Pan-Serotype Diagnostic for Foot-and-Mouth Disease Using the Consensus Antigen of Nonstructural Protein 3B

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An amino acid consensus sequence for the seven serotypes of foot-and-mouth disease virus (FMDV) nonstructural protein 3B, including all three contiguous repeats, and its use in the development of a pan-serotype diagnostic test for all seven FMDV serotypes are described. The amino acid consensus sequence of the 3B protein was determined from a multiple-sequence alignment of 125 sequences of 3B. The consensus 3B (c3B) protein was expressed as a soluble recombinant fusion protein with maltose-binding protein (MBP) using a bacterial expression system and was affinity purified using amylose resin. The MBP-c3B protein was used as the antigen in the development of a competition enzyme-linked immunosorbent assay (cELISA) for detection of anti-3B antibodies in bovine sera. The comparative diagnostic sensitivity and specificity at 47% inhibition were estimated to be 87.22% and 93.15%, respectively. Reactivity of c3B with bovine sera representing the seven FMDV serotypes demonstrated the pan-serotype diagnostic capability of this bioreagent. The consensus antigen and competition ELISA are described here as candidates for a pan-serotype diagnostic test for FMDV infection.

Foot-and-mouth disease (FMD) is the most contagious viral vesicular disease that affects cloven-hoofed livestock species. FMD has significant global socioeconomic consequences, from national livestock industries suffering because of international trade restrictions to subsistence farmers suffering because of losses of stock productivity and livelihoods. Western European, North American, and Far East Asian/Pacific regions and most South American countries have official recognition of freedom-from-FMD status, with or without vaccination, by the World Organization for Animal Health (OIE). Regions in which FMD remains endemic tend to be those of lesser economic capacity, and this limits their ability to control or to eradicate the disease (1, 2). As a result, FMD remains an ongoing problem in regions in which it is endemic, and it is a persistent threat to regions that are free of the disease.

Seven serotypes have been described for foot-and-mouth disease virus (FMDV), i.e., O, A, C, Asia 1, and South African Territories 1 (SAT1), SAT2, and SAT3. The disease forms produced by each serotype are clinically indistinguishable (3); similarly, FMD is clinically indistinguishable from other vesicular lesion-causing diseases, such as vesicular stomatitis, swine vesicular exanthema, and swine vesicular disease, and laboratory-based clinical diagnosis is required (4, 5). FMD infections may be mild or subclinical in ovine or caprine species, further complicating clinical diagnosis (6, 7). Exposure to one serotype does not confer cross-serotype immunity, potentially complicating diagnosis when multiple serotypes are circulating during an outbreak (8). A definitive diagnosis of FMD is possible only with laboratory testing.

Conventional serological assays for FMD, including the virus neutralization test, liquid-phase blocking enzyme-linked immunosorbent assay (ELISA), and solid-phase competition ELISA (cELISA), detect antibodies to structural proteins and are serotype specific (9–11). However, immunoassays based on FMDV nonstructural proteins (NSPs) have two advantages over these conventional assays, namely, (i) detection of multiple serotypes due to their high sequence homology and (ii) differentiation of infected from vaccinated animals (DIVA) when FMDV structural proteins

are used in vaccines (12–14). DIVA tests are important for serological surveys, providing evidence of FMD or freedom from FMD in vaccinated herds (15, 16). Most serodiagnostic DIVA tests for FMD are enzyme-linked immunosorbent assays that use NSP antigens produced in either bacterial or baculovirus-mediated expression systems, in an indirect or competitive format. The NSP intermediate 3ABC is commonly used as an antigen for FMDV DIVA testing because of its high immunogenicity and relatively low abundance in vaccine preparations generated from FMDV-infected cells (4).

The NSP 3B, a constituent of the 3ABC protein, is highly conserved across FMDV serotypes, contains a high density of linear B-cell epitopes, is highly immunogenic, and lacks the autocatalytic activity associated with the 3C component (17). FMDV is the only picornavirus that encodes three similar repeats of 3B in series, essentially tripling the number of potential epitopes (18, 19). These innate characteristics set 3B apart as a prime target for the development of a pan-serotype diagnostic bioreagent. We hypothesized that, through alteration of the amino acid sequence of each triplet to better mimic the sequences present across all FMDV serotypes, an antigenic consensus sequence for 3B suitable for use as a pan-serotype diagnostic reagent for FMDV could be

Received 8 December 2014 Returned for modification 7 January 2015

Accepted 6 March 2015

Accepted manuscript posted online 18 March 2015

Citation Van Dreumel AK, Michalski WP, McNabb LM, Shiell BJ, Singanallur NB, Peck GR. 2015. Pan-serotype diagnostic for foot-and-mouth disease using the consensus antigen of nonstructural protein 3B. *J Clin Microbiol* 53:1797–1805. doi:10.1128/JCM.03491-14.

Editor: M. J. Loeffelholz

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.03491-14>.

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doi:10.1128/JCM.03491-14

developed. This study takes a novel approach to pan-serotype antigen design and applies it to the development of a low-cost sero-diagnostic ELISA with potential DIVA application. We describe the *in silico* identification of a consensus 3B (c3B) sequence, the purification and characterization of recombinant c3B, and its use in the development of a cELISA diagnostic test.

MATERIALS AND METHODS

Sources of materials. The NCBI GenBank accession numbers used to derive FMDV 3B amino acid sequences are listed in Table S1 in the supplemental material. Competent BL21 *Escherichia coli*, pMAL-c5e plasmid, amylose resin, and rabbit anti-maltose-binding protein (MBP) antibody were from New England Biolabs (Genesearch, Australia). Serum samples were obtained from the national serum bank at the CSIRO Australian Animal Health Laboratory and included at least one sample of each FMDV serotype (see Table S2 in the supplemental material). All imported sera were gamma irradiated (50 Gy). Oligonucleotide primers were from Sigma-Aldrich (Australia).

Identification and analysis of FMDV 3B protein consensus sequence. Full-length amino acid sequences were translated from 374 FMDV nonstructural protein 3B gene entries in the NCBI GenBank database, and redundant sequences were removed. A multiple-sequence alignment of the remaining 125 sequences was generated using the L-INS-i algorithm of MAFFT v6.864 (20, 21). The consensus 3B (c3B) sequence was derived from the aligned sequences using WebLogo software v3.0 (22), with the residue with the greatest probability of occurrence at each position being chosen as canonical. Posttranslational modifications and biophysical characteristics of c3B were investigated using a variety of online tools, i.e., phosphorylation by the GPP prediction server (23), O-linked glycosylation by the NetOGlyc 3.1 server (24), N-linked glycosylation by NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>), hydrophobicity by the method described by Welling et al. (25), available on the ProtScale server (<http://web.expasy.org/protscale>), and continuous B-cell epitopes by COBEPro (26); the theoretical molecular mass was calculated using the ExpASY Compute pI/MW tool (27).

Plasmids and cloning. A gene encoding c3B was codon optimized using GeneGPS expression optimization technology (DNA2.0), for expression in *E. coli*. The c3B gene was synthesized and provided in pJ204 (DNA2.0). PCR was used to amplify the c3B gene using the primers 5'-AAGGTACCGGGTCCGTACGCTGGTCT-3' and 5'-CGTCCATGGCC TTATTCCGTGACGATCAGGTTCTT-3' (enzyme restriction sites are underlined). PCR conditions for amplification of c3B were 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 1 cycle of 72°C for 7 min. PCR products were purified from agarose gels using a QIAquick gel extraction kit (Qiagen). The c3B PCR product and pMAL-c5e were digested with KpnI and NcoI (Promega). Digested plasmid was incubated for 15 min at 37°C with 1 U thermosensitive alkaline phosphatase (Promega). Digested products were purified using a QIAquick PCR purification kit (Qiagen) and ligated using T4 DNA ligase (Promega). Chemically competent DH5α *E. coli* (Invitrogen) was transformed with pMAL-c5e containing c3B (pMAL-c3B) was purified from individual colonies using a Wizard Plus SV miniprep DNA purification kit (Promega). DNA sequence integrity of all constructs was confirmed by sequencing (Micromon, Australia) using the vector-specific primers 5'-G GTCTGACACTGTGATGAAGC-3' and 5'-TGTCTACTCAGGAG AGCGTTCAC-3'.

Expression and purification of recombinant proteins. Chemically competent BL21 *E. coli* (NEB) was transformed with 10 ng pMAL-c5e or pMAL-c3B for overexpression of recombinant MBP or MBP-c3B fusion protein, according to the manufacturer's instructions. Cultures of each clone were expanded to 100 ml in Luria-Bertani broth containing 100 μg/ml ampicillin and 0.2% (wt/vol) glucose by incubation at 37°C, with shaking at 225 rpm in an orbital shaking incubator, until the optical density at 600 nm (OD₆₀₀) reached 0.5 to 0.6 units. Protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a

final concentration of 1 mM, and cells were incubated for 3 h at 37°C with shaking at 225 rpm in an orbital shaking incubator. Cells were harvested by centrifugation at 4,000 × g for 10 min. Cell pellets were resuspended in 5 ml BugBuster master mix (Merck Millipore) and allowed to lyse at room temperature (RT) for 20 min on a rocking platform mixer. The lysate was clarified by centrifugation at 16,000 × g for 20 min at 4°C, and the supernatant containing soluble recombinant protein was retained.

Recombinant MBP and MBP-c3B were purified by affinity chromatography on amylose resin, according to the manufacturer's instructions. Clarified lysate was diluted 1:18 in binding buffer (20 mM Tris [pH 7.4], 200 mM NaCl, 1 mM EDTA), and then recombinant proteins were bound to the resin, washed with 10 column volumes of binding buffer, and eluted with binding buffer containing 10 mM maltose. Eluted proteins were dialyzed against 25 mM Tris (pH 8.0), and protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Scientific). Purified protein was diluted to 0.56 mg/ml or 0.05 mg/ml in 50% (wt/vol) glycerol and stored at -20°C.

Recombinant FMDV 3ABC was produced as described above with the following exceptions: chemically competent BL21(DE3) *E. coli* (C6000-03; Invitrogen) was transformed with 10 ng purified pRSETb-3ABC (28). 3ABC inclusion bodies were partially purified using BugBuster master mix (Novagen) containing protease inhibitors (P8849; Sigma), according to the manufacturers' instructions. Inclusion bodies were resuspended and solubilized by being heated to 100°C for 10 min in SDS-PAGE sample reducing buffer (Invitrogen).

3ABC was resolved by SDS-PAGE on NuPAGE Novex 4 to 12% Bis-Tris gels (1 mm; two-dimensional well format), in morpholinepropanesulfonic acid (MOPS) running buffer (Invitrogen), at 200 V for 50 min. Gel segments containing 3ABC were visualized with ice-cold 0.3 M KCl and excised. The protein was passively eluted into phosphate-buffered saline (PBS) with 0.1% (wt/vol) SDS and was dialyzed against PBS.

Immunogen preparation and antiserum production. All procedures involving animals were approved by the Australian Animal Health Laboratory Animal Ethics Committee. Immunogen was prepared as a water-in-oil emulsion of purified 3ABC protein and triple adjuvant, as described previously (29). New Zealand White rabbits were immunized by intramuscular injection on three occasions approximately 3 weeks apart. Each immunization used a total of 75 μg 3ABC in two 0.25-ml doses, with one dose in each hind leg. Sera were obtained by exsanguination and were assessed for immunoreactivity with 3ABC by immunoblotting.

SDS-PAGE and immunodetection. Protein samples were analyzed by SDS-PAGE under reducing conditions, in precast NuPAGE Novex 4 to 12% Bis-Tris gels, in MOPS or morpholineethanesulfonic acid (MES) running buffer (Invitrogen). Protein bands were visualized by staining with Coomassie brilliant blue R-250 (CBB) or silver nitrate. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes by Western blotting, in 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (pH 11) containing 10% (vol/vol) methanol, for immunodetection. Membranes were blocked for 1 h at RT with 5% (wt/vol) skim milk powder in Tris-buffered saline (pH 7.3) containing 0.05% (vol/vol) Tween 20 and then were probed with either rabbit anti-MBP antibody or rabbit anti-3ABC antiserum (prepared in-house) diluted 1:20,000, followed by goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (170-6515; Bio-Rad) diluted 1:40,000. Immunoreactive bands were visualized using ECL Plus substrate (Pierce) and a Typhoon FLA9000 fluorescence laser scanner (GE Healthcare).

Mass spectrometry. Purified proteins were separated by SDS-PAGE and stained with CBB. Protein bands were excised and subjected to in-gel digestion with trypsin or chymotrypsin (30). Extracted peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Dionex UltiMate 3000 nano-high-performance liquid chromatography (HPLC) system connected directly to the nanospray ion source of a Thermo Orbitrap Velos Pro hybrid ion trap-Orbitrap mass spectrometer (Thermo Scientific). Proteins were identified using the Sequest algorithm within the Proteome Discoverer 1.4 platform (Thermo

FIG 1 FMDV 3B amino acid consensus sequence and B-cell epitope predictions. (A) WebLogo graphic depicting amino acid residue conservation of the 3B protein consensus sequence. Letter heights indicate the relative frequency (probability) of each amino acid appearing at that position within the sequence (22). Amino acid properties shown are as follows: green, polar; purple, neutral; blue, basic; red, acidic; black, hydrophobic. (B) Histogram generated by COBEPro (26), mapping predicted antigenic propensity scores (+/-). Published B-cell epitopes of FMDV protein 3B are shown boxed in gray.

Scientific). Briefly, mass spectra of HPLC-separated peptides were matched with theoretical mass spectra produced by *in silico* tryptic or chymotryptic digestion of protein sequences from a custom database. Search results were filtered with a single threshold; matches to peptides were reported only if the Sequest cross-correlation factor (Xcorr) was >1.5 for a singly charged peptide ion, >2.0 for a doubly charged peptide ion, or >2.5 for a triply charged peptide ion.

Enzyme-linked immunosorbent assays. Unless stated otherwise, all assays included a blank control that consisted of blocking buffer (PBS [pH 7.3] with 0.05% [vol/vol] Tween 20 [PBST] plus 5% [wt/vol] skim milk powder) only and a minimum absorbance ($A_{\min}$) control that consisted of all test reagents except primary antibody. All samples were prepared in duplicate in 96-well Nunc MaxiSorb microtiter plates (Thermo Scientific), and all incubations were performed at 37°C on an orbital shaker. Plates were coated for 1 h with 50 μ l/well of antigen diluted in coating buffer (50 mM carbonate-bicarbonate [pH 9.6]), and blocking buffer was applied at 200 μ l/well for 30 min; all antibodies or sera were diluted in blocking buffer and used at 50 μ l/well for 30 min except for HRP-conju-

gated secondary antibody, which was incubated for 1 h. Signal color development was performed by incubation with 50 μ l/well of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) for 10 min at RT, and the colorimetric reaction was stopped by the addition of 50 μ l/well of 1 M sulfuric acid. Absorbance was measured at 450 nm using a microtiter plate reader (MultiSkan EX; Thermo Scientific). Washes were performed in triplicate with wash buffer (200 μ l/well Dulbecco's phosphate-buffered saline [DPBS] with 0.5 mM MgCl₂ and 0.7 mM CaCl₂), using an automated plate washer (ELx405 select deep well microplate washer; BioTek); plates were washed after incubations with antigen, blocking buffer, antiserum or antibody, and HRP-conjugated polyclonal rabbit anti-chicken IgG antibody (Pierce) diluted 1:4,000.

Determination of c3B antigen coating concentration and absorbance maximum by ELISA. Checkerboard titrations of MBP or MBP-c3B against chicken anti-3ABC polyclonal antibody (31) were performed in an indirect ELISA format. Rows of 96-well plates were coated with purified MBP-c3B fusion protein or MBP, serially diluted 2-fold from 70 ng/well to 1 ng/well and incubated for 1 h with chicken anti-3ABC antibody,

FIG 2 Recombinant c3B protein expression, purification, and detection. (A) Coomassie blue-stained polyacrylamide gel of recombinant MBP-c3B protein expressed in BL21 *E. coli* and affinity purified using amylose resin. Lane M, molecular mass markers (Mark 12; Invitrogen); lane 1, preinduction whole-cell extract; lane 2, 2-h postinduction whole-cell extract; lane 3, soluble protein fraction; lane 4, soluble protein fraction after 0.45- μ m filtration; lane 5, unbound protein fraction; lanes 6 and 7, consecutive column wash samples; lane 8, eluted purified protein. Arrow, MBP-c3B at $\sim$ 52 kDa. (B) Coomassie blue-stained polyacrylamide gel (left) and immunoblots (center and right) of purified recombinant MBP (lanes 1, 3, and 5) or MBP-c3B (lanes 2, 4, and 6). Lanes M, molecular mass markers (See Blue Plus 2; Invitrogen). The center panel was probed with rabbit anti-MBP antibody (NEB). The right panel was probed with rabbit anti-3ABC antiserum. The primary antibody and antiserum were diluted 1:20,000, and the secondary HRP-conjugated anti-rabbit IgG antibody was diluted 1:40,000. MBP and MBP-c3B are indicated (arrows) at approximately 42 and 52 kDa, respectively.

seriallyly diluted 2-fold from 1:40 to 1:5,120. Plates were developed, and the absorbance at 450 nm (A_{450}) of each sample was determined. A_{450} readings were plotted as mean $\pm$ standard deviation (SD) and were fitted with a sigmoidal dose-response curve by nonlinear regression analysis using GraphPad Prism (v5.02). The absorbance maximum was confirmed by cELISA, in which titration of chicken anti-3ABC antibody was performed by incubating wells coated with MBP-c3B (50 ng/well) with chicken anti-3ABC antibody diluted 1:300, 1:350, or 1:400 in blocking buffer. Plates were developed, and the A_{450} of each sample was measured.

Development of c3B cELISA. Plates were coated with 50 ng purified MBP-c3B fusion protein per well. Test sera and negative-control serum (C $-$) (naive FMDV antibody-negative serum) were used at a dilution of 1:5. Positive control serum (28-day postinfection positive-control serum, produced by sequential infection with C1 Detmold, 8WPI Asia 1 Shamir, and 12WPI A22 Iraq) was used diluted 1:5 and 1:50, to represent high-positive controls (C $+++$) and low-positive controls (C $+$), respectively. Competing chicken anti-3ABC antibody diluted 1:350 was added to each well (31). All assays included duplicate blank control wells and controls (quadruplicate) that contained all test reagents except test serum, for calculation of the absorbance maximum (A_{max}) of the test matrix. Results were expressed as percent inhibition (PI) using the following formula: $PI = 100 - [(\text{mean } A_{450} \text{ of serum} / \text{mean } A_{max}) \times 100]$.

Preliminary control sample repeatability. Upper and lower limits for the A_{max} values and positive and negative control sera were determined by repeating the cELISA method once per day for 10 days. Limits were expressed as the mean and range.

Analytical sensitivity and specificity of c3B cELISA. Analytical sensitivity (ASe) was determined by endpoint dilution of the positive bovine control serum against chicken anti-3ABC antibody. Positive and negative control sera were serially 2-fold diluted, added to a 96-well plate coated with MBP-c3B, and then titrated against dilutions of chicken anti-3ABC antibody. Results were expressed as PI and were used to construct dose-response curves. Analytical specificity (ASp) was assessed using bovine sera that were positive for bluetongue virus (BTV) antibodies ($n = 19$) or vesicular stomatitis virus (VSV) ($n = 2$), ovine sera that were positive for BTV antibodies ($n = 17$), and porcine sera that were positive for swine vesicular disease virus (SVDV) antibodies ($n = 7$).

Diagnostic sensitivity and specificity. Sera derived from 105 naturally or experimentally infected cattle, including at least one representative of each FMDV serotype, were used to estimate the diagnostic sensitivity (DSe). Seroconversions were confirmed using the AAHL 3ABC cELISA (31). Sera from 184 FMDV antibody-negative bovine samples were used to estimate the diagnostic specificity (DSp). Receiver operating characteristic (ROC) curve analysis using the method of DeLong et al. (32) was performed using MedCalc for Windows v13.2.2.0 (MedCalc Software, Belgium), to identify the optimal cutoff value for declaring positive and negative test results.

Bayesian latent class analysis. The DSe and DSp of the c3B cELISA were compared with those of the AAHL 3ABC cELISA by using Bayesian modeling for two dependent tests for one population with no gold standard (31, 33). Beta distribution parameters were derived using Beta Buster. Bayesian inferences were based on the joint posterior distribution, approximated using OpenBUGS (34) (see Table S3 in the supplemental material). The convergence estimates were derived using 2×10^6 iterations of simulation, with sampling performed every 1×10^5 iterations until the Monte Carlo (MC) error value, defined as the standard deviation divided by the square root of the number of iterations, was $<5\%$ of the standard deviation of the node estimate. Final inferences of DSe and DSp were given as the means of their 95% probability intervals.

RESULTS

Identification and analysis of 3B protein consensus sequence. WebLogo analysis of a multiple-sequence alignment of translated gene sequences of FMDV 3B revealed a 71-amino-acid consensus sequence (c3B); the sequence consisted of the most prevalent amino acids at the given positions within all sequences (Fig. 1A). The regions of c3B that were predicted to be most antigenic were residues 9 to 15, 32 to 38, and 54 to 59, with epitopic propensity scores of 0.90, 0.81, and 0.81, respectively (Fig. 1B). Amino acids 11 to 15 and 34 to 38 correspond to the known B-cell epitope QKPLK, which was found in the first and second repeats of the 3B triplet (17). Residues 54 to 59 (VKKPVA) are present in the third repeat and correspond to a predicted B-cell epitope. The entire

c3B sequence produced a probability of antigenicity score of 0.6118. Analysis of posttranslational modification predictions for the 3B protein indicated that neither O-linked (Thr70) nor N-linked (Asn66) glycosylation was likely, scoring below threshold values. Phosphorylation was predicted for all three tyrosine residues, consistent with known phosphorylation sites (35). The theoretical molecular mass of MBP-c3B was 52.89 kDa.

Expression and characterization of c3B protein. Recombinant MBP and MBP-c3B were expressed in BL21 *E. coli*. SDS-PAGE analysis revealed protein bands corresponding to MBP and MBP-c3B at approximately 42 and 52 kDa, respectively (Fig. 2A). These bands were visible only in the soluble protein fractions of lysates from BL21 *E. coli* cultures that had been treated with IPTG. A total of 24 mg MBP and 14 mg MBP-c3B protein were affinity purified from 100 ml of culture. The identities of purified MBP and MBP-c3B were confirmed by immunodetection with anti-3ABC antibody (Fig. 2B) and LC-MS/MS analysis. Mass spectrometric analysis of MBP-c3B resulted in peptide matches to MBP and c3B. Peptide matches to c3B provided 94% coverage of the protein.

Titration of c3B and anti-3ABC antibody by ELISA. Checkerboard titrations by indirect ELISA of c3B and chicken anti-3ABC antibody gave A_{450} values of approximately 1.5 after development for 10 min using 50 ng/well c3B and antibody diluted 1:640 (Fig. 3A). Titrations by cELISA confirmed that the optimal concentration of MBP-c3B antigen was 50 ng/well and the optimal dilution of chicken anti-3ABC antibody was 1:350. These conditions produced an A_{450} value of 1.42 (95% confidence interval [CI], 1.33 to 1.50) ($n = 28$). Nonspecific binding of chicken anti-3ABC antibody to matrix or MBP under these conditions yielded an A_{450} value of <0.1 (Fig. 3B).

Control sample limits and repeatability. PI limits (ranges) for the high-positive-control (C+++), low-positive-control (C+), and negative-control (C-) sera were determined to be as follows: C+++, 87.8% (85.0 to 90.8%); C+, 66.0% (60.0 to 72.8%); C-, 23.2% (15.0 to 35.0%) ($n = 10$). The absorbance limit for $A_{\max}$ was 1.081 (0.85 to 1.71). Wells without test sera produced background A_{450} readings of <0.06 .

Evaluation of analytical sensitivity and specificity of c3B cELISA. The limit of detection of the positive-control serum was identified at a dilution of 1:80 when diluted with negative-control serum or blocking buffer (Fig. 4A). The choice of diluents had no effect on PI at the optimal dilution of competing antibody (Fig. 4B). At higher dilutions, the degree of inhibition was below the PI cutoff value. Negative-control serum PI values were all below the PI cutoff value, even at the highest dilution (1:350) of chicken anti-3ABC antibody tested (Fig. 4C). Bovine, ovine, and porcine sera from animals infected with BTV, VSV, or SVDV all tested negative using the c3B cELISA.

Determination of diagnostic sensitivity and specificity of c3B cELISA. The DSe and DSp of the c3B cELISA were determined using 289 sera (105 positive and 184 negative, as determined using the AAHL 3ABC cELISA). The cross-classified data showed 93 true-positive, 14 false-positive, 12 false-negative, and 170 true-negative sera. ROC curve analyses of these data gave an area under the curve (AUC) of 0.944 and a Youden index (36) value of 0.8164, indicating that the test has high discriminatory power (37) (Fig. 5A) and a PI cutoff value for a positive sample of $\geq 47\%$ (Fig. 5B). At a PI of 47%, the DSe and DSp were 87.62% (95% CI, 79.8 to 93.2%) and 94.02% (95% CI, 89.6 to 97.00%), respectively. Bayesian anal-

FIG 3 Checkerboard titration of MBP-c3B antigen and chicken anti-3ABC antibody by indirect ELISA. Sigmoidal dose-response curve (variable slope) analyses of MBP-c3B (A) and MBP (B) titrated against a 2-fold dilution series of chicken anti-3ABC antibody by indirect ELISA. Dilutions are indicated (B). Values shown are mean $\pm$ standard deviation of absorbance at 450 nm for each concentration of protein tested ($n = 3$).

ysis of the c3B cELISA gave a sensitivity of 87.22% (95% probability interval, 81.37 to 92.37%) and a specificity of 93.15% (95% probability interval, 89.59 to 96.19%) (Table 1).

Seven FMDV-infected, NSP-antibody-positive bovine serum samples, representing the seven serotypes of FMDV, were analyzed, and all were detected using the c3B cELISA. All samples produced PI values between 71 and 83% (cutoff value, 47%).

DISCUSSION

Immunoassays based on FMDV nonstructural proteins (NSPs) have two significant advantages over those using structural proteins, namely, the capacity to detect multiple serotypes due to their high sequence homology and the ability to differentiate infected from vaccinated animals (12–14). The reference DIVA test adopted by the OIE for FMDV is an ELISA that detects antibodies against the NSP 3ABC (NCPanftosa screening test) complemented by an enzyme-linked immunoelectrotransfer blot assay (12, 38). A problem associated with the use of 3ABC is the presence of vaccine-associated 3A protein, which may complicate DIVA testing due to the presence of 3A-specific antibodies in sera from some vaccinated cattle (15, 39, 40). Furthermore, due to its

FIG 4 Analytical sensitivity of c3B competition ELISA. (A) Analysis of the limit of detection for the positive-control serum serially diluted 2-fold in blocking buffer (left) or negative-control bovine serum (right), performed using chicken anti-3ABC antibody diluted as indicated. (B) Comparison of percent inhibition values for positive serum diluted 2-fold in blocking buffer or negative-control bovine serum, using chicken anti-3ABC antibody diluted 1:350. (C) Percent inhibition of negative-control bovine serum serially diluted 2-fold with blocking buffer, using chicken anti-3ABC antibody diluted as indicated.

expression as insoluble inclusion bodies in *E. coli* and the presence of the autocatalytic 3C protein segment, recombinant 3ABC is difficult to produce (28, 41–43; our unpublished observations). Recombinant 3A, 3B, and 3AB have also been shown to be insoluble when expressed as His-tagged fusion proteins (44); however, others reported expression of proteins in soluble form tagged with 6×His (45), with the solubility-enhancing leader sequence His-patch (HP) thioredoxin (46) or glutathione *S*-transferase (GST) (47). This study described the expression of soluble c3B protein N-terminally fused to the known solubility enhancer MBP and its use in the development of a cELISA suitable for detection of antibodies to NSPs from each of the seven FMDV serotypes.

Previously developed FMDV NSP ELISAs that used FMDV 3B protein as the antigen have capitalized on one of the unique innate properties of the protein, i.e., a triplet of highly conserved B-cell epitopes, one each within the segments 3B₁, 3B₂, and 3B₃ (17, 18, 40). Tests for the detection of FMDV-positive antisera have been

developed using synthetic 3B peptides; however, this is at a cost premium and is limited by the length of peptides that can be produced. A lower-cost alternative is the use of recombinant 3B peptides produced in *E. coli* (17, 40, 46, 48). By extension, the use of tandem repeats of 3B peptides to effectively multiply the number of potential antibody binding sites on the antigen has been used successfully to differentiate infected and vaccinated animals (18, 48). In addition, others have developed DIVA tests by expressing the entire 3B protein individually or as part of a larger FMDV NSP protein, typically 3AB, 2C3AB, or 3ABC (for recent examples, see references 31, 45, 49–51). In each case, however, the sequence used was derived from a single FMDV strain. In contrast, our approach to developing a 3B diagnostic assay was to generate a consensus 3B (c3B) sequence to eliminate potential strain-specific deficiencies in specificity by better mimicking the 3B epitopes of all serotypes. Thus, c3B was representative of all FMDV 3B proteins, irrespective of serotype. It is worth noting that the GenBank database

FIG 5 Receiver operating characteristic (ROC) curve analysis of c3B competition ELISA. (A) ROC curve analysis of c3B cELISA results derived from 289 cattle sera. Dotted lines, 95% confidence intervals. Circle (upper left corner), Youden index of 0.8164. AUC, area under the curve. (B) Plot-versus-criterion analysis for the c3B cELISA. The data set from panel A was used to plot the sensitivity (solid line) or specificity (dashed line) at different cutoff values, and results indicated an optimal PI cutoff value of >47%. Respective 95% confidence intervals are shown.

contains significantly more sequence submissions for type O and A serotypes than the other serotypes, and this is reflected in the consensus sequence. The proportions of FMDV 3B gene sequences by serotype after the removal of redundant sequences were as follows: O, 35.2%; A, 28%; SAT1, 8%; SAT2, 5.6%; SAT3, 2.4%; Asia 1, 17.6%; C, 3.2%. Predicted antigenic regions and B-cell epitopes of c3B were consistent with epitopes of 3B determined experimentally (17, 18, 40). An additional epitope (VKKP VA), not previously described, was predicted in the third repeat of the c3B protein. It remains to be determined whether this prediction translates into a biologically relevant immunogenic site.

The combination of minimal predicted posttranslational modifications, a lack of cysteine residues, and known linear epitopes made c3B ideal for production using a bacterial expression system. Recombinant c3B was generated using a synthetic codon-optimized gene in BL21 *E. coli*. Previous studies that used *E. coli*-expressed proteins to develop FMDV diagnostic tests required the addition of *E. coli* lysates to sample serum diluents, to preabsorb nonspecific background reactivity (39, 52). It has been suggested that this background reactivity may be due in part to inclusion of the N-terminal 3A sequence, which shares sequence homology with a transposase IS4 family protein from *E. coli* (53). In addition, 10% (vol/vol) equine serum has been required in blocking buffers

to minimize background reactivity. The assay described here eliminated these additional requirements by using amylose resin to affinity purify MBP-c3B, thereby removing potential *E. coli* protein contamination. Expression yielded approximately 14 mg of affinity-purified MBP-c3B antigen per 100 ml of culture, i.e., sufficient antigen to coat approximately 2,800 plates with 96 wells, at 50 ng/well. Others have reported yields for 3AB and 3B proteins of 3 and 1 mg/100 ml culture, respectively (44, 54). Coomassie blue staining of MBP-c3B revealed a band at ~52 kDa, consistent with its predicted molecular mass. In addition, antibodies specific for 3ABC and MBP produced similarly sized bands in immunoblotting, confirming the identity of MBP-c3B. Additional smaller protein bands were observed on stained gels and immunoblots, suggesting partial degradation of MBP-c3B. Immunoblotting with anti-MBP and anti-3ABC antibodies indicated that degradation was due to cleavage of the c3B protein rather than MBP. The observed pattern of 3B protein degradation was similar to that shown in a study of a 6×His-tagged 3B protein purified from BL21 *E. coli* (45). Rabbit anti-3ABC antisera and chicken anti-3ABC antibody did not cross-react with the MBP tag or any other assay components in an indirect ELISA.

The c3B cELISA showed high analytical sensitivity by endpoint dilution of positive control sera from 1:5 to 1:320. The analytical specificity was 100%, as PI values observed using sera from animals (cattle, sheep, or pigs) infected with other clinically relevant diseases were all below the 47% cutoff value. The c3B cELISA detected the reference serum representatives of all seven FMDV serotypes, demonstrating the assay's potential as a pan-serotype test. Future work will require testing of a more diverse population of samples of each serotype. The test also discriminated between infected and uninfected animals, based on a PI cutoff value determined using the maximum Youden index, which gives equal weight to the sensitivity and specificity of the test. A validated test would require modifications to the balance between DSe and DS

TABLE 1 Bayesian latent class analysis of c3B and AAHL 3ABC cELISAs

ELISA and parameter ^a	Mean (%)	SD	MC error	Minimum (%)	Median (%)	Maximum (%)
c3B						
DSe	87.22	0.03	2.75E-05	81.37	87.34	92.37
DSp	93.15	0.02	1.83E-05	89.59	93.24	96.19
AAHL						
DSe	90.78	0.03	3.59E-05	84.66	91.01	95.59
DSp	95.69	0.02	3.09E-05	91.25	95.92	98.82

^a DSe, diagnostic sensitivity; DSp, diagnostic specificity.

according to the disease status of the country or region of the test's intended use. Using a cutoff value of 47% means that the DSe and DSp of the c3B cELISA are slightly lower than those of the AAHL cELISA. This may be explained by the fact that the AAHL cELISA was validated using 2,500 samples, compared with the prototype c3B cELISA presented here, which has been assessed using almost 10-fold fewer samples. The diagnostic sensitivity and specificity of the c3B cELISA are anticipated to improve with the evaluation of increasing numbers of samples during validation. In the absence of a gold standard test, we showed, using Bayesian latent class analysis, that the DSe and DSp of our test are similar to those of the AAHL cELISA, with decreases of only 0.40% and 0.86%, respectively.

Herein we have described a novel purified consensus 3B bioreagent expressed in high yield and in soluble form in *E. coli*. We describe its use as an effective pan-serotype FMDV diagnostic reagent in a cELISA format, and we provide preliminary assessment of its analytical sensitivity and diagnostic sensitivity and specificity. Future work will build on these data as part of a comprehensive validation study encompassing testing of all serotypes, multiple host species, ability to differentiate infected from vaccinated animals, and performance against benchmark tests. We are currently seeking access to panels of sera for this purpose.

ACKNOWLEDGMENTS

We thank Ross Lunt for technical assistance with ELISA methods and for access to the serum bank at the CSIRO Australian Animal Health Laboratory, Kim Halpin and Adam Foord for critical reading of the manuscript, Meng Yu for pRSETb-3ABC, the Canadian Food Inspection Agency (Winnipeg, Canada) for positive-control serum and seven experimentally infected bovine serum samples representing the seven FMDV serotypes, the Pirbright Institute (United Kingdom) for proficiency test panel samples from 2012 and 2013, the University of Melbourne Bio21 Institute for mass spectrometric analyses, and Suzanne Wilson (AAHL) for assistance with rabbit anti-3ABC antiserum production.

A.K.V.D. carried out most of the experiments and wrote the manuscript. W.P.M. and G.R.P. designed the study, provided critical input on experimental design, and assisted in manuscript preparation. W.P.M., L.M.M., B.J.S., and N.B.S. assisted with the experiments and data analysis. All authors read and approved the final manuscript.

REFERENCES

- Paton DJ, Sumption KJ, Charleston B. 2009. Options for control of foot-and-mouth disease: knowledge, capability and policy. *Philos Trans R Soc Lond B Biol Sci* 364:2657–2667. <http://dx.doi.org/10.1098/rstb.2009.0100>.
- Rweyemamu MM, Astudillo VM. 2002. Global perspective for foot-and-mouth disease control. *Rev Sci Tech* 21:765–773.
- Geering WA, Lubroth J. 2002. Preparation of foot-and-mouth disease contingency plans: FAO animal health manual. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Ma L, Zhang J, Chen H, Zhou J, Ding Y, Liu Y. 2011. An overview on ELISA techniques for FMD. *Virol J* 8:419. <http://dx.doi.org/10.1186/1743-422X-8-419>.
- Rémond M, Kaiser C, Lebreton F. 2002. Diagnosis and screening of foot-and-mouth disease. *Comp Immunol Microbiol Infect Dis* 25:309–320. [http://dx.doi.org/10.1016/S0147-9571\(02\)00028-0](http://dx.doi.org/10.1016/S0147-9571(02)00028-0).
- Geering WA. 1967. Foot-and-mouth disease in sheep. *Aust Vet J* 43:485–489. <http://dx.doi.org/10.1111/j.1751-0813.1967.tb04774.x>.
- Davies G. 2002. The foot-and-mouth disease (FMD) epidemic in the United Kingdom 2001. *Comp Immunol Microbiol Infect Dis* 25:331–343. [http://dx.doi.org/10.1016/S0147-9571\(02\)00030-9](http://dx.doi.org/10.1016/S0147-9571(02)00030-9).
- Alexandersen S, Mowat N. 2005. Foot-and-mouth disease: host range and pathogenesis. *Curr Top Microbiol Immunol* 288:9–42. http://dx.doi.org/10.1007/3-540-27109-0_2.
- Bronsvort BM, Toft N, Bergmann IE, Sørensen KJ, Anderson J, Malirat V, Tanya VN, Morgan KL. 2006. Evaluation of three 3ABC ELISAs for foot-and-mouth disease non-structural antibodies using latent class analysis. *BMC Vet Res* 2:30. <http://dx.doi.org/10.1186/1746-6148-2-30>.
- Alexandersen S, Zhang Z, Donaldson AI, Garland AJM. 2003. The pathogenesis and diagnosis of foot-and-mouth disease. *J Comp Pathol* 129:1–36. [http://dx.doi.org/10.1016/S0021-9975\(03\)00041-0](http://dx.doi.org/10.1016/S0021-9975(03)00041-0).
- Hamblin C, Barnett IT, Hedger RS. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *J Immunol Methods* 93:115–121. [http://dx.doi.org/10.1016/0022-1759\(86\)90441-2](http://dx.doi.org/10.1016/0022-1759(86)90441-2).
- Bergmann IE, Malirat V, Neitzert E, Beck E, Panizzutti N, Sánchez C, Falczuk AJ. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Arch Virol* 145:473–489. <http://dx.doi.org/10.1007/s007050050040>.
- Brocchi E, Bergmann IE, Dekker A, Paton DJ, Sammin DJ, Greiner M, Grazioli S, de Simone F, Yadin H, Haas B, Bulut N, Malirat V, Neitzert E, Goris N, Parida S, Sørensen KJ, de Clercq K. 2006. Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. *Vaccine* 24:6966–6979. <http://dx.doi.org/10.1016/j.vaccine.2006.04.050>.
- Sørensen KJ, Madsen KG, Madsen ES, Salt JS, Ngindi J, Mackay DK. 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Arch Virol* 143:1461–1476. <http://dx.doi.org/10.1007/s007050050390>.
- Mackay DKJ, Forsyth MA, Davies PR, Berlinzani A, Belsham GJ, Flint M, Ryan MD. 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. *Vaccine* 16:446–459. [http://dx.doi.org/10.1016/S0264-410X\(97\)00227-2](http://dx.doi.org/10.1016/S0264-410X(97)00227-2).
- Paton DJ, de Clercq K, Greiner M, Dekker A, Brocchi E, Bergmann IE, Sammin DJ, Gubbins S, Parida S. 2006. Application of non-structural protein antibody tests in substantiating freedom from foot-and-mouth disease virus infection after emergency vaccination of cattle. *Vaccine* 24:6503–6512. <http://dx.doi.org/10.1016/j.vaccine.2006.06.032>.
- Höhllich BJ, Wiesmüller KH, Schlapp T, Haas B, Pfaff E, Saalmüller A. 2003. Identification of foot-and-mouth disease virus-specific linear B-cell epitopes to differentiate between infected and vaccinated cattle. *J Virol* 77:8633–8639. <http://dx.doi.org/10.1128/JVI.77.16.8633-8639.2003>.
- Sun T, Lu P, Wang X. 2004. Localization of infection-related epitopes on the non-structural protein 3ABC of foot-and-mouth disease virus and the application of tandem epitopes. *J Virol Methods* 119:79–86. <http://dx.doi.org/10.1016/j.jviromet.2004.02.016>.
- Belsham GJ, Martinez-Salas E. 2004. Genome organisation, translation and replication of foot-and-mouth disease virus RNA, p 19–52. In Sobrino F, Domingo E (ed), *Foot-and-mouth disease: current perspectives*. Horizon Bioscience, Wymondham, Norfolk, England.
- Katoh K, Kuma K, Toh H, Miyata T. 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res* 33:511–518. <http://dx.doi.org/10.1093/nar/gki198>.
- Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 9:286–298. <http://dx.doi.org/10.1093/bib/bbn013>.
- Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo generator. *Genome Res* 14:1188–1190. <http://dx.doi.org/10.1101/gr.849004>.
- Hamby SE, Hirst JD. 2008. Prediction of glycosylation sites using random forests. *BMC Bioinformatics* 9:500. <http://dx.doi.org/10.1186/1471-2105-9-500>.
- Stentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, Schjoldager KTBG, Lavrsen K, Dabelsteen S, Pedersen NB, Marcos-Silva L, Gupta R, Bennett EP, Mandel U, Brunak S, Wandall HH, Lavery SB, Clausen H. 2013. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J* 32:1478–1488. <http://dx.doi.org/10.1038/emboj.2013.79>.
- Welling GW, Weijer WJ, van der Zee R, Welling-Wester S. 1985. Prediction of sequential antigenic regions in proteins. *FEBS Lett* 188:215–218. [http://dx.doi.org/10.1016/0014-5793\(85\)80374-4](http://dx.doi.org/10.1016/0014-5793(85)80374-4).
- Sweredoski MJ, Baldi P. 2009. COBPro: a novel system for predicting

- continuous B-cell epitopes. *Protein Eng Des Sel* 22:113–120. <http://dx.doi.org/10.1093/protein/gzn075>.
27. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools in the ExPASy server, p 571–607. In Walker JM (ed), *The proteomics protocols handbook*. Humana Press, New York, NY.
 28. Foord AJ, Muller JD, Yu M, Wang LF, Heine HG. 2007. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. *J Immunol Methods* 321:142–151. <http://dx.doi.org/10.1016/j.jim.2007.01.014>.
 29. Prowse S. 2000. A new adjuvant. *ANZCCART News* 13:3:7.
 30. Shiell BJ, Beddome G, Michalski WP. 2002. Mass spectrometric identification and characterisation of the nucleocapsid protein of Menangle virus. *J Virol Methods* 102:27–35. [http://dx.doi.org/10.1016/S0166-0934\(01\)00441-4](http://dx.doi.org/10.1016/S0166-0934(01)00441-4).
 31. Colling A, Morrissey C, Barr J, Meehan G, Wright L, Goff W, Gleeson LJ, van der Heide B, Riddell S, Yu M, Eagles D, Lunt R, Khounsy S, Than Long NGO, Phong Vu P, Than Phuong N, Tung N, Linchongsubongkoch W, Hammond J, Johnson M, Johnson WO, Unger H, Daniels P, Crowther JR. 2014. Development and validation of a 3ABC antibody ELISA in Australia for foot-and-mouth disease. *Aust Vet J* 92:192–199. <http://dx.doi.org/10.1111/avj.12190>.
 32. DeLong ER, DeLong DM, Clarke-Pearson DL. 1988. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 44:837–845. <http://dx.doi.org/10.2307/2531595>.
 33. Branscum AJ, Gardner IA, Johnson WO. 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Prev Vet Med* 68:145–163. <http://dx.doi.org/10.1016/j.prevetmed.2004.12.005>.
 34. Thomas A, O'Hara B, Ligges U, Sturtz S. 2006. Making BUGS open. *R News* 6:12–17.
 35. Ambros V, Baltimore D. 1978. Protein is linked to the 5' end of poliovirus RNA by a phosphodiester linkage to tyrosine. *J Biol Chem* 253:5263–5266. [http://dx.doi.org/10.1002/1097-0142\(1950\)3:1<32::AID-CNCR2820030106>3.0.CO;2-3](http://dx.doi.org/10.1002/1097-0142(1950)3:1<32::AID-CNCR2820030106>3.0.CO;2-3).
 36. Grzybowski M, Younger JG. 1997. Statistical methodology. III. Receiver operating characteristic (ROC) curves. *Acad Emerg Med* 4:818–826.
 37. Paton DJ, Barnett PV, Ferris NP. 2012. Foot-and-mouth disease, p 1–29. In OIE Biological Standards Commission (ed), *Manual of diagnostic tests and vaccines for terrestrial animals*, 7th ed. World Organisation for Animal Health, Paris, France.
 38. Malirat V, Neitzert E, Bergmann IE, Maradei E, Beck E. 1998. Detection of cattle exposed to foot-and-mouth disease virus by means of an indirect ELISA test using bioengineered nonstructural polyprotein 3ABC. *Vet Q* 20(Suppl 2):S24–S26. <http://dx.doi.org/10.1080/01652176.1998.9694958>.
 39. Shen F, Chen PD, Walfield AM, Ye J, House J, Brown F, Wang CY. 1999. Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. *Vaccine* 17:3039–3049. [http://dx.doi.org/10.1016/S0264-410X\(99\)00148-6](http://dx.doi.org/10.1016/S0264-410X(99)00148-6).
 40. Sariya L, Thangthumnyiom N, Wajjwalku W, Chumsing W, Ramasoota P, Lekcharoensuk P. 2011. Expression of foot-and-mouth disease virus non-structural polyprotein 3ABC with inactive 3C^{pro} in *Escherichia coli*. *Protein Expr Purif* 80:17–21. <http://dx.doi.org/10.1016/j.pep.2011.07.006>.
 41. Srisombundit V, Tungthumnyiom N, Linchongsubongkoch W, Lekcharoensuk C, Sariya L, Ramasoota P, Lekcharoensuk P. 2013. Development of an inactivated 3C^{pro}-3ABC (mu3ABC) ELISA to differentiate cattle infected with foot-and-mouth disease virus from vaccinated cattle. *J Virol Methods* 188:161–167. <http://dx.doi.org/10.1016/j.jviromet.2012.12.016>.
 42. de Diego MP, Brocchi E, Mackay DK, de Simone F. 1997. The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch Virol* 142:2021–2033. <http://dx.doi.org/10.1007/s007050050219>.
 43. Yakovleva AS, Shcherbakov A, Kan'shina A, Mudrak NS, Fomina TA. 2006. Use of the recombinant nonstructural 3A, 3B, and 3AB proteins of foot-and-mouth disease virus in indirect ELISA for differentiation of vaccinated and infected cattle. *Mol Biol* 40:146–151. <http://dx.doi.org/10.1134/S0026893306010195>.
 44. Mohapatra AK, Mohapatra JK, Pandey LK, Sanyal A, Pattnaik B. 2014. Diagnostic potential of recombinant nonstructural protein 3B to detect antibodies induced by foot-and-mouth disease virus infection in bovines. *Arch Virol* 159:2359–2369. <http://dx.doi.org/10.1007/s00705-014-2089-0>.
 45. Oem JK, Chang BS, Joo HD, Yang MY, Kim GJ, Park JY, Ko YJ, Kim YJ, Park JH, Joo YS. 2007. Development of an epitope-blocking-enzyme-linked immunosorbent assay to differentiate between animals infected with and vaccinated against foot-and-mouth disease virus. *J Virol Methods* 142:174–181. <http://dx.doi.org/10.1016/j.jviromet.2007.01.025>.
 46. Silberstein E, Kaplan G, Taboga O, Duffy S, Palma EL. 1997. Foot-and-mouth disease virus-infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. *Arch Virol* 142:795–805. <http://dx.doi.org/10.1007/s007050050119>.
 47. Gao M, Zhang R, Li M, Li S, Cao Y, Ma B, Wang J. 2012. An ELISA based on the repeated foot-and-mouth disease virus 3B epitope peptide can distinguish infected and vaccinated cattle. *Appl Microbiol Biotechnol* 93:1271–1279. <http://dx.doi.org/10.1007/s00253-011-3815-0>.
 48. Dong Y, Xu Y, Liu Z, Fu Y, Ohashi T, Mawatari K, Kitamori T. 2014. Determination of cattle foot-and-mouth disease virus by micro-ELISA method. *Anal Sci* 30:359–363. <http://dx.doi.org/10.2116/analsci.30.359>.
 49. Lu Z, Zhang X, Fu Y, Cao Y, Tian M, Sun P, Li D, Liu Z, Xie Q. 2010. Expression of the major epitope regions of 2C integrated with the 3AB non-structural protein of foot-and-mouth disease virus and its potential for differentiating infected from vaccinated animals. *J Virol Methods* 170:128–133. <http://dx.doi.org/10.1016/j.jviromet.2010.09.016>.
 50. Shao J, Chang H, Lin T, Cong G, Du J, Gao S. 2011. Expression and utilization of 3AB nonstructural protein of foot-and-mouth disease virus in *Escherichia coli*. *Chin J Biotechnol* 27:180–184.
 51. Jaworski JP, Compair D, Trotta M, Perez M, Trono K, Fondevilla N. 2011. Validation of an r3AB1-FMDV-NSP ELISA to distinguish between cattle infected and vaccinated with foot-and-mouth disease virus. *J Virol Methods* 178:191–200. <http://dx.doi.org/10.1016/j.jviromet.2011.09.011>.
 52. He C, Wang H, Wei H, Yan Y, Zhao T, Hu X, Luo P, Wang L, Yu Y. 2010. A recombinant truncated FMDV 3AB protein used to better distinguish between infected and vaccinated cattle. *Vaccine* 28:3435–3439. <http://dx.doi.org/10.1016/j.vaccine.2010.02.072>.
 53. Nanni M, Alegre M, Compair D, Taboga O, Fondevilla N. 2005. Novel purification method for recombinant 3AB1 nonstructural protein of foot-and-mouth disease virus for use in differentiation between infected and vaccinated animals. *J Vet Diagn Invest* 17:248–251. <http://dx.doi.org/10.1177/104063870501700306>.