An improved, rapid competitive ELISA using a novel conserved 3B epitope for the detection of serum antibodies to foot-and-mouth disease virus

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Abstract. The highly contagious foot-and-mouth disease virus (FMDV) afflicts cloven-hoofed animals, resulting in significant costs because of loss of trade and recovery from disease. We developed a sensitive, specific, and rapid competitive ELISA (cELISA) to detect serum antibodies to FMDV. The cELISA utilized a monoclonal blocking antibody specific for a highly conserved FMDV nonstructural 3B epitope, a recombinant mutant FMDV 3ABC coating protein, and optimized format variables including serum incubation for 90 min at 20–25°C. Samples from 16 animals experimentally infected with one FMDV serotype (A, O, Asia, or SAT-1) demonstrated early detection capacity beginning 7 d post-inoculation. All samples from 55 vesicular stomatitis virus antibody-positive cattle and 44 samples from cloven-hoofed animals affected by non-FMD vesicular diseases were negative in the cELISA, demonstrating 100% analytical specificity. The diagnostic sensitivity was 100% against sera from 128 cattle infected with isolates of all FMDV serotypes, emphasizing serotype-agnostic results. Diagnostic specificities of U.S. cattle (n = 1135) and swine (n = 207) sera were 99.4% and 100%, respectively. High repeatability and reproducibility were demonstrated with 3.1% coefficient of variation in percent inhibition data and 100% agreement using 2 kit lots and 400 negative control serum samples, with no difference between bench and biosafety cabinet operation. Negative results from vaccinated, uninfected cattle, pig, and sheep sera confirmed the DIVA (differentiate infected from vaccinated animals) capability. This rapid (<3 h), select agent–free assay with high sensitivity and specificity, DIVA capability, and room temperature processing capability will serve as a useful tool in FMDV surveillance, emergency preparedness, response, and outbreak recovery programs.

Key words: Differentiate infected from vaccinated animals; DIVA; foot-and-mouth disease virus 3ABC protein.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that causes severe economic losses because of trade limitations and recovery efforts. The causative agent, FMD virus (FMDV; order Picornavirales, family Picornaviridae, genus Aphthovirus), is a positive sense, single-stranded RNA virus comprised of 8,400 nucleotides encoding 12 proteins. Four structural proteins, VP 1–4, assemble to form the virus capsid and serve as the main antigenic components in all FMD licensed vaccines. Nonstructural proteins (NSPs) L, 2A–C, and 3A–D are required for viral replication and interactions with host cell factors.

Because highly purified FMDV vaccines lack NSPs, differentiation of infected from vaccinated animals (DIVA) by assays detecting FMDV NSP-specific antibodies can be...
used in endemic regions with active FMDV vaccination programs.\textsuperscript{9,35} NSP-purified vaccines and DIVA assays are crucial to demonstrate FMDV-free status in countries and regions that have, or are interested in, FMDV vaccination-based control programs. However, false-positive reactions may occur in vaccinated animals with no history of FMD, particularly animals vaccinated with less purified vaccines that may contain some immunodominant NSPs, such as 3A.\textsuperscript{19,21,28} Therefore, identification of conserved NSP B-cell epitopes is useful for developing more sensitive and specific DIVA tests. Several B-cell epitopes have been identified in FMDV NSPs, including 2B, 2C, 3A, 3B, and 3D.\textsuperscript{14} DIVA tests have been developed based on some of these epitopes. For example, a 2C epitope (CELHEKVSSHPFKQ) serves as the coating antigen for a synthetic peptide-based indirect ELISA (iELISA).\textsuperscript{25} A 3B core repeat epitope (QKPLK) and an immunodominant motif.\textsuperscript{10,18} However, variable performance of these iELISAs or cELISAs using serum samples from different geographic areas has been reported, and specific DIVA tests utilizing the 3D epitope (MRKT KLAPTVAH-GVF) and the sequence-specific mAb.\textsuperscript{33}

The FMDV NSP 3B has 3 similar but non-identical tandem repeat sequences, 3B1 (VPg1), 3B2 (VPg2), and 3B3 (VPg3). The common core motif for all three 3B proteins is [QKPL(M)K].\textsuperscript{14} Several DIVA tests utilize this highly conserved, immunodominant motif.\textsuperscript{10,18} However, variable performance of these iELISAs or cELISAs using serum samples from different geographic areas has been reported, and can contribute to decision errors in disease response and control.\textsuperscript{2,4,9,10,17,20,24,26,30,35} Therefore, developing new, validated DIVA tests that demonstrate consistent performance across all 7 FMDV serotypes and multiple FMD-susceptible species is crucial for effective control of FMD endemic and epizootic areas. We describe herein a highly specific and sensitive, <3 h assay that can serve as an important companion test during FMD outbreak response, control, and recovery when FMDV vaccination is used.

**Materials and methods**

**Animal studies**

All animal studies were conducted in compliance with the Plum Island Animal Disease Center (PIADC) and VMRD Institutional Biosafety Committees and Institutional Animal Care and Use Committees (IACUC). Serum samples from infected animals were acquired from previous IACUC-approved studies at the institutions or from samples submitted for investigation to the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Foreign Animal Disease Diagnostic Laboratory (USDA-APHIS-FADDL; Greenport, NY). The samples were stored frozen at −20°C or −80°C, and used in the studies described herein.

**Production of mAb hybridoma clone 40C8**

The consensus peptide NH2-GPYAGPLERQKPLK-COOH derived from 3B2 was synthesized and conjugated to the keyhole limpet hemocyanin (KLH) carrier protein. The procedures for mouse immunization and mAb production were performed as described previously.\textsuperscript{34} Culture supernatants of growing hybridomas were screened in an iELISA using a recombinant 3ABC NSP antigen. The positive clone (40C8) was subcloned 3 times, and the IgG1 isotype with kappa light chains was determined using a mouse mAb isotyping kit (Roche, Indianapolis, IN). The mAb produced from clone 40C8 exhibited strong reactivity to the recombinant 3ABC antigen described below. The minimal optimal epitope sequence was determined as GPLERQ in NSP 3B2.

**Preparation of horseradish peroxidase–conjugated mAb 40C8**

Monoclonal Ab 40C8 was conjugated to horseradish peroxidase (HRP) as described previously.\textsuperscript{22} The HRP-conjugated mAb 40C8 was stabilized with a final concentration of 10% heat-inactivated goat serum (VMRD, Pullman, WA), 0.01% thimerosal (Sigma-Aldrich, St. Louis, MO), and 0.03% 4-aminoantipyrine (Thermo Fisher Scientific, Waltham, MA), and stored at −20°C.

**Production of recombinant mutant 3ABC NSP**

FMDV O1 Campos RNA was extracted from FMDV-infected BHK-21 cells (baby hamster kidney; RNeasy kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. Cloning and expression of His-tagged recombinant mutant 3ABC protein were performed as described previously.\textsuperscript{7,8} Briefly, site-directed mutagenesis (*) using an overlapping PCR technique was carried out to replace the nucleotides encoding for cysteine at position 163 with the nucleotides encoding for arginine at the active site of the 3C\textsubscript{pro} viral protease. Sense (5′-CAATTCCTTCCCAAAAATCT-3′) and antisense (5′-GTGGTGTTGTCGGGGTCAA-3′) primers were used to amplify and clone 3ABC\* into the pET30c plasmid containing a 6× His-tag sequence at the N-terminus of the cloned gene. The cloning procedure was done using BamHI/HindIII restriction endonuclease digestion. Sequencing (Big Dye terminator cycle sequencing kit, Applied Biosystems, Foster City, CA; PRISM 3700 automated sequencer, Thermo Fisher Scientific) verified the presence of the correct mutant gene sequence. Recombinant 3ABC\* protein was induced in pET30c-3ABC plasmid-transformed *Escherichia coli* (Rosetta DE3 pLysS, Novagen, Darmstadt, Germany) using a final concentration of 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) for 3 h at 37°C with shaking. The recombinant His6-3ABC\* was extracted (BugBuster reagent, 25 U/mL, Novagen). Briefly, bacterial cells were pelleted and washed once in 200 mL of 10 mM Tris and 1.0 mM EDTA. The cells were suspended in 5 mL of BugBuster reagent (supplemented with lysozyme, leupeptin, and pepstatin to 1× concentration)/gram wet weight of cells. The cell suspension was incubated at room temperature (20–25°C) for 20 min with intermittent stirring. The cell suspension was...
centrifuged at 1,864 × g for 20 min at 4°C. The pellet containing inclusion bodies was washed 3 times in 0.1× Bug-Buster reagent and solubilized with 8 M urea in 100 mM NaH₂PO₄, 10 mM Tris–HCl, and 12 mM β-mercaptoethanol (pH 8.0). Glycerol (10%) was added prior to storage at −80°C.

Development of the 3B cELISA

The concentrations for recombinant 3ABC* protein and HRP-conjugated mAb were determined using checkerboard titrations of known strongly positive, weakly positive, and negative sera provided by the U.S. Department of Homeland Security Science and Technology Directorate at PIADC from historical FMDV cattle studies. Other assay functional components and parameters, including type of antigen coating plate (Immuno plate, Nunc, Roskilde, Denmark), serum dilution factor (1:2), serum incubation time (90 min), HRP-conjugate incubation time (30 min), types of serum dilution buffer (phosphate-buffered saline [PBS] containing 0.09% sodium azide), conjugate dilution buffer (1:2 dilution of Stabilzyme, SurModics, Eden Prairie, MN), plate wash buffer, and washing method (PBS containing 0.1% Tween 20, 3×) were thoroughly compared to choose the combination of conditions that supported the best early detection capacity and analytical specificity. The 3B cELISA was performed in 96-well microtiter plates coated with 50 µL/well of recombinant 3ABC* diluted in 0.1 M carbonate and bicarbonate buffer (pH 9.6). Briefly, the plates were filled with 50 µL of antigen per well and incubated in a humid chamber overnight (i.e., 16–20 h) at 4°C. Plates received 200 µL/well of blocking buffer (VMRD) for 2 h at 37°C. The plates were dried overnight at 30°C and 25% humidity and stored at 4°C. The following 6-step assay protocol was used: 1) add 50 µL of serum (diluted 1:2 with serum dilution buffer) to each well and incubate for 90 min at room temperature; 2) wash plates 3 times with 250 µL per well of wash buffer; 3) add 50 µL of HRP-conjugated mAb 40C8 (1:100 dilution at the conjugate diluting buffer, VMRD) to each well and incubate at room temperature for 30 min; 4) wash plates 3 times with 250 µL per well of wash buffer; 5) add 50 µL of substrate solution (TM blue chromogen, BioFX Laboratories, Owings Mills, MD) to each well and incubate for 20 min at room temperature; and 6) add 50 µL of Stop solution (VMRD) to each well and read optical density (OD) at 450 nm. Results are expressed as percent inhibition.

Bovine and porcine positive control serum samples were prepared by immunizing U.S.-origin, FMDV-negative cattle and pigs with recombinant 3ABC* antigen emulsified in complete Freund adjuvant. Animals were boosted with the same antigen emulsified in incomplete Freund adjuvant 21 d after the primary immunization. Sera were collected 14 d after the boost. Negative control serum samples were collected from U.S.-origin, FMDV-negative cattle.

Comparator FMDV NSP antibody cELISA

Testing using the comparator cELISA (PrioCHECK FMDV NS antibody ELISA, Thermo Fisher Scientific) was performed per the manufacturer’s instructions. The cutoff for positive samples was ≥50% inhibition. Three samples were also tested in the ID Screen FMD NSP competition cELISA (ID.vet, Grabels, France).

Serum samples used for evaluation of 3B cELISA and comparator assay

Sera for evaluating analytical specificity and sensitivity. To evaluate analytical specificity (ASp), USDA-APHIS-FADDL–collected sera were confirmed as negative for antibodies to FMDV but positive for antibodies to FMD look-alike diseases. Fifty-five sera from vesicular stomatitis virus (VSV) antibody-positive cattle were included in the set causing a look-alike disease that cannot be differentiated clinically from FMD. Another set of 44 sera from 8 different animal species with uncharacterized, clinical look-alike vesicular diseases were included to evaluate ASp: alpaca (n = 2), bison (n = 1), cattle (n = 2), buffalo (n = 1), goat (n = 4), sheep (n = 2), pig (n = 4), and yak (n = 2). Cattle sera sequentially collected during the first 35–36 d post-infection (dpi) following intradermal infection with 1 of 4 FMDV serotype isolates (n = 4 cattle per serotype) were used as the check set to evaluate onset of positive cELISA detection.

Sera for evaluating diagnostic specificity and sensitivity. Diagnostic specificity (DSP) was evaluated using 486 serum samples from southern U.S. cattle herds and 649 serum samples from northwestern U.S. cattle herds. Diagnostic sensitivity (DSe) was assessed with serum samples collected from cattle infected with each of the 7 FMDV serotypes (n = 128 total). Additional evaluation of DSP and DSe was carried out by testing porcine serum samples (n = 272: 65 positives, 207 negatives).

Sera for evaluating repeatability and reproducibility. Eighteen bovine sera (designated as the Check Set) encompassing weak, moderate, and strong FMDV antibody-positive samples collected from cattle immunized with the recombinant 3ABC* protein were included to evaluate repeatability and reproducibility of the 2 assays. Another 400 negative serum samples from northwestern U.S. cattle were used to evaluate intra-run and inter-run variability of the assay and reproducibility of assay results.

Data analysis

The DSP of the 3B cELISA and the comparator cELISA were calculated as the percentage of U.S.-origin, FMDV-negative sera that were negative in each assay. DSe was the percentage of the virus neutralization test (VNT)-defined,
Table 1. Evaluation of the 3B competitive ELISA (cELISA) using sera from confirmed foot-and-mouth disease virus (FMDV) nonstructural protein antibody-negative and -positive animals, a sensitivity Check Set from FMDV serotype A24–infected cattle, and positive and negative control sera used in the 3B cELISA. Samples were tested at least twice.

| Source                  | OD1\(_{450}\) | OD2\(_{450}\) | Mean OD\(_{450}\) | % inhibition |
|-------------------------|---------------|---------------|------------------|--------------|
| U.S. field serum sample |               |               |                  |              |
| 1                       | 0.76          | 0.76          | 0.76             | 0.5          |
| 2                       | 0.91          | 0.85          | 0.88             | −15.3        |
| 3                       | 0.66          | 0.68          | 0.67             | 12.1         |
| FMDV A24 positive serum |               |               |                  |              |
| 1:5                     | 0.15          | 0.15          | 0.15             | 80.6         |
| 1:10                    | 0.22          | 0.22          | 0.22             | 71.2         |
| 1:20                    | 0.38          | 0.29          | 0.34             | 55.7         |
| 1:40                    | 0.33          | 0.36          | 0.34             | 55.2         |
| 1:80                    | 0.40          | 0.42          | 0.41             | 46.2         |
| 1:160                   | 0.51          | 0.53          | 0.52             | 31.8         |
| Sample buffer test      |               |               |                  |              |
| 1                       | 0.90          | 0.88          | 0.89             | −16.0        |
| 2                       | 0.84          | 0.96          | 0.90             | −17.7        |
| Negative control test   |               |               |                  |              |
| 1                       | 0.78          | 0.76          | 0.77             | 0.0          |
| 2                       | 0.78          | 0.74          | 0.76             | 0.0          |
| Positive control test   |               |               |                  |              |
| 1                       | 0.15          | 0.14          | 0.14             | 82.1         |
| 2                       | 0.17          | 0.17          | 0.17             | 79.1         |
| 3                       | 0.30          | 0.29          | 0.29             | 63.4         |
| 4                       | 0.20          | 0.16          | 0.18             | 77.7         |

OD = optical density.

FMDV antibody-positive sera having a positive result in the assay being evaluated. Receiver operating characteristic (ROC) curve and scatter plot analysis were performed using spreadsheet software (Excel, Microsoft, Seattle, WA) and R software (http://www.r-project.org/) to evaluate the cutoff for positive and negative detection by the newly developed 3B cELISA through comparison with the VNT-positive and -negative serum reference panels described above. A binomial test was used to determine if there were significant \( p \leq 0.05 \) differences in sensitivity and specificity between the comparator cELISA when testing various sets of serum samples. All statistical analyses were performed using R software. The coefficient of variation (CV) was determined based on the ratio of the standard deviation of sample OD data to OD mean, and used to evaluate intra- and inter-run variability of the 3B cELISA. Reproducibility of the 3B cELISA was determined by the percent correlation of test results obtained using 3B cELISA in different laboratories using different operators and equipment against predetermined positive and negative samples.

Results

Optimization of the 3B cELISA and determination of the cutoff using ROC curve and scatter plot analysis

The 3B cELISA was optimized by evaluation of several functional components and parameters including the antigen-coating procedure, serum incubation time, wash buffer, and HRP-conjugated anti-FMDV 3B 40C8 used to detect FMDV antibody binding. Optimization utilized a set of samples that included 3 known U.S. field negative serum samples, 6 serial dilutions (in negative serum) of FMDV serotype A24 antibody-positive serum ranging above and below the anticipated end-point of positive detection, sample diluting buffer, and negative and positive control sera used in the kit (Table 1). The optimized format included the use of 50 μL of 1:2 diluted serum to maximize the combination of assay sensitivity, specificity, and simplicity.

Using the optimized 3B cELISA format, percent inhibitions were determined for 614 bovine sera (known positive samples from cattle that we had experimentally infected with FMDV) that were previously characterized as either positive or negative by FMDV VNT. Scatter plot and ROC curve analysis were carried out using the percent inhibition data and categorization (Fig. 1A). Based on a cutoff of 45% inhibition, this analysis resulted in a maximum value for a combined 100% DSe and 99.8% DSp. The area under the ROC curve with this cutoff was 1.0. A score of 1.0 indicates perfect discriminatory power, indicating high accuracy of the 45% inhibition cutoff for classifying serum samples into positive or negative, providing optimal DSe and DSp. The CV for intra- and inter-run variability was 3.1% among 3 independent runs by several operators in 2 laboratories using the Check Set \( (n = 18) \). Percent reproducibility, intra-run assay CV, and inter-run CV of test results in a representative laboratory were 100%, 3.4%, and 6.2% using two 3B cELISA kit
A rapid sensitive specific cELISA detects antibodies to FMDV 703 lots and 400 negative serum samples. Furthermore, there was no significant (≥10%) variability in the results between bench and biosafety cabinet operation, meeting different operational needs in foreign animal disease diagnostic and surveillance laboratories.

Diagnostic specificity of the 3B cELISA and the comparator cELISA in cattle

All but 1 of 486 sera from southern U.S. herds were negative in the 3B cELISA, producing a DSp of 99.8% (Fig. 1A). However, the comparator cELISA generated a false-positive rate of 3.5% and a resultant DSp of 96.5% when evaluated with a subset of sera (n = 200) from the 486 sera (Fig. 1B). Among 649 serum samples from northwestern U.S. herds, the 3B cELISA had 6 false positives and a resultant DSp of 99.1%; the comparator cELISA was not used to test this set. Across the 1,135 bovine sera tested using the 3B ELISA, overall DSp was 99.4%.

DSe of the 3B cELISA and the comparator cELISA in cattle

The 3B cELISA detected FMDV NSP antibodies in all of the FMDV VNT-positive sera collected from 128 cattle experimentally infected with 1 of 7 FMDV serotypes, resulting in a DSe of 100% (Fig. 1A). However, the comparator cELISA generated 2 false negatives and a resultant DSe of 98.4% (Fig. 1B).

Evaluation of the 3B cELISA and the comparator cELISA on DSe and DSp in pigs

In pigs, among 65 VNT positives and 207 VNT negatives, the DSe and DSp of the 3B cELISA were 96.9% and 100%, respectively. For the comparator cELISA, the DSe and DSp against the same set of sera was 76.9% and 100%, respectively.

Early detection capacity of the 3B cELISA against sera from FMDV-infected cattle

The timing of initial positive antibody detection occurred 7–11 dpi in animals infected with FMDV Asia 1 Shamir, O Israel 2008, A Iraq 2009, or SAT 1 (Fig. 2). All of these animals reached peak levels of antibody response by 14 dpi, and these peak responses were maintained longer than 5 wk in all animals.

ASp of the 3B cELISA against sera from non-FMD vesicular diseased animals

The ASp of the 3B cELISA was evaluated with 55 sera positive for antibodies to VSV. All 55 sera were negative by the 3B cELISA and the comparator cELISA, demonstrating optimal (e.g., >99%) ASp against VSV. Additionally, 44
sera collected from FMD-negative alpaca, bison, cattle, buffalo, goat, sheep, pig, and yak that had vesicular lesions were negative by the 3B cELISA, the comparator cELISA, and in the FMDV 3D VIAA AGID (virus infection–associated antigen assay, agar gel immunodiffusion) test, demonstrating ideal ASp against non-FMD vesicular diseases in 8 animal species.

Evaluation of the 3B cELISA and the comparator cELISA on DIVA feasibility in bovine, porcine, and ovine herds using Ad5-FMD and inactivated FMDV vaccines

All sera from 24 cattle immunized with serotype 5 adenovirus-vectored recombinant subunit FMDV vaccines (Ad5-FMD) constructed with 1 of 12 different FMDV strains encompassing 5 serotypes were negative by both the 3B cELISA and the comparator cELISA (Table 2). Additionally, sera from 28 cattle immunized with binary ethylenimine (BEI)-inactivated FMDV high potency vaccines covering 6 FMDV strains and 5 serotypes were negative by both cELISAs resulting in a DIVA specificity of 100% (Table 2). These 2 data sets demonstrate the DIVA capability in bovine herds vaccinated with Ad5-FMD and BEI-inactivated FMDV. However, using another set of sera collected from 33 cattle vaccinated with different BEI-inactivated vaccines including 11 FMDV strains in 6 serotypes, both cELISAs had 3 false positives, resulting in a DIVA specificity of 90.9% (Table 2). Two of the 3 false positives had identical results in both cELISAs. The 3 false positives in the 3B cELISA were also positive in the ID Screen FMD NSP cELISA, suggesting incomplete elimination of NSPs in the inactivated vaccines used for immunizing cattle. None of the 46 porcine sera collected from Ad5-FMD- and BEI-inactivated FMDV-vaccinated herds reacted positively in either cELISA (Table 2), demonstrating DIVA capability in porcine herds that use subunit or inactivated vaccines. Sera from 42 sheep vaccinated with a high potency inactivated FMDV vaccine were all negative by the 3B cELISA, but one was positive by the comparator cELISA (Table 2).

### Table 2. Evaluation of DIVA capability of the 3B competitive ELISA (cELISA) against bovine, porcine, and ovine sera that were collected prior to foot-and-mouth disease virus (FMDV) challenge.

| Source                          | 3B cELISA | Comparator cELISA |
|--------------------------------|-----------|-------------------|
|                                | No. positive/ total | Mean % inhibition | No. positive/ total | Mean % inhibition |
| Bovine (domestic)              |           |                   |                   |
| Non-vaccinated                 | 0/200     | 0.14 (17)         | 7/200             | 21 (15)          |
| Vaccinated with Ad5-FMD vaccine, 5 serotypes (12 strains), 7–29 dpv | 0/24     | 9.8 (13)          | 0/24             | 27 (11)          |
| Vaccinated with inactivated FMDV vaccine, 5 serotypes (6 strains), 7–15 dpv | 0/28     | 11 (11)           | 0/28             | 34 (11)          |
| Vaccinated with inactivated FMDV vaccine, 6 serotypes (11 strains), 21 dpv | 3/33     | 12 (18)           | 3/33             | 32 (11)          |
| Porcine (domestic)             |           |                   |                   |
| Non-vaccinated                 | 0/99      | −16 (14)          | 0/99             | 13 (7.8)         |
| Vaccinated with inactivated FMDV monovalent vaccine, 3 serotypes (4 strains), 14–42 dpv | 0/18     | −16 (11)          | 0/18             | 19 (5.1)         |
| Vaccinated with Ad5-FMD vaccine, 2 serotypes (2 strains), 21 dpv | 0/28     | −25 (12)          | 0/28             | 13 (12)          |
| Ovine (domestic)               |           |                   |                   |
| Non-vaccinated                 | 0/50      | 0.20 (14)         | 0/50             | 22 (9.2)         |
| Vaccinated with high potency inactivated FMDV vaccine, 1 serotype (1 strain), 7–14 dpv | 0/42     | 2.0 (16)          | 1/42             | 21 (9.1)         |

dpv = day post-vaccination. Numbers in parentheses are standard deviations.

### Discussion

We developed a novel, sensitive, and specific cELISA for rapid (<3 h) and room temperature processing capability. This new cELISA with DIVA capability used a novel 3B2 epitope (GPLERQ)-specific mAb as the epitope-blocking detection agent. A mutant 3ABC recombinant protein (3ABC*) on solid phase served as the antigen, capturing FMDV NSP–specific immunoglobulins. We evaluated sera from 3 FMD-susceptible livestock species (cattle, pigs, and sheep) that were naturally or experimentally infected, uninfected, or vaccinated. DSp and DSe of the 3B cELISA with bovine sera were 99.4% and 100%, respectively, with the capability of detecting antibodies against all 7 FMDV serotypes. In addition, the 3B cELISA exhibited the same DSp (100%) and higher DSe (96.9%) against porcine sera than the comparator cELISA (100% DSp and 76.9% DSe). The higher DSe of the new 3B ELISA is arguably more important in countries and regions with high pork production and/or per capita consumption (e.g., China, European Union, North America, Taiwan, South Korea, Japan, and Brazil). For example, in the United States, daily interstate mass movement of pigs is vital to the sustainability of the pork
industry. Having a FMDV test with significantly higher DSe and shorter assay result times will allow earlier detection and quicker decision-making to help mitigate widespread dissemination in the event of a large FMD outbreak. The 3B cELISA displayed highly repeatable and reproducible results using different kit lots and operators in multiple labs. High DIVA assay performance was demonstrated against sera from cattle and pigs vaccinated with Ad5-FMD vaccines as well as conventional, inactivated, NSP-purified vaccines, and for sheep vaccinated with a NSP-purified inactivated vaccine.

Prescribed effective control of FMD outbreaks and requalification of FMD-free status in affected countries require systematic monitoring of serologic evidence of FMDV replication in all herds, accompanied by enforcement of a DIVA-feasible vaccination program. For this FMD control procedure, a reliable and DIVA-suitable NSP serology assay is essential. However, suboptimal sensitivity and specificity of some DIVA assays hinders timely and accurate decision between prompt initiation of emergency control efforts and release of false positives because of FMD look-alike diseases in animals or serologic cross-reactivity. Delayed responses to true FMD outbreaks may cause major socioeconomic damage in the case of rapid spread of FMD and large losses of animals as a result of depopulation and trade restrictions of animals and animal products. False positives resulting from misidentification may also cause substantial financial and human resource burdens in animal disease testing laboratories. Therefore, a rapid DIVA assay with high analytical and detection accuracy is necessary for outbreak preparedness in FMD-free or non-endemic countries.

The level of performance for the new 3B cELISA was higher than other DIVA NSP cELISAs and iELISAs. The new 3B cELISA exhibited higher DSe and DSp against bovine sera, well-characterized by reference assays, than the licensed comparator cELISA, used in many FMDV testing laboratories to date. In addition, test resolution against negative and positive samples was superior with the new 3B cELISA compared to the comparator assay. Furthermore, the 3B cELISA exhibited the same 100% DSp against porcine negative sera, but higher DSe (96.9%) with positive sera than the comparator cELISA (76.9%), demonstrating a higher positive predictive value. The feasibility of the 3B cELISA for other species including caprine and ovine was tested satisfactorily with limited numbers of samples (data not shown). However, formal validation in these 2 species using an appropriate number of well-characterized samples is under consideration. A validated test in sheep and goats with improved DSe and DSp would be important to FMDV control measures in countries in which FMD is endemic.

The higher DSe and DSp of the 3B cELISA versus the comparator could be related to differences in the epitope sequence, functional avidity of the epitope-blocking detection mAb, or the optimal epitope frequency in the coating antigen. Our 3B cELISA uses a 3B2 epitope GPLERQ-specific mAb as the blocking detection antibody. Other assays use different mAbs recognizing similar epitope sequences in FMDV NSP 3B2, such as CGPYAGPLERQKPLK-specific mAb, GPY-AGPMER-specific mAb, and QKPLK-specific mAb. The differences among such mAbs in functional avidity with epitope sequences has not been empirically determined, but could be a factor for the lower ASe of the assays. Relatively lower DSe of another 3ABC cELISA using 3B1,2,3 epitope QKPLK-specific mAb as the blocking detection antibody and recombinant 3ABC as the coating antigen could be related to the high frequency of a tandem repeat sequence in three 3B subunit proteins. Limited anti-FMDV immunoglobulins in sera of FMDV-infected animals may not sufficiently compete for the excessive number of epitope sequences in the coating antigen. In cELISA testing, an optimal balance among the number of available epitopes on capture antigens, immunodominance of epitope-specific antibody responses in host animals, and/or functional avidity, may be required for a highly sensitive and specific assay than simply high levels of these 3 factors.

Our data reported herein demonstrate that the 3B cELISA is compatible with conventional FMDV vaccines as well as next-generation molecular subunit FMDV vaccines. Since ~1990, the DSp of DIVA NSP tests has often been compromised because of incomplete elimination of NSPs from inactivated FMDV-based vaccines. The lack of a complete NSP 3A and 3B allows Ad5-FMD vaccines full compatibility with the 3B DIVA cELISA. Furthermore, the 3B cELISA kit is manufactured without FMDV, a select agent, and can therefore be used safely for FMDV surveillance testing in FMDV-free countries. This novel, rapid, accurate, DIVA 3B cELISA could be a crucial FMD countermeasure tool to be used in conjunction with global FMDV control and eradication programs in both endemic and non-endemic countries.

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Declaration of conflicting interests
A Clavijo, MA Bounpheng, E Rieder, S Uddowla, A Sayed, and B Dancho are inventors on a patent application filed in the United States. Other authors declare no competing interests.

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