent sera. We also thank Paul R. Adams at Faculty of Tropical Medicine, Mahidol University for the English editing.

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