

SWINE HEALTH INFORMATION CENTER
FINAL RESEARCH GRANT REPORT FORMAT

Project Title and Project identification number: Development of a FMDV 3ABC antibody ELISA for swine oral fluid specimens (#17-191)

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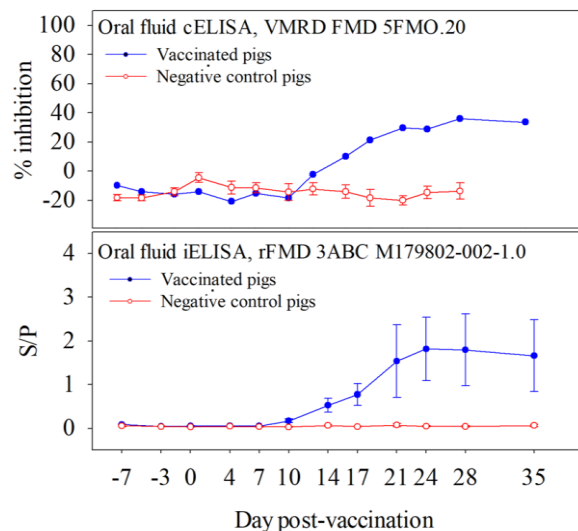
Date Report Submitted July 06, 2018

Industry Summary:

Foot-and-mouth disease virus (FMDV) remains uncontrolled in most of the world, with circulation of multiple serotypes in endemic areas. Actually, North America is among the few "FMDV-free without vaccination" areas of the world. The current massive level of global trade and traffic means that FADs anywhere in the world present a credible risk to U.S. agriculture. Our recent experience with PEDV is witness to that fact.

Preparing an effective response to the introduction of FMDV is the responsible thing to do. In the event of an FMDV outbreak in North America, effective control and elimination will require rapid detection. In turn, rapid detection will rely on an (1) efficient surveillance sampling technology and (2) immediate access to accurate diagnostic assays. Therefore, the long-term objective of this project is to create a FMD 3ABC antibody indirect ELISA (iELISA) for use with swine oral fluids.

In this study (Year One), serum and oral fluid samples of precisely known 3ABC antibody status were used to optimize FMDV competitive (cELISA) and indirect (iELISA) ELISAs for the detection of antibody in serum and oral fluids. Both optimized tests provided clear discrimination between negative and positive control oral fluid samples (See figure). That is, vaccinated pigs showed a detectable antibody response in serum at DPV ≥ 7 and in oral fluid at DPV ≥ 14 . The antibody responses in serum and oral fluid persisted through the end of the study.



Diagnostic testing of swine oral fluid samples has proven to be an effective and reliable method for the surveillance of endemic infectious diseases. Expanding this methodology to include FMDV will help provide FMDV-infected countries a new tool to control the infection and prepare the U.S. industry for a "worst-case" scenario.

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Keywords: foot-and-mouth disease, ELISA

Scientific Abstract:

Foot-and-mouth disease virus (FMDV) remains uncontrolled in most of the world, with circulation of multiple serotypes in endemic areas. Actually, North America is among the few "FMDV-free without vaccination" areas of the world. The current massive level of global trade and traffic means that FADs anywhere in the world present a credible risk to U.S. agriculture. The major barrier to efficient sampling is the high cost of collecting statistically adequate numbers of individual pig samples. Realistically, the rapid collection and testing of individual pig serum samples is logistically and fiscally impossible on a regional or national scale. Oral fluid-based diagnostics provide a solution to this problem. Oral fluids are routinely collected and accurately reflect the disease status of the population.

The objective of this project was to create FMD 3ABC antibody competitive and indirect ELISAs for use with swine oral fluids. In this study, the VMRD FMD 3ABC competitive ELISA (cELISA) was optimized to detect FMDV-specific antibodies in oral fluid. Thereafter, an indirect FMD 3ABC ELISA (iELISA) was developed to detect FMDV-specific IgG in serum and oral fluid using a recombinant FMD 3ABC protein provided by VMRD.

The ELISAs were optimized using serum and oral fluid samples of known FMD antibody status. In brief, the originally experimental design included samples from 13 FMDV antibody-negative pigs vaccinated (2 doses) with a Synthetically Modified Alpha Replicon Technology (SMART) RNA vector expressing the 3ABC polyprotein; 3 pigs inoculated (one dose) with an FMDV 3B peptide subunit vaccine (positive controls); and 3 uninoculated pigs (negative controls). Serum and oral fluid samples were collected from all pigs on day post-vaccination (DPV) -7, 0, 4, 6, 8, 10, 12, 14, 17, 21, and 35 used in the ELISAs optimization.

Serum samples from animals receiving the FMDV 3B peptide subunit vaccine (positive controls) produced a positive response on both 3ABC cELISA and iELISA (Figure 1, page 4 and Figure 4, page 6). In contrast, no serum antibody was detected in negative control animals or in the 13 animals receiving the SMART RNA vector expressing the 3ABC polyprotein. Therefore, oral fluid ELISA optimization was based on samples from the positive and negative control animals.

Variables taken into consideration in the optimization of the oral fluid ELISAs included sample volume, sample dilution, incubation time, incubation temperature, conjugate formulation, conjugate incubation time, conjugate incubation temperature, substrate, substrate incubation time, stop solution, and washing protocol. This approach was consistent with our previous work on PRRSV and Influenza A virus oral fluid ELISA development (Kittawornrat et al., 2012, 2013; Panyasing et al., 2013, 2014). The optimized procedures for the FMDV 3ABC cELISA and iELISA provided clear discrimination between negative and positive control oral fluid samples (Figures 2, 3, 6).

Thus, the results of this study demonstrated that FMDV antibody in oral fluid and could be detected using a FMDV 3ABC ELISA. These results are consistent with previous reports of detectable pathogen-specific antibody in swine oral fluids (Bjuström Kraft et al., 2018; Rotolo et al., 2018).

1. **INTRODUCTION** Foot-and-mouth disease virus (FMDV) remains uncontrolled in most of the world, with circulation of multiple serotypes in endemic areas. Actually, North America is among the few "FMDV-free without vaccination" areas of the world. Although the introduction of FMDV into North America would devastate the industry, the fiscal and economic constraints of the current era suggest that the likelihood of securing public funding for the elimination of the disease would be remote. Therefore, it is in the best interest of livestock producers to take charge of their future and facilitate the development of effective response strategies.
2. **OBJECTIVES** The long-term objective of this project is to create a FMD 3ABC antibody ELISA for use with swine oral fluids. In order to achieve an on-the-shelf product, we worked in coordination with VMRD, the manufacturer of a competitive FMD 3ABC ELISA (cELISA, catalog no. 5FMO.20). VMRD's 3ABC assay has been shown to detect all 7 FMDV serotypes (A, O, C, Asia 1, SAT 1, SAT 2, and SAT 3) and has been validated for use in bovine, ovine, and porcine sera. USDA-approved commercial FMD 3ABC cELISA and recombinant FMD 3ABC protein were used to develop the assays in this study.
3. **MATERIALS & METHODS** A step-wise approach was used to achieve the project objectives:

STEP 1. Create FMD 3ABC antibody-positive serum and oral fluid samples.

Samples of precisely known 3ABC antibody status were needed to develop a FMDV 3ABC oral fluid cELISA. Creating FMDV 3ABC antibody-positive samples by infecting animals with FMDV in biocontainment systems is impractical and expensive. In this study, the problem was solved using alternative approaches. 13 FMDV antibody-negative pigs were vaccinated (2 doses) with a Synthetically Modified Alpha Replicon Technology (SMART) RNA vector expressing the 3ABC polyprotein; 3 pigs were inoculated (one dose) with an FMDV 3B peptide subunit vaccine (positive controls); and 3 pigs remained uninoculated (negative controls). Serum samples were collected from all pigs on day post-vaccination (DPV) -7, 0, 4, 6, 8, 10, 12, 14, 17, 21, and 35. Oral fluid samples were collected daily.

STEP 2. Develop a prototype FMDV 3ABC antibody competitive ELISA (cELISA) for oral fluids.

Serum and oral fluid samples derived from the negative and positive control animals in Step 1 were used to optimize the VMRD FMD cELISA for the detection of antibody in oral fluids. Specifically, the FMD competitive ELISA (Catalog no. 5FMO.20, VMRD, Pullman WA) was adapted to the oral fluid matrix by optimizing the following variables:

Oral fluid sample volume, sample dilution, incubation time, incubation temperature, conjugate formulation, conjugate incubation time, conjugate incubation temperature, substrate, substrate incubation time, stop solution, washing protocol.

The oral fluid samples of known negative or positive 3B antibody status generated in Step 1 were used to optimization process.

STEP 3. Develop a prototype FMDV 3ABC antibody indirect ELISA (iELISA) for swine serum.

A recombinant FMD 3ABC protein (Lot M179802-002-1.0) from VMRD was used to develop the iELISA. Assembled strip/breakable 96-well plates (Microtiter™, Thermo Fisher Scientific, Waltham,

MA) were coated with the protein diluted 1/3000 with carbonate-bicarbonate buffer (0.1 M, pH 9.6). The plates were then blocked, dried, and kept at 4°C until used.

Serum samples derived from the negative and positive control animals in Step 1 were used to optimize the iELISA for the best detection of antibody. Developmental processes were performed as described in STEP 2 using 1/30,000 goat anti-pig IgG Fc fragment HRP conjugated antibody.

STEP 4. Develop a prototype FMDV 3ABC antibody indirect ELISA (iELISA) for oral fluids.

The FMD 3ABC iELISA plates prepared in STEP 3 were optimized for antibody detection in oral fluid specimens. Oral fluid samples derived from the negative and positive control animals in Step 1 were used to optimize the oral fluid FMD 3ABC iELISA for the detection of antibody in oral fluids. Developmental processes were performed as described in STEP 2 using 1/3,000 goat anti-pig IgG Fc fragment HRP conjugated antibody.

4. RESULTS

STEP 1. Create FMD 3ABC antibody-positive serum and oral fluid samples.

Serum samples from vaccinates were negative on the 3ABC cELISA following 2 doses of the RNA vector. Serum samples from animals receiving the FMDV 3B peptide subunit vaccine (positive controls) produced a positive response on the 3ABC cELISA (Figure 1). No serum antibody was detected in negative control animals (Figure 1).

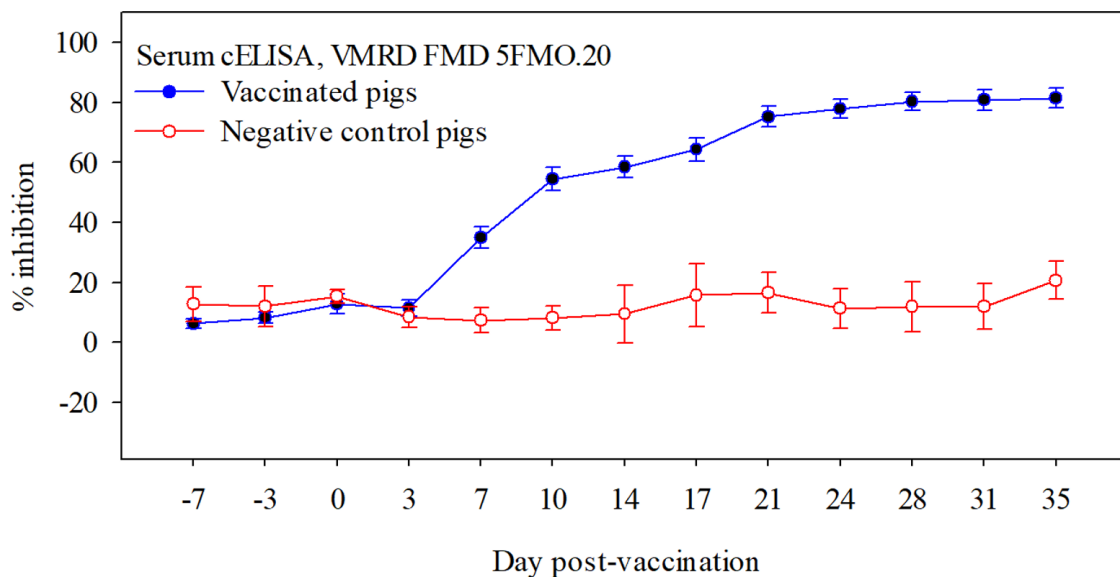


Figure 1. Serum antibody responses (positive and negative controls) on the FMD 3ABC cELISA

The results of testing sera from negative and positive controls demonstrated that the test functioned correctly. Therefore, it was concluded that the RNA inoculate did not effectively express the 3ABC polyprotein and samples from the 13 pigs in the SMART group were eliminated from the remainder of the experiment. Subsequent developmental steps were based on samples from the negative and positive control animals.

STEP 2. Develop a prototype FMDV 3ABC antibody competitive ELISA (cELISA) for oral fluids.

The optimized procedure for the FMDV 3ABC provided clear discrimination between negative and positive control oral fluid samples, i.e., negative oral fluid antibody was lower than 10.16% and positive oral fluid antibody was higher than 14.84% inhibition (Figures 2 and 3).

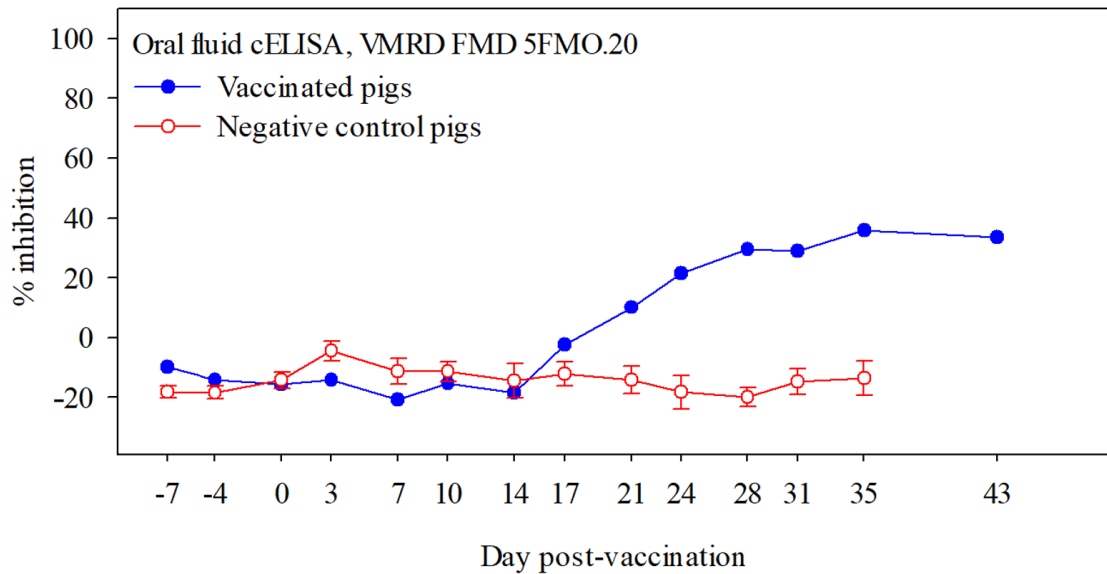


Figure 2. FMDV 3ABC cELISA oral fluid antibody response in positive and negative controls

Oral fluid cELISA (VMRD FMD 5FMO.20) final procedure

Sample preparation

- A) Warm reagents: Bring oral fluid samples, reagents, and plates to room temperature ($23 \pm 2^\circ\text{C}$) at least 30 minutes prior to starting the test.
- B) Prepare samples: 250 μl test oral fluid samples must be added to a transfer plate. Positive and negative oral fluid samples of known status must be included as controls.
- C) Prepare conjugate: Prepare 1X VMRD antibody-peroxidase conjugate by diluting one part of the 100X VMRD antibody-peroxidase conjugate with 99 parts of VMRD conjugate diluting buffer.
- D) Prepare wash solution: Prepare 1X VMRD wash solution by diluting 1 part of the 10X VMRD wash solution concentrate with 9 part of sterilized deionized water.

Test procedure

- A) Load 200 μl of oral fluid samples from the transfer plate into the VMRD FMD cELISA plate using a multichannel pipette.
- B) Cover the plate with sealing film and incubate at 4°C for 16 hours.
- C) After incubation, wash the plate 3 times with 300 μl 1X wash buffer, then tap the plate on clean paper towel to remove excess fluid.

- D) Add 50 μ l of diluted (1X) VMRD antibody-peroxidase conjugate to each well. Tap the side of the loaded plate several times to make sure the conjugate coats the bottom of the wells.
- E) Cover the plate with sealing film and incubate at 37°C for 5 minutes
- F) After incubation, wash the plate 3 times with 300 μ l 1X wash buffer, then tap the plate on clean paper towel to remove excess fluid.
- G) Add 50 μ l of TMB One component substrate (Bethyl Laboratories, Inc., TX) to each well. Tap the side of the loaded plate several times to make sure the substrate coats the bottom of the wells.
- H) Incubate the plate the plate for 20 minutes in dark place at room temperature (23 \pm 2°C).
- I) Add 50 μ l of TMB stop solution (Bethyl Laboratories, Inc., TX) to each well. Tap the side of the leaded plate several times to mix the substrate solution and the stop solution.
- J) Read the test results using a microplate absorbance spectrophotometer at optical density reading wavelength 450 nm.
- K) Calculate percent inhibition:

$$\text{Percent inhibition} = 100 \times [1 - (\text{Sample OD} \div \text{Negative control OD})]$$

STEP 3. Develop a prototype FMDV 3ABC antibody indirect ELISA (iELISA) for swine serum.

S/Ps of negative serum samples (n = 51) ranged from -0.003 to 1.2 throughout the study period. Vaccinated pigs showed a detectable antibody response in serum at DPV \geq 7 that persisted through the end of the study (Figure 4). S/Ps of positive serum samples (n = 21) ranged from 2.11 to 3.67.

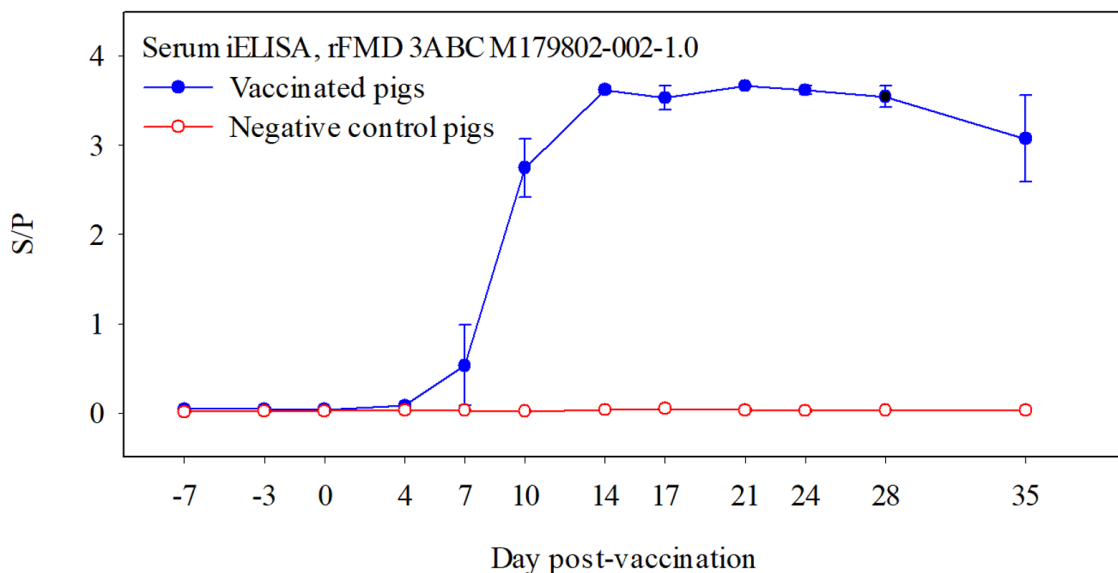


Figure 4. Serum antibody responses (positive and negative controls) on the FMD 3ABC iELISA

Serum FMD 3ABC indirect ELISA (rFMD 3ABC M179802-002-1.0) final procedure

ELISA plate

- 1) ELISA plate: Assembled strip/breakable 96-well plates

- 2) ELISA plate coating
 FMD 3ABC Antigen: VMRD recombinant FMD 3ABC antigen (Lot. M170802-002-1.0)
 Antigen concentration: 1/3,000
 Coating buffer: Carbonate-bicarbonate (0.1 M, pH 9.6)
- 3) ELISA plate blocking
 Blocking buffer: VMRD blocking buffer

Sample preparation

- A) Warm reagents: Bring serum samples, reagents, and plates to room temperature ($23 \pm 2^\circ\text{C}$) at least 30 minutes prior to starting the test.
- B) Prepare samples: Mix 1.2 μl test serum samples with 120 μl serum diluent in a transfer plate. Positive and negative serum samples of known status must be included as controls.

Test procedure

- A) Load 100 μl of serum samples from the transfer plate into the FMD 3ABC indirect ELISA (rFMD 3ABC M179802-002-1.0) plate using a multichannel pipette.
- B) Cover the plate with sealing film and incubate at 37°C for 1 hours.
- C) After incubation, wash the plate 5 times with 300 μl 1X wash buffer, then tap the plate on clean paper towel.
- D) Add 100 μl of goat anti-pig IgG Fc fragment antibody HRP conjugate (1/30,000) into each well.
- E) Cover the plate with sealing film and incubate at 37°C for 1 hour
- F) After incubation, wash the plate 5 times with 300 μl 1X wash buffer, then tap the plate on clean paper towel.
- G) Add 100 μl of TMB One component substrate (Bethyl Laboratories, Inc., TX) to each well.
- H) Incubate the plate the plate for 5 minutes in dark place at room temperature ($23 \pm 2^\circ\text{C}$).
- I) Add 100 μl of TMB stop solution (Bethyl Laboratories, Inc., TX) to each well. Tap the side of the leaded plate several times to mix the substrate solution and the stop solution.
- J) Read the test results using a microplate absorbance spectrophotometer at optical density reading wavelength 450 nm.
- K) Calculate S/P:

$$S/P = (\text{Read OD-negative control OD})/(\text{positive control OD/negative control OD})$$

STEP 4. Develop a prototype FMDV 3ABC antibody indirect ELISA (iELISA) for oral fluids.

Negative oral fluids (n = 54): S/P of negative oral fluid samples ranged between 0.01 and 0.15 throughout the study period. Positive oral fluid (n = 18): the vaccinated pig showed a detectable antibody response in oral fluid at $\text{DPV} \geq 7$ that persisted through the end of the study (Figure 5). The positive oral fluid antibody S/P ranged between 0.21 and 3.16.

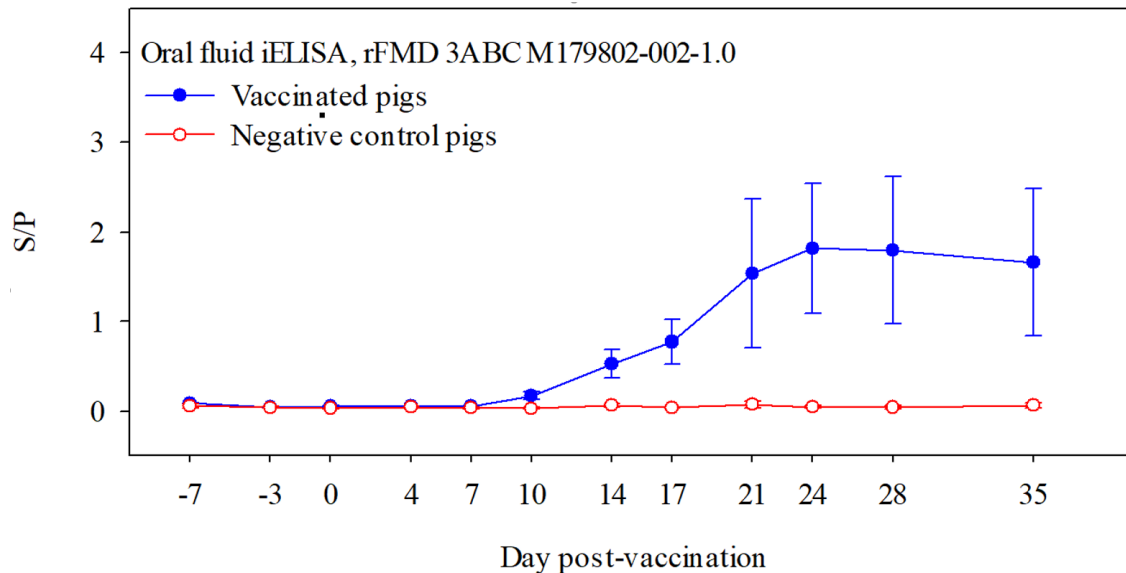


Figure 5. Oral fluid antibody response in positive and negative controls

Oral fluid FMD 3ABC indirect ELISA (rFMD 3ABC M179802-002-1.0) final procedure

Oral fluid preparation

- A) Warm reagents: Bring oral fluid samples, reagents, and plates to room temperature ($23 \pm 2^\circ\text{C}$) at least 30 minutes prior to starting the test.
- B) Prepare samples: Mix 60 μl test oral fluid samples with 60 μl serum diluent in a transfer plate. Positive and negative oral fluid samples of known status must be included as controls.

Test procedure

- A) Load 100 μl of oral fluid samples from the transfer plate into the FMD 3ABC indirect ELISA (rFMD 3ABC M179802-002-1.0) plate using a multichannel pipette.
- B) Cover the plate with sealing film and incubate at 37°C for 1 hour.
- C) After incubation, wash the plate 5 times with 300 μl 1X wash buffer, then tap the plate on clean paper towel to remove excess fluid.
- D) Add 100 μl of goat anti-pig IgG Fc fragment antibody HRP conjugate (1/3,000) into each well.
- E) Cover the plate with sealing film and incubate at 37°C for 1 hour
- F) After incubation, wash the plate 5 times with 300 μl 1X wash buffer, then tap the plate on clean paper towel to remove excess fluid.
- G) Add 100 μl of TMB One component substrate (Bethyl Laboratories, Inc., TX) to each well.
- H) Incubate the plate the plate for 5 minutes in dark place at room temperature ($23 \pm 2^\circ\text{C}$).
- I) Add 100 μl of TMB stop solution (Bethyl Laboratories, Inc., TX) to each well. Tap the side of the leaded plate several times to mix the substrate solution and the stop solution.
- J) Read the test results using a microplate absorbance spectrophotometer at optical density reading wavelength 450 nm.

K) Calculate S/P:

$$S/P = (\text{Read OD-negative control OD})/(\text{positive control OD/negative control OD})$$

5. DISCUSSION

Previously, replicon particle technology was successfully used to produce antibody responses against African swine fever virus proteins 30, 54, and 72 (Giménez-Lirola et al., 2016). In this study, the RNA vector unexpectedly failed to produce detectable 3ABC antibody. Despite this set-back, FMDV 3ABC competitive and indirect ELISAs were developed and shown capable of detecting antibody in the 3B subunit vaccinated animals (Figure 2).

These results are consistent with previous reports in the the refereed literature. Earlier workers reported that FMDV-specific IgA could be detected in esophageal-oro-pharyngeal or oral fluid samples for up to 182 DPI in cattle and 112 DPI in pigs (Eblé et al., 2007; Mohan et al., 2008). Subsequently, virus neutralization assays and IgA-specific ELISAs for esophageal-oro-pharyngeal samples or oral fluid samples were shown to detect FMDV antibody in cattle and swine (Pacheco et al., 2010; Parida et al., 2006; Stenfeldt et al., 2016). Using an experimental FMDV serotype O-specific IgA cELISA, antibody was detected in oral fluids from pigs by 14 DPI (Senthilkumaran et al., 2017).

Based on previous research in our laboratories and reports in the literature, future research should focus on assessment and comparison of 3ABC IgG and IgA prototype indirect ELISAs (iELISAs) using samples collected from FMDV infected and/or FMDV vaccinated animals. Future research should take the following into consideration:

- Given that vaccine will undoubtedly be used in the event of an FMDV introduction, the 3ABC ELISA is the best choice because of its proven DIVA application.
- Focus on the iELISA format is justified by the higher analytical sensitivity of the indirect vs competitive format. High analytical sensitivity is necessary to detect antibody in aggregate samples, i.e., samples from pens of pigs. For this reason, all current commercial oral fluid antibody ELISA are based on the indirect format.
- Evaluation of IgG and IgA ontogeny is justified by the fact that direct comparisons showed that IgG oral fluid ELISAs provided higher diagnostic sensitivity than IgA oral fluid ELISAs, with the sole exception of PEDV (Bjuström Kraft et al., 2016).

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