RESEARCH REPORT
SWINE HEALTH

Title: Development and validation of two anti-CSFV-specific antibody competitive ELISAs with an emphasis on the differentiation of infected from C-strain vaccinated animals - **NPB #18-059**

Investigator: Dr. Jishu Shi

Institution: Kansas State University

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Industry Summary:

Classical swine fever (CSF) is a highly contagious swine viral disease. To date, CSF is still listed as a highly contagious swine viral disease by the World Organization for Animal Health (OIE). An outbreak of CSF impairs domestic and international trade and the movement of pigs and pig products. C-strain vaccines are considered to be safe and effective against all CSFV genotypes, and are widely used for CSF control worldwide. However, no corresponding serological DIVA (differentiate infected from vaccinated animals) test for C-strain vaccine is available, which hampered the application of C-strain based DIVA approach in CSF control.

In this project, to make the well-known safe and efficacious C-strain vaccine DIVA compatible, two competitive ELISAs (cELISA) with an emphasis on the differentiation of infected from C-strain vaccinated animals were developed and validated. After comprehensive assessment and validation by Western blotting, indirect fluorescent antibody assay (IFA) and testing different categories of pig sera, Horseradish Peroxidase (HRP)-mAb 1504 (against C-strain E^{rns} protein) based cELISA showed very high specificity, sensitivity and reproducibility. The established HRP-mAb 1504 based cELISA can efficiently differentiate C-strain or C-strain E^{rns} immunized pigs from other CSFVs or other viruses (PRRSV, PRV, BVDV) infected pigs. It can detect C-strain induced antibodies as early as 7-14 days post vaccination (DPV) with neutralizing titer 1:5 to 1:15. The diagnostic sensitivity and specificity of the cELISA were 100% (95% confidence interval: 91.2 to 100%) and 100% (95% confidence interval: 99.3 to 100%), respectively.

The novel HRP-mAb 1504 based cELISA established in this project is a valuable tool for measuring and differentiating immune responses to C-strain vaccination in pigs. It is applicable as an accompanying DIVA assay to C-strain originated vaccines (live-attenuated or E2 subunit), can be safely manufactured in the United States and will facilitate the implementation of “vaccination to live” strategy for CSF outbreak control.

Keywords: Classical swine fever (CSF), C-strain, DIVA, competitive ELISA (cELISA), E^{rns} protein, monoclonal antibody

Contact information: Dr. Jishu Shi, (785) 532-4506, jshi@ksu.edu, Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, 232 Coles Hall, 1620 Denison Ave, Manhattan, KS, 66506.

Scientific Abstract:

Introduction

Currently, CSF continues to cause severe economic losses to the swine industry worldwide. The conventional Chinese vaccine strain (C-strain) is the most frequently used vaccine for CSF control and prevention, and has showed outstanding efficacy and safety after many years of use. However, no reliable DIVA (differentiate pigs infected with wildtype CSFV from vaccinated animals) diagnostic assay is available for use in conjunction with C-strain vaccination. In this study, we developed and validated two competitive ELISAs (cELISA) with an emphasis on the differentiation of infected from C-strain vaccinated animals.

Method

For generating suitable capture recombinant antigens, E2 protein and E^{rns} protein of C-strain were expressed in insect cells by using Bac-to-Bac® Baculovirus Expression System. For generating suitable competitive monoclonal antibodies (mAbs), purified E2 and E^{rns} of C-strain were used as immunogens to inject Balb/c mice. The generated mAbs were comprehensively assessed by testing insect cell expressed E2 or E^{rns} proteins of CSFVs from genotypes:1.2,1.3,1.4, 2.1a, 2.1b, 2.1c, 2.1g, 2.1h, 2.1i, 2.1j, 2.2, 2.3, 3.1, 3.2, 3.4) using Western blotting, by testing CSFVs (n=111) from genotypes: 1.1, 2.1a, 2.1b, 2.1c, 2.1g, 2.1h, 2.1i, 2.1j, 2.2, 2.3 and testing BVDVs from genotypes: 1 and 2 using indirect fluorescent antibody assay (IFA). cELISAs were established based on the strategy that C-strain specific mAbs will compete with the C-strain vaccine induced antibodies in the pig serum to bind the capture antigens (C-strain E2 or C-strain E^{rns}). The cELISAs were optimized and were further evaluated by testing different categories of pig sera.

Results

i) C-strain E2 and E^{rns} proteins were successfully expressed. The purified native C-strain E2 protein mainly existed as homodimer. The purified native C-strain E^{rns} protein existed as homodimer and monomer; **ii)** Two panels of mAbs were generated. One mAb against E2 protein and one mAb against E^{rns} showed the best differential characteristics; **iii)** Two cELISAs based on these two mAbs were developed. After comprehensive assessment and validation, the HRP-mAb 1504 (against C-strain E^{rns} protein) based cELISA showed high specificity, sensitivity and reproducibility; **iv)** The established HRP-mAb 1504 based cELISA can efficiently differentiate C-strain or C-strain E^{rns} immunized pigs from other CSFVs or other viruses (PRRSV, PRV, BVDV) infected pigs. It can detect C-strain induced antibodies as early as 7-14 days post vaccination (DPV) with neutralizing titer 1:5 to 1:15. The diagnostic sensitivity and specificity of the cELISA were 100% (95% confidence interval: 91.2 to 100%) and 100% (95% confidence interval: 99.3 to 100%), respectively.

Conclusion

The novel HRP-mAb 1504 based cELISA established in this project is a valuable tool for measuring and differentiating immune responses to C-strain vaccination in pigs. It is applicable as an accompanying DIVA assay to C-strain originated vaccines (live-attenuated or E2 subunit), can be safely manufactured in the United States and will facilitate the implementation of “vaccination to live” strategy for CSF outbreak control.

Keywords: Classical swine fever (CSF), C-strain, DIVA, competitive ELISA (cELISA), E^{rns} protein, monoclonal antibody

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Introduction:

Despite the enormous control efforts, classical swine fever (CSF) continues to cause significant economic losses to the swine industry worldwide and remains as an important agro-security threat to CSF free countries such as the United States. Outbreaks and heavy losses have occurred in other parts of the world where CSF was eradicated in the last few decades. The legal movements of live animals or their products, byproducts, or animal feed, the illegal movement of live animals and their products, or an intentional viral release in an act of bioterrorism are all channels through which CSF is likely to occur in the U.S. United States must be well prepared to protect its swine industry from the threat of either an inadvertent or deliberate introduction of CSF.

C-strain derived vaccines are considered to be safe and effective against all genotypes of CSF viruses, and are widely used for CSF control globally. If a CSF outbreak were to occur in the U.S., emergency vaccination with the C-strain vaccine is preferred to a large-scale pre-emptive culling for controlling outbreaks of CSF. However, because C-strain companioning DIVA (differentiate infected from vaccinated animals) test is not commercially available right now, C-strain vaccinated pigs would eventually be treated as CSFV-infected pigs and culled. The currently available serological DIVA assays of CSF are designed for E2-subunit vaccines and cannot be used for C-strain vaccines due to their intrinsic deficiency in sensitivity and specificity. Thus, reliable C-strain compatible DIVA diagnostic assays are critically needed in order to keep C-strain vaccinated pigs in the production cycle after a CSF outbreak in CSF free countries such as the U.S.

Objectives:

Objective 1: Development and optimization of C-strain specific cELISA systems;

Objective 2: Validation of the optimized C-strain specific cELISA systems.

Materials & Methods:

Generation of monoclonal antibody to C-strain E2 and E^{rns} protein

C-strain E2 and E^{rns} protein were expressed by using the baculovirus expression system. The expressed proteins were purified using Ni-NTA Agarose (Novex™), were concentrated using Amicon Ultra Centrifugal Filters 30,000 NMWL (Millipore, Billerica, USA) and were measured using BCA assay kit (Pierce, USA) according to the manufacture's recommendations.

Animal care and protocols of monoclonal antibody generation were approved by Institutional Animal Care and Use Committee (IACUC#3517) at Kansas State University. Briefly, 50 µl (1µg/µl) purified E2/E^{rns} protein plus equal volume of 2% Alhydrogel (Invitrogen, CA, USA) was used as an immunogen to inject each of five female Balb/c mice (purchased from Charles River Laboratories, Inc. Wilmington, MA, U.S.A) via intraperitoneal injection for mAb production. Three booster immunizations with same dose were conducted at two week intervals. Three days after the final booster injection, the mice were euthanized and spleen cells were fused with the mouse myeloma partner SP2/0-Ag14 (ATCC, MD, *USA*) by using polyethylene glycol 1500 (Boehringer Mannheim, IN, USA) at a ratio of 10:1. The hybridoma cells were maintained in

RPMI1640 medium (Gibco, NY, USA) with 20% fetal bovine serum (FBS, Hyclone, UT, USA). Supernatants from growing hybridomas were screened by ELISA. The positive hybridoma clones were subcloned three times by limiting dilution until monoclones were obtained. Isotyping was performed with an antibody-isotyping kit (Roche Diagnostics Corporation, IN, USA).

Indirect fluorescent antibody assay (IFA) test

The reactivity of mAbs with different CSFVs and BVDVs was tested by IFA. Briefly, PK-15 cells (porcine kidney cell) grown in 96-well plate were infected with viruses (CSFVs and BVDVs) at a MOI of 0.1 for 3 days, respectively. Cells were fixed in cold acetone and washed two times with cold phosphate buffered saline (PBS). Supernatant of hybridomas (1:50 diluted) was added and plates were incubated at 37℃ for 1 hour (hr). Plates were washed three times with PBS and Alexa Fluor 488 goat anti-mouse IgG (H+L) (Life Technologies, MA, USA) was added at 1:200 dilution to each well and incubated at 37℃ for 1 hr. Finally, the plate was washed three times with PBS and examined under a fluorescence microscope.

Western blotting

Insect cell expressed CSFV E2 or E^{rns} proteins (0.1μg) from different CSFVs were mixed with SDS loading buffer (Bio-Rad, CA, USA) and were subjected to Mini-Protean TGX Gels (Bio-Rad, CA, USA). Proteins were electrotransferred to Amersham Protran NC nitrocellulose Western blotting membranes (GE Healthcare Bio-Sciences, PA, USA). The membranes were probed with different mAbs followed by a secondary Horseradish Peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc. PA, USA). After three washes with PBS containing 0.05% Tween 20 (PBST), reactive proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, NJ, USA).

Competitive enzyme-linked immunosorbent assay (cELISA)

The mAbs were purified by HiTrap™ Protein G column (GE Healthcare Life Sciences, PA, USA) followed by conjugating with Horseradish Peroxidase (HRP) using EZ-Link™ Plus Activated Peroxidase (Thermo Scientific, NJ, USA) according to the manufacturer's instruction. The HRP-mAbs were dialyzed with Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, NJ, USA) against PBS and stored in Pierce™ Peroxidase Conjugate Stabilizer (Thermo Scientific, NJ, USA).

The systematic checkerboard procedure was used to optimize the concentration of capture antigen and HRP-mAbs. The optimal dilution of serum and blocking solution were determined experimentally. The established cELISA was performed in Corning® 96 Well Clear Flat Bottom Polystyrene High Bind Microplate (Corning, NY, USA). Briefly, plates were coated overnight with antigens (100μl/well) in PBS (without calcium and magnesium, pH7.4, Thermo Scientific, NJ, USA) at 4°C. After washing three times with washing buffer (PBS containing 0.05% Tween 20, PBST), the plates were blocked with blocking buffer by incubating at 37°C 1 hr; After washing, 50 μl of diluted serum samples and 50 μl of diluted HRP-mAbs were added to each well and mixed well by pipetting. The plates were incubated at 37°C 1 hr. After five times washing, 100 μl of room-temperature TMB Stabilized Chromogen (Invitrogen, CA, USA) were added and incubated at room temperature (RT) for 10 min; after adding 100 μl/well of 2N Sulfuric Acid (Ricca Chemical Company, TX, USA), the absorbance at 450 nm were obtained using SpectraMAX microplate reader (Molecular Devices, CA, USA). The OD450 of the samples were converted to a percent inhibition (PI) value using the following formulation: PI (%) = (OD450 value of negative controls − OD450 value of sample)/OD450 value of negative controls × 100%.

The cut-off value that served as the threshold to separate positive from negative sera was determined by testing negative sera of unvaccinated pigs and positive sera of C-strain or C-strain E^{rns} vaccinated pigs.

Reproducibility and statistical analysis of the cELISA

Inter-assay and intra-assay reproducibility for the established cELISA was evaluated by testing CSFV antibody negative (n=20) serum samples and CSFV antibody positive samples (n=20). For the intra-assay reproducibility, each serum sample (in duplicate) was detected by the same batch of pre-coated ELISA plates. For the inter-assay reproducibility, each serum sample was detected by three batches of pre-coated ELISA plates. Statistical analysis of

reproducibility was carried out by calculate the mean PI value and coefficient of variation (CV) of replications of each test.

Results:

Objective 1: Development and optimization of C-strain specific cELISA systems;

1) Expression of suitable capture recombinant antigens

For developing C-strain cELISAs as accompanying C-strain DIVA assays that can be safely manufactured and used in the United States, E2 protein and E^{rns} protein of C-strain CSFV (as capture recombinant antigens of cELISAs) were expressed in insect cells by using Bac-to-Bac® Baculovirus Expression System. The expressed E2 protein and E^{rns} protein of C-strain were purified by using the His-tag/Ni-NTA system. The purified native C-strain E2 protein mainly existed as homodimer under non-reducing conditions with a molecular weight of ~90 kDa (Figure 1). The purified native C-strain E^{rns} protein existed as homodimer and monomer under non-reducing conditions with a molecular weight of ~74 kDa and 37 kDa, respectively (Figure 1).

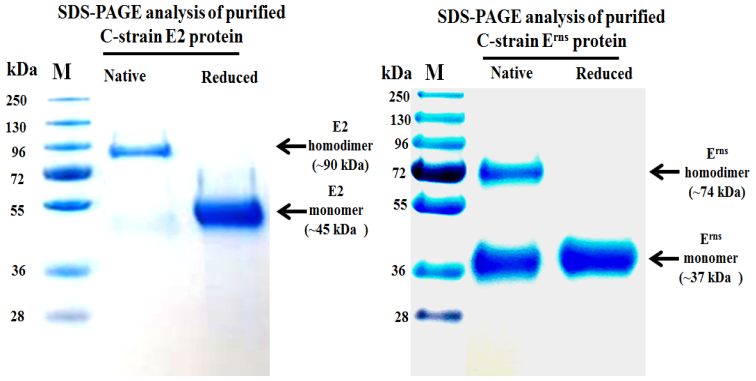


Figure 1. SDS-PAGE analysis of insect cell expressed E2 protein and E^{rns} protein of C-strain. Reduced : E2 or E^{rns} protein treated with Laemmli sample buffer with addition of reducing reagent β-mercapto-ethanol (β-ME); Native: E2 or E^{rns} protein treated with Laemmli sample buffer without β-ME.

2) Generation of suitable competitive monoclonal antibodies

For generating suitable competitive monoclonal antibodies, purified E2 and E^{rns} protein of C-strain were used as immunogens to inject Balb/c mice for monoclonal antibody (mAb) production. Two panels of mAbs against E2 and E^{rns} protein were generated, respectively. After assessment by ELISA, western blotting and indirect fluorescent antibody assay (IFA), one mAb (6B211) against E2 protein and one mAb (1504) against E^{rns} showed differential characters i.e. could recognize C-strain E2 or E^{rns}, but not react with other CSFVs (Figure 2) or BVDVs in genus *Pestivirus* (Figure 3). The mAb 6B211 and mAb 1504 are IgG1 (light chain: kappa) isotype. Both mAbs target conformational epitopes (Figure 4) and showed very high sensitivity (could get positive fluorescence signal at dilution 1:16,000 for ascites when tested by IFA).

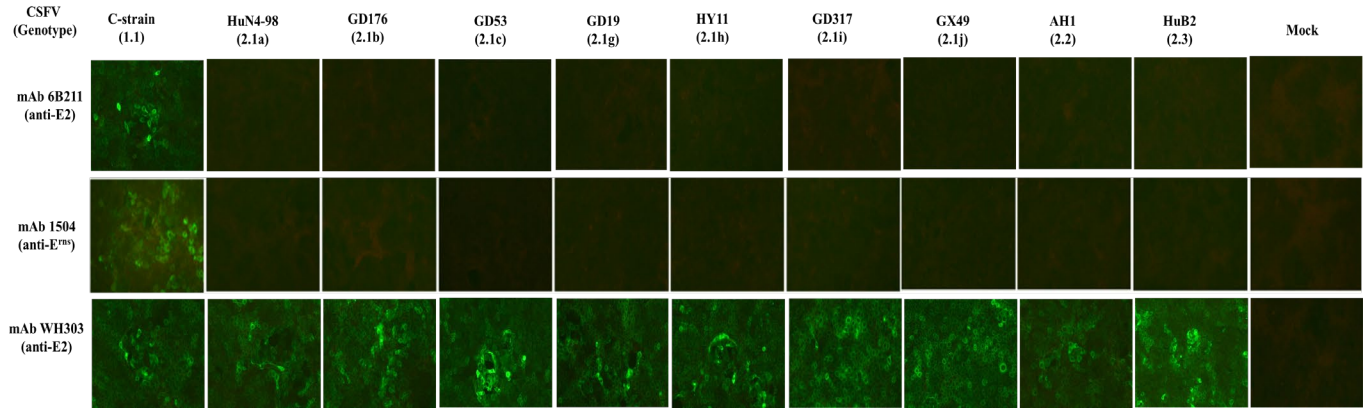


Figure 2. IFA testing the specificity of mAb 6B211 and mAb 1504. Cells: PK-15; Inoculated viruses: CSFV isolates (genotype.subgroup); 3 days post infection (DPI).

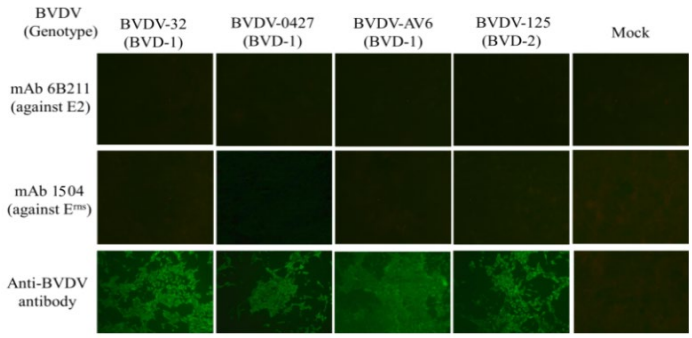


Figure 3. mAb 6B211 and mAb 1504 do not have cross reactivity to bovine viral diarrhea virus (BVDV) by IFA testing. Cells: PKWRL; inoculated viruses: BVDV-32 (genotype 1), BVDV-0427 (genotype 1), BVDV-AV6 (genotype 1), and BVDV-125 (Genotype 2). 3 days post infection (DPI).

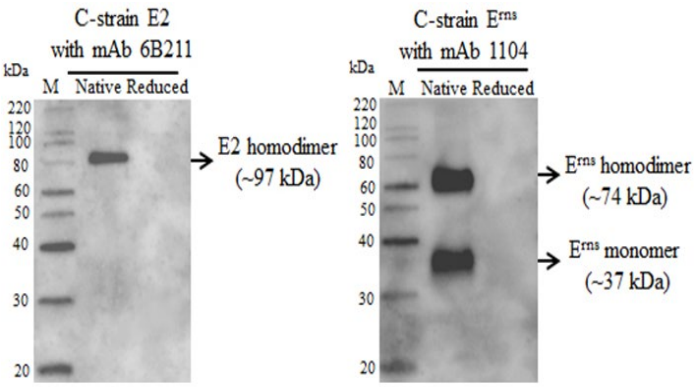


Figure 4. mAb 6B211 and mAb 1504 recognize conformational epitopes of CSFV proteins by western blotting analysis. Insect cell expressed CSFV E2 or E^{rns} protein treated without (Native) or with (Reduced) β-mercaptoethanol. None of the mAbs could recognize the reduced proteins, which indicate that these mAbs recognize conformational epitopes.

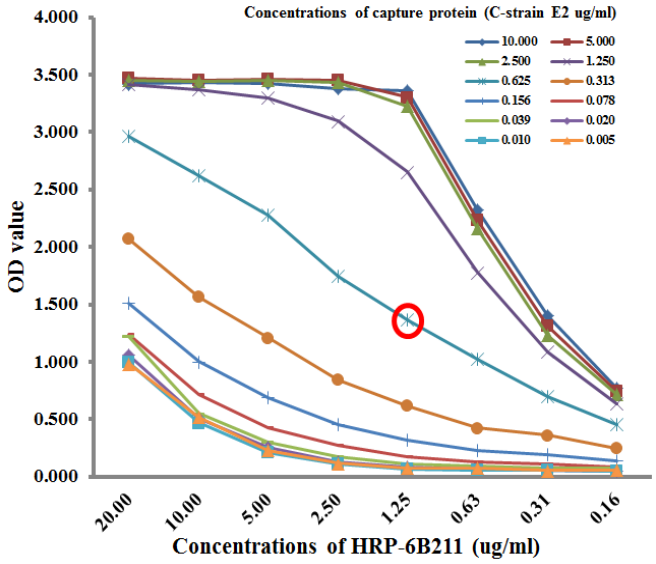
3) Purify and label mAbs with horseradish peroxidase (HRP)

Hybridomas of 6B211 and 1504 were scale up cultured. Supernatant from cultured hybridomas of 6B211 and 1504 were harvested. mAbs were successfully purified by using the HiTrap™ Protein G HP (GE Healthcare Bio-Sciences Corp). The purified mAbs were dialyzed in PBS. The concentration of the purified mAbs were measured by Pierce BCA Protein Assay Kit. Then the mAbs were labeled by HRP with EZ-Link™ Plus Activated Peroxidase kit and dialyze with Slide-A-Lyzer Dialysis Cassettes.

4) Determine optimal concentrations of the coating antigen and HRP-mAbs

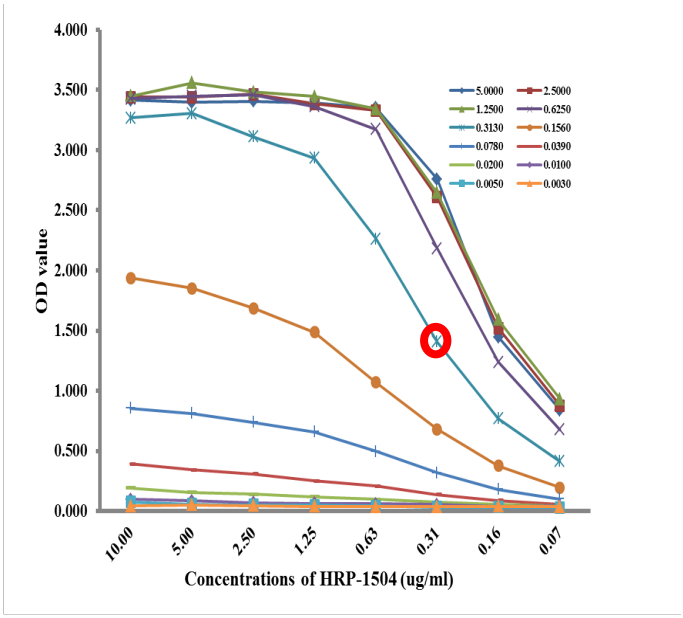
The optimal concentrations of antigen (C-strain E2) and detection mAb (HRP-6B211) were determined by means of checkerboard titration (Figure 5): Considering strong signal versus low background and use as few as possible for the capture proteins, 0.625μg/ml for capture protein (100μl/well) and 1.25μg/ml (50ul/well) for detection mAb were selected for cELISA.

Figure 5. Determination of the optimal concentrations of the coating antigen (C-strain E2) and HRP-mAb (HRP-6B211). ELISA plate was coated with different concentrations of capture proteins (C-strain



The optimal concentrations of antigen (C-strain E^{rns}) and detection mAb (HRP-1504) were determined by means of checkerboard titration (Figure 6): Considering strong signal versus low background and use as few as possible for the capture proteins, 0.31μg/ml for capture protein (100μl/well) and 0.31μg/ml (50μl/well) for detection mAb were selected for cELISA.

Figure 6. Determination of the optimal concentrations of the coating antigen (C-strain E^{rns}) and HRP-mAb (HRP-1504). ELISA plate was coated with different concentrations of capture proteins (C-strain E^{rns}, 100μl/well) overnight; After washing, the plate was blocked with 2% FBS (in PBST) by incubating at 37℃ 1 hour; After washing, 50 μl of different concentrations of detecting Ab (HRP-1504) were added to each well. The plates were incubated at 37℃ 1 hour. After washing, 100 μl of room-temperature TMB Stabilized Chromogen were added and incubated at RT for 10 min; after adding 100 μl/well of 2N Sulfuric Acid, the absorbance at 450nm were obtained using SpectraMAX microplate reader.



5) Select optimal blocking buffer

Different blocking buffers (nonfat milk, FBS and BSA) were tested with C-strain vaccinated swine serum samples and negative swine serum samples by cELISAs (Table 1). Considering high ratio value of (Negative serum OD)/(C-strain vaccinated serum) and low background, 2%FBS in PBST was selected as the optimal blocking buffer for cELISAs.

Table 1. Selection of optimal blocking buffer by testing different blocking buffers.

	1	2	3	4	5	6	7	8	9	10	11	12	
HRP-6B211	**anti-E2 positive serum (327, 1:5)**	**anti-E2 positive serum (327, 1:10)**	**anti-E2 positive serum (327, 1:50)**	**anti-E2 positive serum (327, 1:100)**	**anti-E2 negative serum (355, 1:5)**	**anti-E2 negative serum (355, 1:10)**	**anti-E2 negative serum (355, 1:50)**	**anti-E2 negative serum (355, 1:100)**	**Blocking buffer**	**Blocking buffer**	**PBS**	**PBS**	
1.25ug/ml HRP-6B211 100ul/well	0.184	0.296	0.503	0.577	0.668	0.701	0.705	0.657	0.663	0.671	0.667	0.618	**Blocked by 2%FBS in PBST**
	0.171	0.295	0.578	0.602	0.624	0.680	0.706	0.676	0.687	0.674	0.676	0.682	
	0.254	0.424	0.645	0.699	0.725	0.758	0.750	0.741	0.746	0.753	0.745	0.747	Blocked by 3% BSA in PBST
	0.259	0.433	0.696	0.765	0.764	0.730	0.784	0.759	0.693	0.676	0.738	0.743	
	0.400	0.580	0.865	0.915	1.120	1.126	1.057	1.093	1.060	1.078	1.115	1.070	Blocked by 5% nonfat milk in PBST
	0.420	0.568	0.943	0.913	1.189	1.179	1.164	1.097	1.084	1.063	1.074	1.104	

Note: Capture protein: 0.625μg/ml C-strain E2; Detection mAbs: 1.25μg/ml HRP-mAb6B211; mixed 50μl HRP-mAb6B211 and 50μl diluted serum/blocking buffer/PBS were disperse in each well of the detection plate.

6) Determine optimal dilutions of serum samples

The optimal dilution of serum was determined by testing different dilutions of C-strain vaccinated serum and non-vaccinated serum (Figure 7&8). Considering high ratio value of (Negative serum OD)/(C-strain vaccinated serum), low background and using less serum, 1:5 dilution of serum was selected for cELISAs.

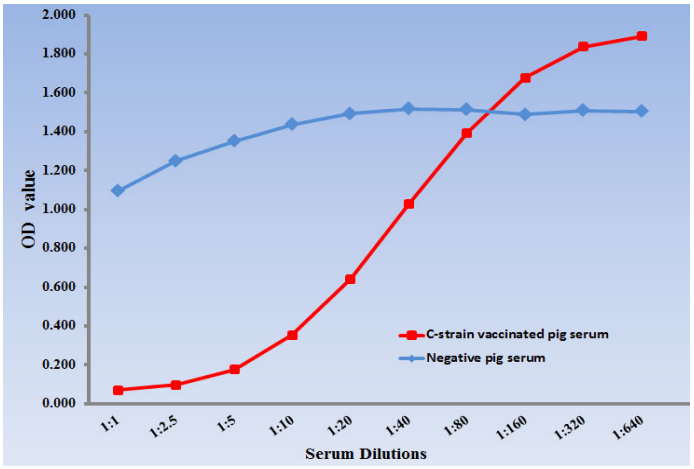


Figure 7. mAb 6B211 based cELISA could differentiate C-strain vaccinated pigs from non-vaccinated pigs over broad range of serum dilutions. Insect cell expressed C-strain E2 were used as capture proteins. 50μl horseradishperoxidase (HRP)-conjugated mAbs compete with the C-strain induced specific antibodies in 50 μl diluted pig serum to bind the capture proteins. The higher the C-strain specific antibody concentration in the pig serum, the lower OD value will be obtained. Pig serums were collected on 56 days post vaccination (DPV).

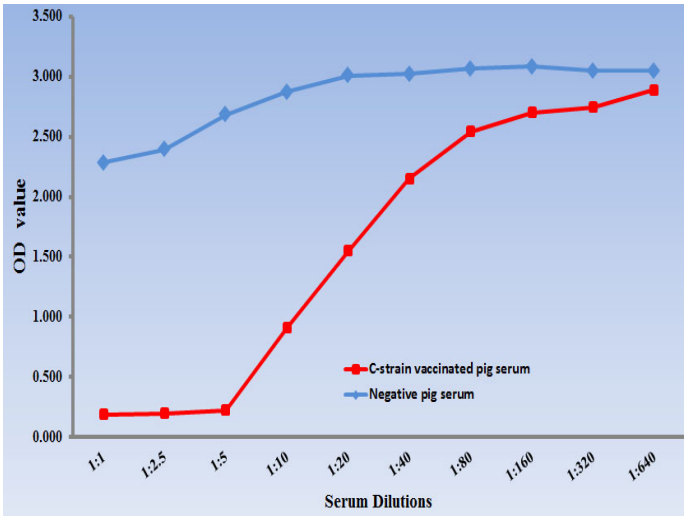


Figure 8. mAb 1504 based cELISA could differentiate C-strain vaccinated pigs from non-vaccinated pigs over broad range of serum dilutions. Insect cell expressed C-strain E^{rns} were used as capture proteins. 50μl horseradishperoxidase (HRP)-conjugated mAbs compete with the C-strain induced specific antibodies in 50 μl diluted pig serum to bind the capture proteins. The higher the C-strain specific antibody concentration in the pig serum, the lower OD value will be obtained. Pig serums were collected on 56 days post vaccination (DPV).

Objective 2: Validation of the optimized C-strain specific cELISA systems.

1) Comprehensive validate the specificity of mAb6B211 and mAb1504 by IFA

The reactions between mAb 6B211/mAb 1504 and 111 CSFVs (genotypes 1.1, 2.1, 2.2 and 2.3) were tested by IFA. The results showed that mAb6B211 can recognize some strains from CSFV genotypes 1.1, 2.1c and 2.2. mAb1504 did not show any cross-reaction with any strains from different CSFV genotypes (Table 2).

Table 2. Specificity testing of mAb6B211 and mAb1504 by IFA

CSFVs and BVDVs	Genotyes	Monoclonal antibodies		
		mAb WH303	mAb 6B211	mAb 1504
CSFV_C-strain	1.1	++++	+++	+++
CSFV_Shimen	1.1	++++	+++	-
CSFV_XD-1-5	1.1	++++	++	-
CSFV_XD-1-6	1.1	++++	+++	-
CSFV_CN001	2.1a	++++	-	-
CSFV_CN021	2.1a	++++	-	-
CSFV_CN023	2.1a	++++	-	-
CSFV_CN079	2.1a	++++	-	-
CSFV_CN080	2.1a	++++	-	-
CSFV_CN147	2.1a	++++	-	-
CSFV_CN148	2.1a	++++	-	-
CSFV_CN174	2.1a	++++	-	-
CSFV_CN024	2.1b	++++	-	-
CSFV_CN025	2.1b	++++	-	-
CSFV_CN069	2.1b	++++	-	-
CSFV_CN081	2.1b	++++	-	-
CSFV_CN091	2.1b	++++	-	-
CSFV_CN093	2.1b	++++	-	-
CSFV_CN094	2.1b	++++	-	-
CSFV_CN096	2.1b	++++	-	-
CSFV_CN112	2.1b	++++	-	-
CSFV_CN115	2.1b	++++	-	-
CSFV_CN117	2.1b	++++	-	-
CSFV_CN118	2.1b	++++	-	-
CSFV_CN120	2.1b	++++	-	-
CSFV_CN123	2.1b	++++	-	-
CSFV_CN125	2.1b	++++	-	-
CSFV_CN139	2.1b	++++	-	-
CSFV_CN160	2.1b	++++	-	-
CSFV_CN169	2.1b	++++	-	-
CSFV_CN173	2.1b	++++	-	-
CSFV_CN153	2.1b	++++	-	-
CSFV_CN151	2.1c	++++	-	-
CSFV_CN032	2.1c	++++	-	-
CSFV_CN033	2.1c	++++	-	-

CSFV_CN034	2.1c	++++	-	-
CSFV_CN038	2.1c	++++	-	-
CSFV_CN041	2.1c	++++	-	-
CSFV_CN042	2.1c	++++	-	-
CSFV_CN044	2.1c	++++	-	-
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CSFV_CN046	2.1c	++++	-	-
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CSFV_CN101	2.1c	++++	-	-
CSFV_CN106	2.1c	++++	++	-
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CSFV_CN069	2.1h	++++	-	-
CSFV_CN077	2.1h	++++	-	-
CSFV_CN107	2.1h	++++	-	-
CSFV_CN111	2.1h	++++	-	-
CSFV_CN122	2.1h	++++	-	-
CSFV_CN124	2.1h	++++	-	-
CSFV_CN144	2.1h	++++	-	-
CSFV_CN152	2.1h	++++	-	-
CSFV_CN158	2.1h	++++	-	-
CSFV_CN163	2.1h	++++	-	-
CSFV_CN164	2.1h	++++	-	-
CSFV_CN171	2.1h	++++	-	-
CSFV_CN052	2.1h	++++	-	-
CSFV_CN142	2.1h	++++	-	-
CSFV_CN145	2.1h	++++	-	-
CSFV_CN063	2.1i	++++	-	-
CSFV_CN068	2.1j	++++	-	-
CSFV_CN002	2.2	++++	-	-
CSFV_CN004	2.2	++++	-	-
CSFV_CN005	2.2	++++	-	-
CSFV_CN006	2.2	++++	-	-
CSFV_CN007	2.2	++++	-	-
CSFV_CN008	2.2	++++	-	-
CSFV_CN011	2.2	++++	-	-

CSFV_CN014	2.2	++++	+++	-
CSFV_CN010	2.2	++++	+++	-
CSFV_CN013	2.2	++++	+++	-
CSFV_CN020	2.2	++++	-	-
CSFV_CN070	2.2	++++	-	-
CSFV_CN102	2.2	++++	+	-
CSFV_CN108	2.2	++++	++	-
CSFV_CN141	2.2	++++	-	-
CSFV_CN149	2.2	++++	-	-
CSFV_CN150	2.2	++++	-	-
CSFV_CN165	2.2	++++	-	-
CSFV_CN170	2.2	++++	-	-
CSFV_CN172	2.2	++++	+	-
CSFV_CN175	2.2	++++	-	-
CSFV_CN154	2.2	++++	+++	-
CSFV_CN103	2.2	++++	-	-
CSFV_CN016	2.3	++++	-	-
CSFV_CN017	2.3	++++	-	-
CSFV_CN018	2.3	++++	-	-
CSFV_CN019	2.3	++++	-	-
CSFV_CN071	2.3	++++	-	-
CSFV_CN072	2.3	++++	-	-
CSFV_CN073	2.3	++++	-	-
CSFV_CN075	2.3	++++	-	-
CSFV_CN076	2.3	++++	-	-
CSFV_CN078	2.3	++++	-	-
CSFV_CN105	2.3	++++	-	-
CSFV_CN140	2.3	++++	-	-
CSFV_CN157	2.3	++++	-	-
CSFV_CN162	2.3	++++	-	-
CSFV_CN071	2.3	++++	-	-
BVDV_32	1	-	-	-
BVDV_0427	1	-	-	-
BVDV_AV6	1	-	-	-
BVDV_125	2	-	-	-

Note: "++++" means very strong positive; "+++" means strong positive; "++" means positive; "+" means weak positive; "-"means negative.

To further validate the specificity of the mAb1504, E^{rns} protein from CSFV genotype 1.2, 1.3, 1.4, 2.1a, 2.1b, 2.1c, 2.1g, 2.1h, 2.1i, 2.1j, 2.2, 2.3, 3.1, 3.2, and 3.4 were expressed in insect cells. The reactivity between mAb 1504 and the purified E^{rns} proteins were tested by western-blotting (Figure 9). The results confirmed that mAb 1504 lacks cross reactivity to other CSFVs. Based on these results, mAb 1504 based cELISA was selected for following further validations.

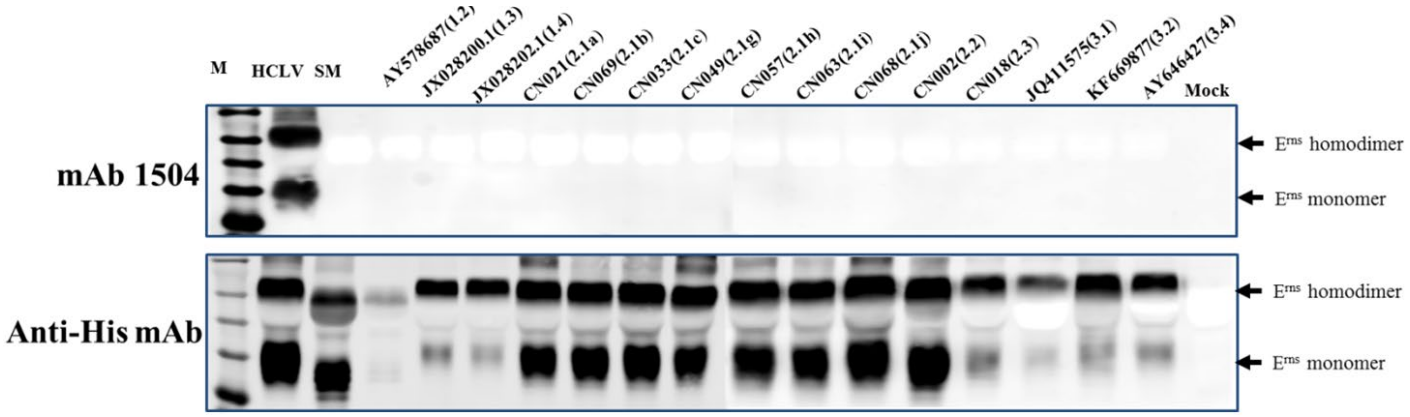

Figure 9. mAb 1504 lack cross reactivity to other CSFVs by WB testing. E^{rns} proteins of CSFVs were expressed in insect cells by using Bac-to-Bac® Baculovirus Expression System. Purified E^{rns} proteins (natural) were loaded on SDS-PAGE gels. The proteins were then transferred to PVDF and the membranes were blocked and incubated with mAb1504 and anti-His mAb, respectively.

2) Sensitivity analysis of HRP-1504 based cELISA by testing serial serum samples (from 0DPV to 56 DPV) of C-strain immunized pigs

Serial serum samples derived from 3 pigs vaccinated with C-strain were tested by the established cELISA. The results showed that HRP-1504 based cELISA can detect anti-C-strain antibodies between 7 DPV and 14 DPV (neutralizing titer 1:5 to 1:15) (Figure 10).

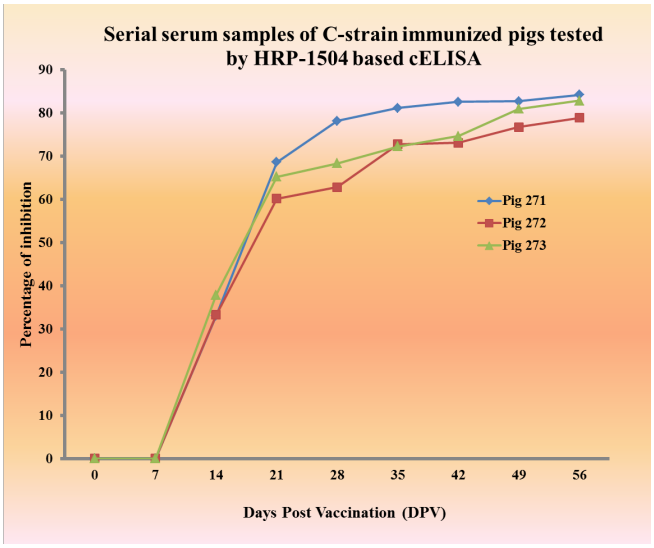

Figure 10. Sensitivity analysis of HRP-1504 based cELISA. Purified C-strain E^{rns} was used as the capture protein. 50µl HRP-conjugnated mAb 1504 mixed with 50 µl diluted (1:5) pig serum to bind the capture protein. C-strain MLV vaccinated pig (three pigs) serum samples were collected from 0DPV, 7DPV, 14DPV, 28DPV, 35DPV, 42DPV, 49DPV and 56 DPV. PI (%) = (OD450 value of negative control samples − OD450 value of sample)/OD450 value of negative control samples × 100 %.

3) Validate the cut-off value of HRP-1504 based cELISA

Total of 548 serum samples were used to validate the cut-off value of the established cELISA. Among them, 504 samples were CSFV antibody negative and 44 samples were C-strain E^{rns} antibody positive when tested by ELISA and VNT. After testing these samples by the established cELISA, distributions of the cELISA PI values showing the frequency of positive and negative samples are calculated and shown in Figure 11. The mean PI value (x-axis) of the negative sera detected by cELISA was -0.12 %. When mean PI of negative sera plus two SD was used as threshold, the sensitivity and specificity of the cELISA were 59.7% (95% confidence interval: 47.0 to 71.51%) and 100% (95% confidence interval: 99.23 to 100%), respectively. When mean PI of negative sera plus three SD was used as threshold, the

sensitivity and specificity of the cELISA were 100% (95% confidence interval: 91.2 to 100%) and 100% (95% confidence interval: 99.3 to 100%), respectively. Depending on the results, the mean PI of negative sera plus three SD was selected as the cut-off value.

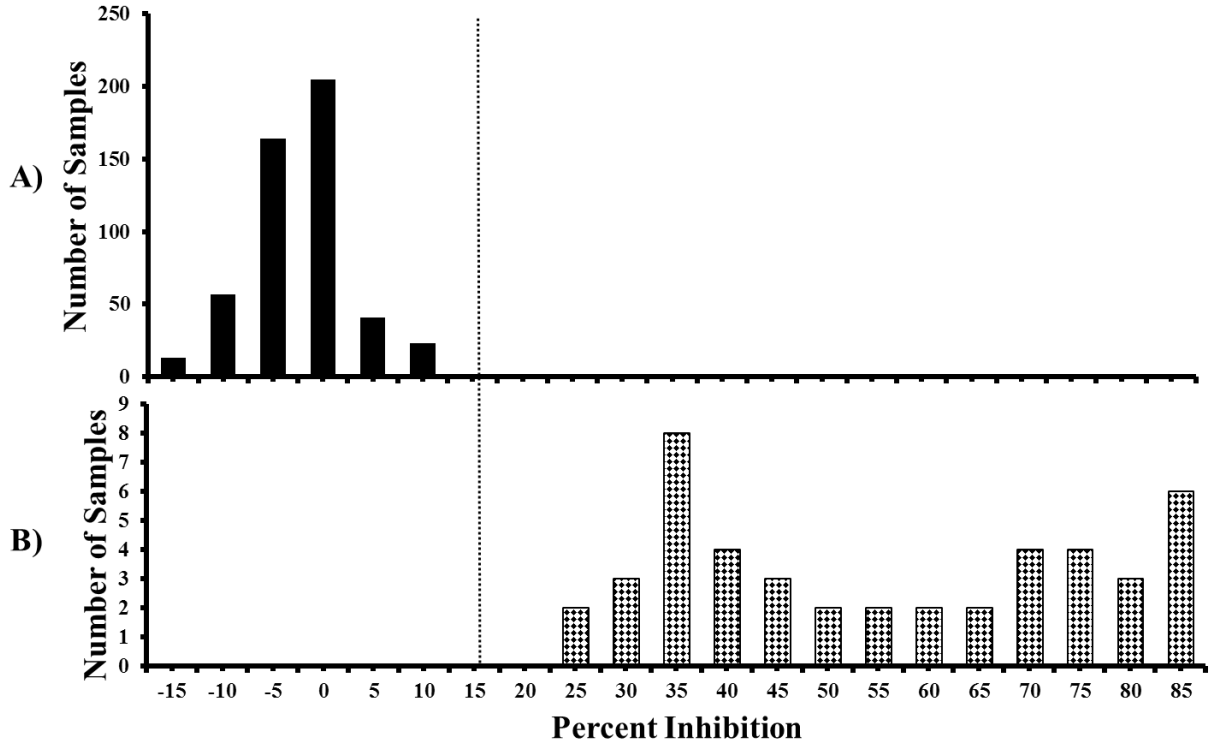

Figure 11. Validation of cut-off value of the cELISA. A) CSFV antibody negative serum samples (N=504); **B)** C-strain E^{rns} antibody positive serum samples (n=44). The dotted line represents cut-off value of 15.99% inhibition when using the mean PI of negative sera plus three SD as the threshold.

3) Reproducibility analysis of the cELISA

The reproducibility of the cELISA was determined by calculating the coefficient of variation (CV) of the PI values by testing 20 negative serum samples and 20 CSFV E^{rns} antibody positive serum samples. The intra-assay CVs of the 20 CSFV E^{rns} antibody positive samples ranged from 0.15 to 2.82%. The inter-assay CVs of those samples ranged from 1.30 to 8.79% (Table 3). The inter-assay and intra-assay of the 20 negative samples also exhibited excellent repeatability, showing 0.30–1.54% and 0.86–6.35%, respectively.

Table 3. Coefficient values of the samples tested by the cELSIA

Inhibition range (%)	No. of sera tested	CV range (%)	
		Intra-assay	Inter-assay
30-75	20	0.15-2.82	1.30-8.79
<5	20	0.30-1.54	0.86-6.35

4) Validate the HRP-1504 based cELISA by testing different categories of pig sera

Nine categories of pig serum samples were tested by the established HRP-1504 based cELISA. These include C-strain immunized pig serum samples (n=26), C-strain E^{rns} protein immunized pig serum samples (n=12), CSFV E2 immunized pig serum samples (n=191), CSFV Honduras challenged pig serum samples (n=59), CSFV Alford challenged pig serum samples (n=223), Porcine reproductive and respiratory syndrome virus (PRRSV) infected pig serum samples (n=107), Bovine Viral Diarrhea Virus (BVDV) neutralization positive control serum from National Veterinary Services Laboratories (n=2), Pseudorabies virus (PRV) antiserum from NVSL (n=1), PBS inoculated control pig serum samples (n=159). The results (Figure 12)

showed that HRP-1504 based cELISA can efficiently differentiate C-strain or C-strain E^{rns} immunized pigs from other CSFVs or other viruses (PRRSV, PRV, BVDV) infected pigs.

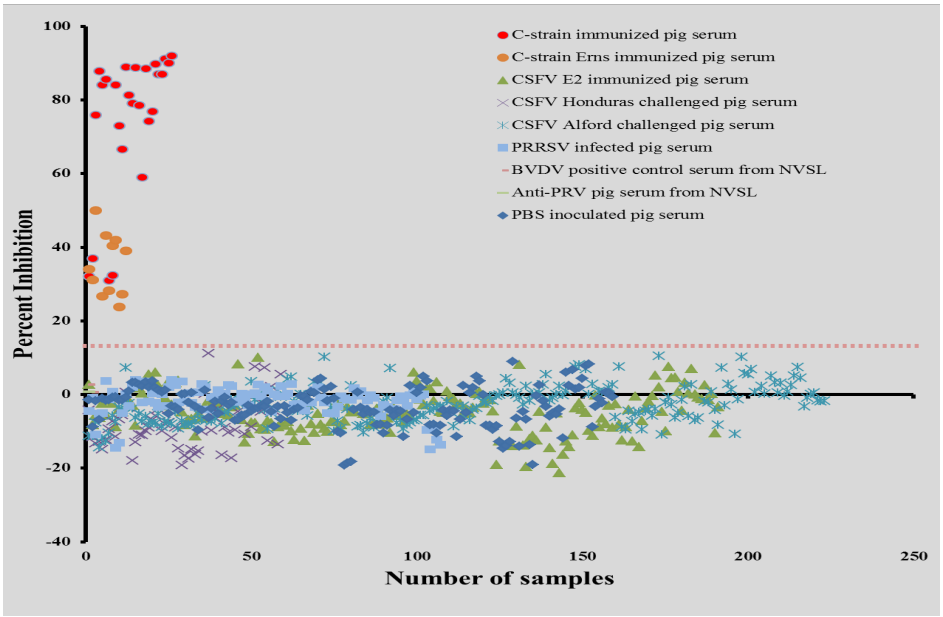

Figure 12. Validate the HRP-1504 based cELISA by testing different categories of pig sera. Purified C-strain E^{rns} (62.5ng) were used as capture proteins. 50µl HRP-conjugnated mAb 1504 (0.625µg/ml) mixed with 50 µl diluted (1:5) serum to bind the capture proteins. The OD450 of the samples were converted to a percent inhibition (PI) value using the following formulation: PI (%) = (OD450 value of negative controls − OD450 value of sample)/OD450 value of negative controls × 100%. Each sample was tested in duplicate. The dotted line represents cut-off value of 15.99 % inhibition when using the mean PI of negative sera plus three SD as the threshold.

5) Presentation

The 1st Annual Veterinary Diagnostic Conference; Shanghai, China; Jun 2-4, 2019; Poster: "Development of DIVA Diagnostic Assays for the Classical Swine Fever C-strain Vaccine".

Discussion:

Improved eradication strategies and emergency vaccination for controlling outbreaks of CSF are dependent on an effective vaccine and a reliable accompanying diagnostic assay that allows DIVA. C-strain vaccine is considered to be safe and effective against all genotypes, and is widely used for CSF control worldwide. However, it is not a DIVA vaccine currently. A reliable C-strain compatible DIVA diagnostic assay is critically needed in order to keep C-strain vaccinated pigs in the production cycle after a CSF outbreak in CSF free countries such as the U.S. Competitive ELISA assays have been shown previously to increase specificity and reduce the cross-reactivity. In this study, we developed two cELISAs with an emphasis on the differentiation of infected from C-strain vaccinated animals. cELISAs were established based on the strategy that C-strain specific mAbs will compete with the C-strain vaccine induced antibodies in the pig serum to bind the capture antigens (C-strain E2 or C-strain E^{rns}).

After comprehensive assessment and validation by western blotting, IFA and testing different categories of pig sera, we found that mAb 6B211 showed cross-reactivity with several strains from CSFV genotypes 1.1, 2.1c and 2.2. In contrast, HRP-mAb 1504 (against C-strain E^{rns} protein) based cELISA showed very high specificity, sensitivity and reproducibility. The HRP-mAb 1504 based cELISA can efficiently differentiate C-strain or C-strain E^{rns} immunized pigs from other CSFVs or other viruses (PRRSV, PRV, BVDV) infected pigs. It can detect C-

strain induced antibodies as early as 7-14 days post vaccination (DPV) with neutralizing titer 1:5 to 1:15. The diagnostic sensitivity and specificity of the cELISA were 100% (95% confidence interval: 91.2 to 100%) and 100% (95% confidence interval: 99.3 to 100%), respectively. The intra-assay CVs and the inter-assay CVs of the HRP-mAb 1504 based cELISA are lower than 10% when tested the negative and E^{rns} antibody positive pig serum samples, which indicate that the established cELSIA is repeatable with acceptable variations.

The novel HRP-mAb 1504 based cELISA established in this project is a valuable tool for measuring and differentiating immune responses to C-strain vaccination in pigs. It is applicable as an accompanying DIVA assay to C-strain originated vaccines (live-attenuated or E2 subunit), can be safely manufactured in the United States and will facilitate the implementation of "vaccination to live" strategy for CSF outbreak control.