NPB Final Research Grant Report

Project Title:	Development of a CSFV Erns IgG AlphaLISA® assay capable of differentiating infected from CSFV-vaccinated animals (DIVA)
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INDUSTRY SUMMARY:

Classical swine fever (CSF) is a highly contagious viral disease causing significant economic losses to swine producers in most parts of the world. Although the US is free of classical swine fever virus (CSFV), we should be prepared for its introduction from outside our borders. Previously, pseudorabies virus (PRV) was eliminated from US commercial swine herds using gene-deleted vaccines and assays able to differentiate infected from vaccinated animals (DIVA). Modeling on this experience, we developed an assay able to differentiate CSFV-infected pigs from pigs vaccinated with a modified-live CSFV marker vaccine (Suvaxyn® CSF Marker). In brief, the CSFV Erns IgG AlphaLISA® detects specific antibody against a CSFV envelope glycoprotein (Erns) present in wild-type viruses. In contrast, the Erns glycoprotein is absent in the CSFV marker vaccine, hence vaccinated pigs are negative on the CSFV Erns AlphaLISA®. Importantly for ease of use in the field and in the laboratory, the assay is able to test both serum and oral fluid samples. In this study, the diagnostic sensitivity and specificity of the assay was estimated at 97.9% and 99.8%, respectively, for serum and 99.7% and 100% for oral fluids. The combined use of a marker vaccine to protect against clinical disease and a serum/oral fluids DIVA assay able to identify wild-type CSFV infections will greatly improve the capacity of the US pork industry to deal quickly and decisively with CSFV.

Key findings:

- The DIVA CSFV Erns IgG AlphaLISA® assay developed in this project was rapid (one hour), and technically simple to perform, and able to detect CSFV Erns IgG in serum and oral fluid samples using the same protocol.
- An evaluation based on serum (*n* = 760) and individual (*n* = 528) or pen-based (*n* = 30) oral fluid samples of known CSFV infection and/or vaccination status found that The CSFV Erns IgG AlphaLISA® was diagnostically sensitive and specific when testing either serum or oral fluid samples.
- The ability to differentiate vaccinated from wild-type CSFV-infected animals will greatly expedite the control and elimination of CSFV in the event of its introduction into the US.

Keywords: AlphaLISA®, classical swine fever virus, DIVA, Erns, IgG, oral fluid, serum

SCIENTIFIC ABSTRACT:

Classical swine fever virus (CSFV) is an OIE-listed disease in need of more effective diagnostic tools for its control and detection. In particular, the availability of diagnostically sensitive and specific DIVA vaccine-compatible tests would increase the chance of successful control of the disease. The aim of this study was to develop a CSFV Erns IgG AlphaLISA® for serum and oral fluids that would complement currently available CSFV E2 DIVA vaccines. After assay development, its performance was evaluated using a panel of well-characterized serum (*n* = 760) and individual (*n* = 528) or pen-based (*n* = 30) oral fluid samples from 4 groups of animals: (1) unvaccinated and uninoculated; (2) unvaccinated and inoculated with wild-type CSFV (ALD strain); (3) uninoculated and vaccinated with a live CSFV (LOM strain) vaccine; and (4) uninoculated and vaccinated with live CSFV marker vaccine. Overall, the CSFV Erns IgG AlphaLISA® showed diagnostic sensitivity and specificity of 97.9% and 99.8% for serum and 99.7% and 100% for oral fluids, respectively. Thus, the assay combined DIVA capability with rapid turnaround and simplicity of use.

INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease of swine with a significant economic impact. Classical swine fever virus (CSFV) is a member of the genus *Pestivirus* in the family *Flaviviridae*, along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV). Occasionally pigs may be infected with BVDV or BDV, but these infections in pigs are usually mild and self-limiting. CSFV is an OIE-listed agent because of direct losses, losses in trade on pigs and derived products, and the substantial costs of maintaining immunization or eradication programs. The potential for CSFV to spread over long distances to areas previously free of CSFV is well-documented, e.g., Israel (David et al., 2011), and South Africa (Sandvik et al., 2005). These events further underline the fact that current surveillance programs are not capable of preventing all movement of CSFV across borders and justifies preparations for dealing with incursions of CSFV.

The highly variable clinical presentation of CSFV infections precludes a diagnosis based on clinical signs and lesions. The list of differential diagnoses varies with the presentation, but classical swine fever may resemble African swine fever, salmonellosis, erysipelas, eperythrozoonosis, pasterurellosis, actinobacillosis, *Haemophilus parasuis*, PRRSV, and PCV2 infections. Thus, accurate and rapid laboratory methods are mandatory for the detection, control, and/or elimination of CSFV. CSFV diagnostic methods include immunohistochemical and fluorescent antibody techniques for the direct detection of CSFV antigen in tissues, antigen-capture ELISAs, virus isolation in cell culture, and reverse transcription-polymerase chain reaction (RT-PCR). Serum antibody assays include the fluorescent antibody, virus-neutralization test (VNT), the neutralizing peroxidase-linked assay (NPLA), and antibody ELISAs.

Generally, there are no legal obligations to use a certain type of vaccine for an emergency vaccination situation, but because of the trade restrictions imposed on pigs vaccinated with conventional live-attenuated vaccines, only marker vaccines are a viable option for the immunization of domestic pigs. After a review of the data and supplementary trials, the vaccine candidate “CP7-E2alf” (Suvaxyn® CSF Marker, Zoetis) was licensed as the first live marker vaccine against classical swine fever (Blome et al., 2017). CP7-E2alf is a chimeric Pestivirus vaccine based on an infectious cDNA clone of BVDV strain CP7. In this backbone, the BVDV

E2 was exchanged for that of CSFV Alfort/187. The resulting virus, CP7_E2alf, replicates mainly in porcine cells.

The CSFV genome contains a single open reading frame encoding a polyprotein of 3898 amino acids that undergoes co- and post-translation processing by cellular and viral proteases, giving rise to four structural proteins (C, Erns, E1, E2) and seven non-structural proteins (Npro, p7, NS2-3, NS4A, NS4B, NS5A, NS5B) (Rümenapf et al., 1993; Meyers and Thiel, 1996; Tautz et al., 1997; Zhang et al., 2011).

The E2 protein possesses an immunogenic domain located in the N-terminal region of about 120 amino acids, while Erns contains an immunodominant region encompassing three overlapping antigenic regions that induce antibody responses during CSFV infection (Lin et al., 2000). Antibody kinetics (IgA, IgG) in serum and oral fluid against E2 and Erns immunogenic regions have been described (Panyasing et al., 2018). Following CSFV infection, antibodies are developed against structural proteins E2, Erns, and the non-structural protein NS3. Neutralizing antibodies that confer protective immunity are induced by E2 and Erns (König et al., 1995). Therefore, the serological diagnosis of CSF is mainly based on the detection of E2- or Erns-specific antibodies (Moser et al., 1996; Moormann et al., 2000; Clavijo et al., 2001).

Erns-based ELISAs have been developed for the evaluation of CSF E2-based DIVA vaccines. That is, animals vaccinated with marker vaccine will produce antibodies against CSFV E2 but not CSFV Erns. In contrast, field virus-infected animals produce antibodies against both E2 and Erns. Thus, an animal with Erns antibodies has been infected with wild-type virus.

Currently, commercially available DIVA-ELISA kits include PrioCHECK™ CSFV Erns (Thermofisher, USA) and the Pigtype® CSF marker (Qiagen, Germany). The available validation data show good overall sensitivity and specificity of the Erns serum ELISAs (Schroeder et al., 2012; Pannhorst et al., 2015; Meyer et al., 2017). However, successful CSFV surveillance requires superb assay performance because both false positives and false negatives will quickly erode confidence in a control/elimination program. In addition, because oral fluids are used routinely for surveillance by North American swine veterinarians and producers, assays need to be able to test oral fluids for Erns antibody.

Objective

This project addressed the NPB 2020 RFP on Foreign Animal Disease Surveillance and, more specifically, the call for "*validated classical swine fever antibody detection tests with increased diagnostic sensitivity and specificity that complement DIVA-capable vaccines*".

Specifically, the objective of this project was to develop a diagnostically sensitive and specific CSFV antibody assay (for both serum and oral fluid) to complement CSFV DIVA vaccines using the AlphaLISA® platform. AlphaLISA® (**A**mplified **L**uminescent **P**roximity **H**omogenous **A**ssay) differs from standard the ELISA format in the following ways: (1) the presence of the target is indicated by a luminescent/fluorescent signal; (2) the assay provides higher analytical sensitivity that standard ELISAs; (3) turn-around time is short.

MATERIALS AND METHODS

Experimental design

We developed and evaluated the performance of a CSFV AlphaLISA®. Assay evaluation was based on testing serum and oral fluid samples from pigs of known status. The performance of the CSFV AlphaLISA® for serum and oral fluid samples was evaluated using receiver operating characteristic (ROC) analyses.

Biological samples

A total of 1,318 well-characterized serum *(n* = 760) and oral fluid (*n* = 558) samples were used to evaluate the test performance from the following groups:

1. Unvaccinated and uninoculated pigs (serum *n* = 160, oral fluid *n* = 160);
2. Unvaccinated pigs inoculated with CSFV ALD strain (serum *n* = 100, oral fluid *n* = 184);
3. Uninoculated pigs vaccinated with live CSFV LOM strain vaccine (serum *n* = 140, oral fluid *n* = 184);
4. Samples from uninoculated pigs vaccinated with live CSFV marker vaccine (serum *n* = 360 and pen-based oral fluids *n* = 30). Group 4 pigs were intramuscularly vaccinated with live CSFV marker vaccine (Suvaxyn® CSF Marker, Zoetis) at 9 weeks of age. Serum and pen-based oral fluid samples were collected at day post vaccination (DPV) 14, 30, and 60. Pen-based oral fluids were collected from 10 pens (50 to 60 pigs per pen).

CSFV Erns IgG AlphaLISA®

CSFV Erns antigen. Erns antigen was produced as previous described (Panyasing et al., 2018). In brief, the codified region of the truncated N-terminal CSFV Erns gene was synthetically produced with the addition of a 5′ GP67 terminal signal peptide for expression (Bac-to-Bac™ Baculovirus Vector System, Invitrogen), followed by a hexa-histidine tag and a "GS linker" (encoding GSGS) for protein purification by affinity chromatography. Recombinant baculovirus was generated and amplified according to the manufacturer's instructions (Bac-to-Bac™ Baculovirus Expression Systems, Invitrogen). The amplified Erns region was cloned into pFastBac1 (Invitrogen) using EcoRI and XhoI restriction enzymes. The construct was confirmed by digestion and sequencing. The pFastBac1 vector carrying Erns was transformed into E. coli DH10Bac-competent cells to regenerate bacmid-expressing Erns. CSFV Erns protein was purified from culture supernatant by Ni-chelating SFF affinity chromatography (GE Healthcare), according to the manufacturer's instructions. Protein elution was dialyzed against 10mM Tris, 150mM NaCl, pH 8.0 and analyzed by SDS-PAGE and Western blot. The amino acid sequence of the recombinant CSFV Erns protein (169 amino acids). Erns molecular weight measured by SDS-PAGE was 25-35 KDa. The final product had a concentration of 0.75 mg/mL.

CSFV Erns AlphaLISA® bead conjugation. The protein buffer was exchanged with PBS (0.1 M $NaKPO_4$ pH 8.0) on a Zeba^{TM} spin column (ThermoFisher) and concentrated up to 1 mg/mL protein (BCA protein assay). Acceptor beads were washed with the same pH 8 buffer as the protein buffer following the manufacturer's protocol. The Erns protein was conjugated to raw (aldehyde) AlphaLISA® Acceptor and Donor beads via reductive amination, as described by the manufacturer (PerkinElmer Health Sciences, Inc., Boston, USA). Briefly, 0.1 mg of Erns protein (1 mg/mL, 100 µl) was mixed with 1 mg AlphaLISA® beads in a 1.5 mL microtube and

reaction buffer (100 mM HEPES pH 7.4) to a final reaction volume of 200 µl. Then, 1.25 µl of 10% Tween-20 (Sigma, ST. Louis, MO, USA) and 10 µl of a 400 mM solution of the reducing agent sodium cyanoborohydride (NaBH3CN; Sigma) were added to the microtube. The tube was incubated for 18-24 h at 37ºC with mild agitation (6-10 rpm) using a rotary shaker. Thereafter, unreacted aldehyde groups on the beads were blocked by adding 10 µl of 65 mg/mL solution of carboxymethoxylamine (CMO, Sigma) in 800 mM sodium hydroxide (NaOH; Sigma) followed by a 60 min incubation at 37ºC under mild agitation. The uncoupled Erns protein was removed by centrifugation (16,000 x *g* for 15 min at 4ºC) followed by washing two times with 200 µl of 100 mM Tris-HCL pH 8. The supernatant was then removed with a micropipette and the bead pellet resuspended in 200 µl of storage buffer (PBS + 0.05% Proclin-300). The bead solution was briefly sonicated with ultrasonic homogenizer (UX-050) (Mitsui-EC Corp., Japan) to ensure that the beads are not aggregated then stored at 4ºC until use.

CSFV Erns IgG AlphaLISA optimization Sample dilution, incubation time, Erns protein-conjugated AlphaLISA® acceptor bead concentration, streptavidin donor bead concentration, and biotinylated goat anti-swine IgG (Fc) concentrations for serum and oral fluid samples were evaluated to derive the optimum conditions. The final one-hour procedure consisted of two steps. In brief, incubations were performed at room temperature (22-25ºC) in half-area 96-well white plates (PerkinElmer). During the process, plates were protected from light and held on a rotating shaker. In the first step, 5 µl of sample (serum or oral fluid) or control (negative and positive) was incubated for 30 min with 20 µl of a mix containing 25 µg/mL of Erns protein-conjugated AlphaLISA® acceptor beads and 80 nM biotinylated goat anti-swine IgG (Fc) (Bethyl Laboratories Inc., Montgomery, TX, USA) in assay buffer 1x (AlphaLISA® buffer 10x; PerkinElmer). In the second step, 25 µl of 80 µg/mL streptavidin donor beads (PerkinElmer) were added to each well and incubated for 30 min. In the presence of CSFV-Erns-specific IgG antibodies, acceptor and donor beads come into close proximity. Laser irradiation (680 nm) of donor beads releases singlet oxygen molecules that trigger a cascade of chemical events in nearby acceptor beads which, in turn, produces a chemiluminescent emission (615 nm) detected by the plate reader. In valid runs, negative control signals should range from 0 - 400 and positive controls from 4000 - 7000. Results were converted to sample-to-positive (S/P) ratios:

$$\text{S/P ratio} = \frac{\text{Sample signal - Negative control signal (mean)}}{\text{Positive control signal (mean) - Negative control signal (mean)}}$$

CSFV Erns IgG ELISA

For comparison with the CSFV Erns IgG AlphaLISA®, serum and oral fluid samples were tested with a CSFV Erns indirect ELISA (Panyasing et al., 2018). Briefly, 96-well microtitration plates (Thermo Scientific) were manually coated with Erns protein (250 ng per well), incubated at 4°C for 16 ± 2h, and then washed 5 times (350 µl) with washing buffer. Next, 300 µl of blocking buffer (1% BSA-PBS) was added to each well and the plates incubated at room temperature (25–27°C) for 2h. After discarding the blocking buffer without washing, the plates were dried at 37°C for 3h and then stored at 4°C in a sealed bag with desiccant packs until used. Plate lots with a coefficient of variation ≥10% were excluded.

Serum and oral fluid Erns ELISAs were performed using the same plates, but procedures differ by specimen. Serum ELISAs are performed using samples diluted 1:50 with sample diluent (1%

BSA-PBS+0.05% Tween-20) and pre-diluted serum negative and positive controls. Oral fluid ELISAs are performed using samples diluted 1:2 with sample diluent and pre-diluted oral fluid negative and positive controls. For both serum and oral fluid ELISAs, 100 µl of sample was added to each well and the plates incubated at 37°C for 1h. Thereafter, plates are washed 5 times with 350 µl of washing buffer (0.05% Tween-20 in PBS). For serum ELISAs, 100 µl of HRP-IgG (1:20,000) conjugate was then added and the plates incubated at 37°C for 1h. For oral fluid ELISAs, 100 µl of HRP IgG (1:3000) conjugate was added and the plates incubated at 37°C for 1h. The washing step was then repeated, after which 100 µl of TMB substrate (T0440, Sigma-Aldrich) was added to each well and the plates incubated at room temperature for 5 min in the dark. Thereafter, 100 µl of stop solution (S5814, Sigma-Aldrich) was added to each well and the plates read (450 nm) using an ELISA plate reader. Sample-to-positive (S/P) ratios were calculated for each sample:

$$\text{S/P ratio} = \frac{\text{Sample OD - Negative control OD (mean)}}{\text{Positive control OD (mean) - Negative control OD (mean)}}$$

As reported by Panyasing et al. (2018), the cutoff for the Erns serum and oral fluid IgG indirect ELISAs was S/P ≥ 0.5 and ≥ 0.3, respectively.

Statistical analysis

The diagnostic performance of the serum and the oral fluid CSFV Erns IgG AlphaLISA® were evaluated by receiver operating characteristic (ROC) analysis. Serum and oral fluid S/P responses were assessed for each group using Wilcoxon signed-rank test (PROC UNIVARIATE). Qualitative differences (positive, negative) by group were assessed using McNemar's test (PROC FREQ) for pairwise comparisons. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A p-value < 0.05 was considered statistically significant. The cumulative proportion of positive samples over the observation was the response, the specimen-assay combination was the covariate, and pig ID was the random effect. Subsequently, pairwise comparisons were performed among assays with Tukey adjustment.

RESULTS

CSFV Erns IgG AlphaLISA® diagnostic performance

The diagnostic performance of the CSFV Erns IgG AlphaLISA® for serum and oral fluid samples was evaluated by receiver operating characteristic (ROC) curve analysis. Samples collected ≥ 14 days post inoculation (DPI) or vaccination (DPV) were used in ROC analysis. Based on the ROC, the optimum cutoff for the serum Erns IgG AlphaLISA was S/P ≥ 0.3. This cutoff produced a diagnostic sensitivity and specificity of 97.9% and 99.8%, respectively. The optimum cutoff for oral fluid Erns IgG AlphaLISA was S/P ≥ 0.10, with diagnostic sensitivity and specificity of 99.7% and 100%, respectively.

Performance comparisons

Comparisons of the rate of Erns antibody positivity (%) by group, specimen, and assay are shown in Table 1. All serum (n = 160) and oral fluids (n = 160) from uninoculated and unvaccinated pigs were Erns antibody negative by both AlphaLISA® and indirect ELISA. In inoculated pigs, the serum AlphaLISA® detected significantly more Erns antibody positives than the indirect ELISA (96.0% vs. 92.0%), whereas all oral fluid samples (n = 184) were positive by both assays. In pigs vaccinated with the live CSFV LOM strain vaccine, no significant difference in the rate of positivity was detected between the two assays. In the marker vaccinated group, the AlphaLISA® showed higher diagnostic specificity than the Erns indirect ELISA for both serum and oral fluids.

Table 1. Erns antibody positivity (%) by group, specimen, and assay

Group	Specimen (no.)	CSFV Erns AlphaLISA[1] (%)	CSFV Erns indirect ELISA[2] (%)
1. Unvaccinated and uninoculated pigs	Serum (160)	0.0	0.0
	Oral fluid (160)	0.0	0.0
2. Pigs inoculated w/ ALD strain	Serum (100)	96.0[a]	92.0[b]
	Oral fluid (184)	100	100
3. Pigs vaccinated w/ LOM vaccine strain	Serum (140)	94.3	94.3
	Oral fluid (184)	99.5	98.9
4. Pigs vaccinated w/ live CSFV marker vaccine	Serum (360)	1.1[a]	8.9[b]
	Pen-based oral fluid (30)	0.0	3.3

[1] CSFV Erns IgG AlphaLISA® - Cutoff for serum was S/P ≥ 0.3 (diagnostic sensitivity and specificity 97.9% and 99.8%, respectively) and cutoff for oral fluid was S/P ≥ 0.1 (diagnostic sensitivity and specificity 99.7% and 100%, respectively).

[2] CSFV Erns IgG indirect ELISA (Panyasing et al., 2018) - cutoff for serum was S/P ≥ 0.5 (diagnostic sensitivity and specificity 98.7% and 100%, respectively) and cutoff for oral fluid was S/P ≥ 0.3 (diagnostic sensitivity and specificity 97.5% and 100%, respectively).

[ab] Within rows, significant differences ($p < 0.05$) in the proportion of positive responses between assays indicated by superscripts.

DISCUSSION

CSFV Erns-based ELISAs have been developed for use with CSF E2-based DIVA vaccines, such as the CSF marker vaccine CP7_E2alf evaluated in this study. However, studies on the diagnostic performance of commercially-available DIVA CSFV ELISAs suggest that performance (false negatives and false positives) is still an issue (Floegel-Niesmann, 2001; Schroeder et al., 2012; Pannhorst et al., 2015; Meyer et al., 2017). Based on the data generated in the present study, the CSFV Erns IgG AlphaLISA® provided an improvement in this respect,

producing a diagnostic sensitivity and specificity of 97.9% and 99.8% for serum and 99.7% and 100% for oral fluids.

AlphaLISA® assays have been developed and implemented in human medicine to detect and quantify targets in serum, urine, or blood, e.g., Shiga toxin 2 (Armstrong et al., 2018) and SARS-CoV-2 (Gorshkov et al., 2020). AlphaLISA® has been previously described for detection of swine infectious agents, i.e., porcine epidemic diarrhea (PED) for antibody detection (Kimpston-Burkgren, et al., 2020) and African swine fever (ASF) for antigen detection (Chen et al., 2021). AlphaLISA® uses fast mix-and-measure protocols that significantly reduce hands-on and total assay times. Further, AlphaLISA® was reported to provide lower inter-assay, batch-to-batch, and day-to-day variation (Beaudet et al., 2008; Peters et al., 2012). Specific strengths of the CSFV Erns IgG AlphaLISA® described in this study would include the following:

1. The AlphaLISA® was simple to perform and required a total turn-around time of 1 hour.
2. The AlphaLISA® was compatible with oral fluid specimens and the identical "no-wash" protocol was used for both serum and oral fluid specimens. This is important because oral fluids are easily collected, routinely used by pork producers and swine veterinarians, and would facilitate surveillance in the event of a CSFV incursion.

Notably, the use of samples that are more easily and rapidly collected in the field, i.e., oral fluids, and are compatible with high-throughput testing, e.g., oral fluid have been reported to be detected CSFV-E2 and Erns specific antibodies (Panyasing et al., 2018). The detectable levels of CSFV specific antibody generally appear 10-15 days after exposure (Moennig, 2000; Panyasing et al., 2018).

In conclusion, we developed and optimized an AlphaLISA® for the detection of CSFV Erns IgG in swine serum and oral fluid. The assay provided excellent diagnostic sensitivity and specificity and the strong potential for a DIVA strategy. In the future, repeatability, reproducibility, and cross-reactivity with other pestiviruses and other pathogens of swine should be evaluated.

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