Original Article

Correlation of Classical Swine Fever (CSF) Antibody Protective Level Detected by Enzyme-Linked Immunosorbent Assay and Serum Neutralization Test

Chairani Ridha Maghfira[1] Nguyen Thanh Che[1] Apisit Kittawornrat[2]
Roongroje Thanawongnuwech[3] Yaowalak Panyasing[3*]

Abstract

Protective antibody against classical swine fever virus (CSFV) is an important parameter used for disease monitoring in CSFV-endemic areas. Enzyme-linked immunosorbent assay (ELISA) is a simple and practical serological assay for CSFV antibody detection. However, the use of CSFV ELISAs in CSFV antibody-positive herds, i.e., CSFV-vaccinated or -previously infected herds, was limited by an application of the test results. This study aimed to evaluate the correlation and estimation of protective antibody levels detected by ELISA and serum neutralization assay. A total of 522 negative and positive serums were tested by SN and ELISA in parallel. Comparisons of sample-to-positive (S/P) values among the level of SN titers, correlation, and agreement between two assays were evaluated. There were statistically significant differences (p<0.001) between the mean S/P values among three distinct levels of SN titers, i.e., negative (SN titer <2), below protective level (SN titer <32), and at protective level (SN titer ≥32). There was a strong positive relationship (rs = 0.89; p<0.001) and excellent agreement between the S/P values and SN titer (Kappa value = 0.91). The correlated S/P values at 1.767±0.479 are suggested to be at the protective level. Therefore, ELISA S/P results could provide an estimation of the protective antibody levels that correlated with the serum neutralization assay.

Keywords: Antibody, Classical Swine Fever, Correlation, ELISA, Serum Neutralization

[1]*The International Graduated Program of Veterinary Science and Technology (VST), Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*CPF (Thailand) Public Company Limited, Bangkok, 10120 Thailand*
[3]*Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand*
Correspondence: *yaowalak.p@chula.ac.th* (Y. Panyasing)

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Introduction

Classical swine fever (CSF) is an important viral disease that has a serious impact on the swine industry worldwide (WOAH, 2022b). Serologically, antibody detection against classical swine fever virus (CSFV) is used for disease diagnosis, surveillance, monitoring, and evaluating immune status post-vaccination. Serological assays for CSFV diagnosis in different regions were determined by CSFV-disease status. For instance, the serum neutralization (SN) test is generally implemented in CSFV-endemic areas for detecting herd protective immunity (Moser *et al.*, 1996; Santana-Rodríguez *et al.*, 2022; WOAH, 2022b), whereas enzyme-linked immunosorbent assay (ELISA) is frequently used in CSFV-free areas for surveilling and monitoring the herd.

CSFV antibodies that confer protective immunity are induced by E2 and Erns glycoproteins (König *et al.*, 1995). The serum neutralization test is considered a gold standard and a confirmatory test for CSFV antibodies (Moser *et al.*, 1996; Vengust *et al.*, 2006). CSFV protective immunity was correlated with the level of neutralizing antibody titers measured by the SN test (Moormann *et al.*, 2000; Santana-Rodríguez *et al.*, 2022; WOAH, 2022a). The antibody SN titers were detected to monitor the herd's immune status and determine the protective antibody after vaccination (Sailasuta *et al.*, 2006). Pigs with SN titer $\geq$32 (the $\log_2$ of antibody titer $\geq$5) are considered to provide the least adequate protection to the individual pig and the herd population (Terpstra and Wensvoort, 1988). Maternal-derived antibody SN titers $\geq$64 may inhibit the efficacy of vaccination (Suradhat and Damrongwatanapokin, 2003), whereas SN titers <4 is the proper time for CSF vaccination (Direksin *et al.*, 2016). However, the SN test is a time-consuming procedure that requires cell culture and live virus manipulation (Moser *et al.*, 1996; Vengust *et al.*, 2006); therefore, this assay has a limitation for testing with a high number of samples.

CSFV antibody ELISA is a simple and rapid serological assay used for the detection of CSFV-specific antibodies in various sample types, i.e., serum (Meyer *et al.*, 2017) and oral fluids (Panyasing *et al.*, 2018). Several CSFV antibody ELISAs were developed to detect antibodies against CSFV E2 and Erns protein. The E2 antibody ELISA is frequently used to monitor CSFV infection during and after outbreaks and test after vaccination with conventional attenuated or E2 subunit vaccines. CSFV Erns antibody ELISA is established for differentiating infected and vaccinated animals (DIVA), targeted to the detection of antibodies against Erns glycoprotein (Schroeder *et al.*, 2012; Pannhorst *et al.*, 2015; Meyer *et al.*, 2017; Wang *et al.*, 2020a; WOAH, 2022a), and used as a companion diagnostic test to identify CSFV-infected pigs and pigs vaccinated with the E2-based subunit or marker vaccines (Meyer *et al.*, 2017).

The use of antibody ELISA in CSFV-positive herds is limited due to the lack of useful, informative data for protective immunity. A stand-alone ELISA for monitoring herd status against CSFV has rarely been specified (Moser *et al.*, 1996; Vengust *et al.*, 2006; Choori *et al.*, 2015). Therefore, this study aimed to evaluate the correlation between CSFV antibody responses detected by ELISA and SN assay and to estimate protective antibody level by ELISA for herd monitoring.

Materials and Methods

Experimental design: A total of 522 serum samples submitted for a routine CSFV sero-monitoring from commercial swine herds in Thailand were included in this study. All serum samples were tested by serum neutralization (SN) assay and a commercial CSFV antibody ELISA (Pigtype® CSFV Erns Ab, Indical Bioscience, GMBH Leipzig, Germany). Serum neutralization was performed to determine the sample status (positive, SN titer ≥2; negative, SN titer <2) and neutralizing antibody titer. The neutralizing antibody titers were categorized into 3 levels, i.e., negative (SN titer <2), below protective level (SN titer <32), and at protective level (SN titer ≥32). The antibody results from SN and ELISA were analyzed for correlation and agreement of the assays. The estimation of the relationships between ELISA S/P values and the SN titers was analyzed using regression analysis.

Serum neutralization: The serum neutralization test was performed in 96-well flat-bottomed microtiter plates. Prior to testing, serum was inactivated at 56ºC for 30 min. The CSFV ALD strain (10^3 $TCID_{50}$/20 µl) and the growth medium (MEM) were used as positive and negative controls, respectively. The serum sample (50 µl) was serially 2-fold diluted with MEM (50 µl, Life Technologies, UK) in the well. Then, 50 µl of prediluted virus suspension in 100 $TCID_{50}$ growth medium was added to the wells. The plates were incubated in a 5% CO_2 incubator at 37 ºC for 1 h. After that, 100 µl of growth medium suspension containing 3 x 10^5 SK6 cells/ml was added to each well, and incubation was carried out at 37ºC in 5% CO_2 incubator for 72 h. After discarding the medium, the cell monolayers were fixed with 100 µl of 0.4% formaldehyde (in 0.5% PBST with 1%BSA) for 30 min and then washed 3 times with 200 µl of 0.5% PBST. The virus was visualized by adding 50 µl prediluted 1:1000 WH303 CSF-specific monoclonal antibody (RAE0826, APHA Scientific, Surrey, UK) in 0.5% PBST with 1% BSA, followed by incubation at 37 ºC for 90 min. The plates were washed 3 to 5 times with 200 µl of 0.5% PBST. Then, 50 µl of prediluted 1:300 polyclonal rabbit anti-mouse IgG/HRP (P0161, Dako Denmark A/S, Denmark) in 0.5% PBST with 1% BSA was added to the wells, followed by incubation for 1 h at room temperature. Subsequently, the plate was washed, and 50 µl of chromogen–substrate solution was added to each well and stained for 1 h at room temperature. After that, the supernatant was discarded and washed once with deionized water. The test plate was read with a light microscope. The presence of the virus was indicated by the reddish-brown coloration in the cytoplasm of the cells. At the low-power microscopy, the monolayer was examined to determine the endpoint of the titration. The neutralizing antibody titers were expressed as the reciprocal of the highest dilution of serum that neutralizes the virus and shows a lack of staining in the cell. Samples with neutralization titer $\geq$2 (the $\log_2$ of antibody titer $\geq$1) were assigned positive, and <2 (the $\log_2$ of antibody titer <1) were negative.

Enzyme-linked Immunosorbent Assay (ELISA): The ELISA (Pigtype® CSFV Erns Ab) procedure was performed in 96-well microtiter plates following the manufacturer's protocol. Positive and negative controls were included on each test plate. Positive and negative control (100 µl) were applied in duplicates. Serum samples were prediluted in a dilution plate with a ratio of 1:10 to sample diluent prior to being transferred to the coated plate. On the coated plate, the diluted serum sample (100 µl) was incubated for 60 min at 37 ºC. Then, the mixture was removed, followed by washing step 3 times using 400 µl of diluted 1:10 wash solution, and the conjugate was added at an amount of 100 µl. The plates were incubated for 60 min at 37ºC, and the washing step was repeated. Subsequently, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added to 100 µl, followed by incubation for 10 min at room temperature in the dark. Finally, the well mixture ended with 100 µl stop solution, and plates were read using a microplate reader (Envision® Multimode Microplate Reader, PerkinElmer, Waltham, MA, USA). The reactions were measured as optical density (OD) at a wavelength of 450 nm, and the values were converted to the sample-to-positive ratio (S/P). Validation criteria were determined with the mean value of the measured optical density (OD) for the positive control (PC) $\geq$0.7 and $\leq$0.3 for the negative control (NC). Data interpretations were applied following manufacturing protocol. That is, samples with S/P ratio <0.3 are considered as negative, S/P-ratio $\geq$0.5 are positive, and S/P-ratio $\geq$0.3 and/or <0.5 are suspected.

Statistical analysis: Statistical analyses were conducted using the SAS® 9.4 version (SAS® Institute Inc., Cary, NC, USA), and *p*-values of less than 0.05 were considered statistically significant. Comparisons of S/P values among the antibody SN titer levels were analyzed using Welch's ANOVA and Tukey's post hoc test. Correlation between S/P values and SN titers was examined using Spearman's-rank correlation coefficient. The concordance between the two assays was analyzed using Cohen's kappa method. A linear regression model was analyzed to estimate the 95% prediction intervals as well as the standard error (SE) of the predicted SN titer for an individual according to the Bland approach explained elsewhere (Bland, 2004).

Table 1 CSFV antibody responses detected by serum neutralization (SN titers) and ELISA (S/P values)

SN titer	n	Mean S/P	SD
< 2	208	-0.034[a]	0.173
2 to <32	116	1.132[b]	0.587
≥32	198	1.767[c]	0.479

[a,b,c] Within a column, different superscripts indicate the differed significantly in mean S/P among the SN titer levels (p<0.0001); n, amount of serum samples; S/P, sample-to-positive ratio; SD, standard deviation.

Table 2 Contingency table of CSFV Erns antibody detection by ELISA and SN assay

ELISA	Serum neutralization (SN)		Total
	Positive	Negative	
Positive	298	6	304
Negative	16	202	218
Total	314	208	522

Result

Antibody responses detected by serum neutralization and ELISA: Out of 522 samples, 314 (60.2%) were positive (the SN titer $\geq$2), and 208 (39.8%) were negative (the SN titer <2) by serum neutralization test. In the positive group, including those below and at the protective level of SN titers, the neutralizing antibody titers varied from 1 to 12 ($\log_2$), and the average titer was 3.367 $\pm$ 3.380 ($\log_2$). Within the same sample set, 304 (58.2%) out of 522 samples were positive, and 218 (41.8%) were negative by ELISA. The sample-to-positive (S/P) values of negative samples ranged between -0.143 and 0.258, with a mean $\pm$ SD of -0.053 $\pm$ 0.064. The S/P values of positive samples ranged between 0.520 and 2.859, with a mean $\pm$ SD of 1.598$\pm$ 0.514.

The S/P values (mean±SD) grouping by the level of the SN titers (negative, below protective levels, and at protective level) are shown in Table 1. The results showed statistically significant differences between the mean S/P value in the negative group and every level of positive groups (p<0.0001), i.e., below and at the protective levels. The categorical results (negative, positive) of CSFV ELISA and SN are presented in Table 2.

Correlation and agreement of antibody responses: The scatter plot and correlation of CSFV antibodies detected by CSFV ELISA and SN assay are presented in Fig. 1. Correlation analysis between S/P values and SN titers using Spearman's-rank correlation coefficient test revealed a strong positive relationship. The value of r_s was 0.89 (p<0.0001), indicating a statistically significant association between S/P values and antibody SN titers. The agreement between the two assays showed near perfect agreement with the value of 0.913 (95% CI 0.8772 – 0.9484).

Regression analysis: Linear regression analysis was performed according to Bland (2004). Predictions of antibody responses between S/P values and SN titers are shown in Fig. 2. In this study, the standard error (SE) of the predicted SN titers by ELISA in individual data was estimated to be 0.908. The predicted SN values by S/P values of CSFV ELISA were given as 3.3306x + 0.3413. The calculation of 95% limits for the prediction can be measured by the regression line ±1.96 standard errors (SE) or written as follows,

$$= (3.3306x + 0.3413) \pm 1.96 * 0.908$$

From the formula above, the upper and lower 95% prediction limits for serum neutralization (log_2) were ±1.96 times standard errors (SE) or equal to ±1.78 of the predicted SN titers, respectively.

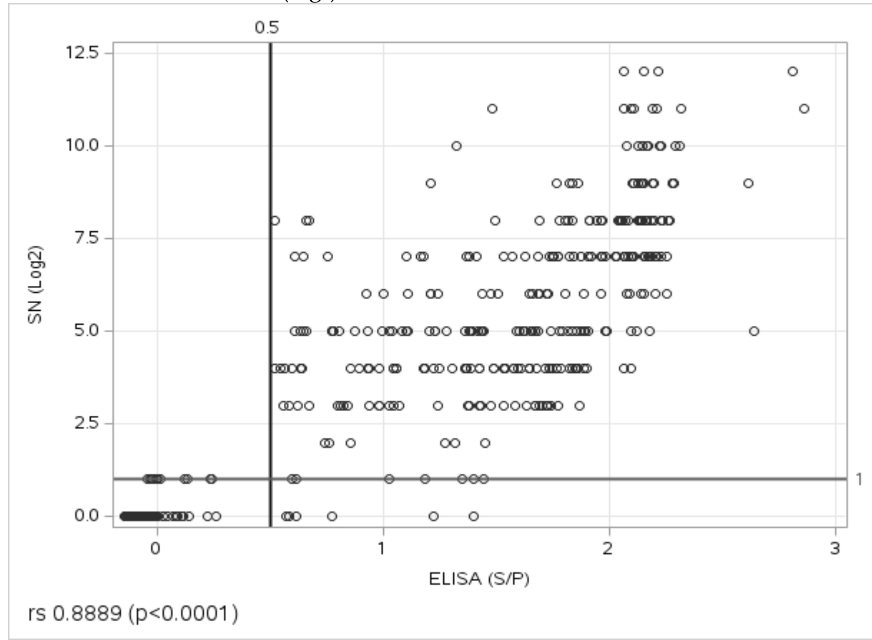

Figure 1 Correlation of antibodies response detected by ELISA towards serum neutralization. The horizontal and vertical lines represent positivity cut-offs of SN assay and ELISA, respectively. rs, Spearman-rank order correlation coefficient.

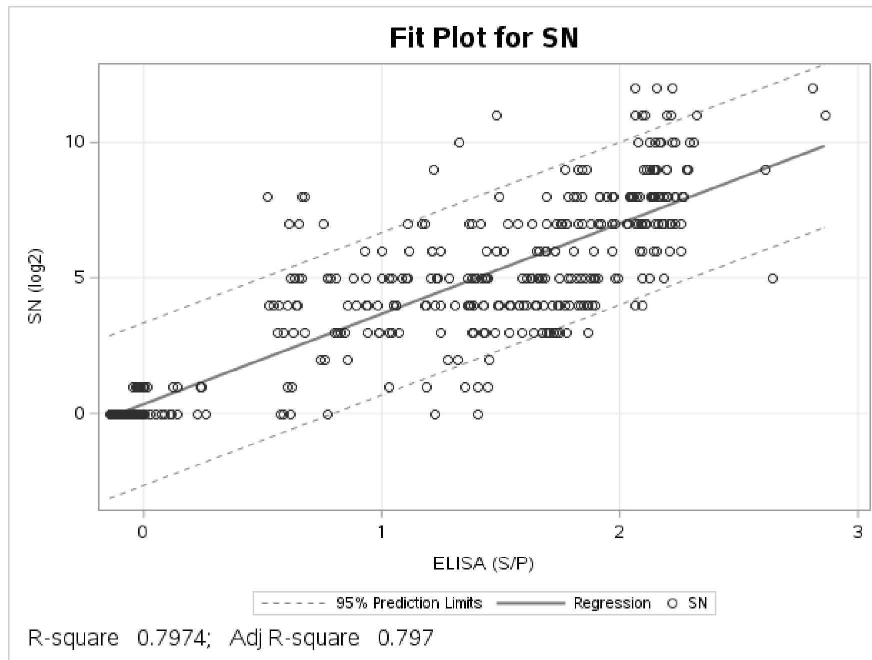

Figure 2 Scatter plots and regression analysis displaying CSF-specific antibody predictions between the sample-to-positive ratio of CSF Erns ELISA and base 2 log scale titers of serum neutralization (SN). Circle hollows represent individual antibody levels. The solid line represents the regression or predicted mean for SN measurement by ELISA. Dashed lines represent the 95% prediction limits, which indicate the interval for a single SN value.

Discussion

This study evaluated the correlation of CSFV antibody levels detected by ELISA and its antibody standard assay and SN test using S/P values and SN titers, respectively. The serum neutralization assay as a CSFV gold standard for antibody detection was performed to determine the antibody status of the samples (negative, positive) and to categorize the group of the positive samples by their protective levels (below protective level, at protective level). Several serological approaches involving ELISA toward a gold standard assay for antibody detection against animal or human diseases have been reported (Graham *et al.*, 1997; Paudel *et al.*, 2014; Nyiro *et al.*, 2019; Wang *et al.*, 2020b; Bonifacio *et al.*, 2022; Dolscheid-Pommerich *et al.*, 2022; Lee *et al.*, 2022). Neutralizing capacity from serum neutralization test was previously demonstrated to correlate with the protection level in

CSFV-challenged pigs (Terpstra and Wensvoort, 1988; Santana-Rodríguez *et al*., 2022).

The strong correlation between the commercial ELISA and SN results suggests a high potential to estimate neutralizing antibody titers by the observed S/P values. Commercial ELISA (Pigtype® CSFV Erns Ab) performed in this study was designed as an accompanying assay for marker vaccines providing DIVA properties. The assay was formatted to detect antibodies against the Erns protein, the CSFV-specific immunogen glycoprotein, which also generated antibodies with a neutralizing capacity (Meyer *et al*., 2017). The viral envelope Erns is one of the CSFV structural proteins, membrane-bound, and a second major target for neutralizing-antibody. Among the structural proteins of CSFV, Erns is highly glycosylated with N-linked glycan (Ruggli *et al*., 2005). Although E2 antibodies were the primary neutralizing factor for CSFV, the strong correlation between S/P values and SN titers presented in this study was contributed by the detection of Erns antibodies, which also have neutralizing properties.

There were significant differences in S/P responses among groups by the antibody levels (negative, below protective level, and at protective level). The group of positive samples, with S/P values of 1.132±0.587 and 1.767±0.479, were estimated to have antibody titers below and at the protective level, respectively. This result could suggest the applicability of the observed S/P for estimating protectivity status on a herd basis. In addition, regression analysis showed that the corresponding SN titer values estimated the observed S/P ELISA at 95% prediction limits within a width of 3.56 $\log_2$ SN titer. Prediction limits represent the maximum and minimum of SN predicted value. This result indicated that the estimated SN titer of a given serum sample by the observed S/P value would probably fall within 1.78-fold higher or lower than the true SN value. The adjusted R-square (0.797) showed a moderate accuracy of the regression model between S/P values and SN titers. Thus, the variability (79%) of SN titers is fairly influenced by the S/P value. This finding is likely due to the high variability of serum sample characteristics, which makes the accuracy of antibody titer estimation difficult. Therefore, careful consideration is needed for a direct estimation using an individual value in the formula. Alternatively, we could use the predicted SN titer based on the observed S/P value to help the functional standard assay determine the level of protective immunity (negative, below protective level, at protective level). Likewise, in another study, a high correlation between the inhibition rate from competitive E2-based ELISA and antibody titers from the SN test (r^2 =0.903, p<0.001) demonstrated a potential use of the cELISA as an alternative assay for sero-monitoring of C-strain vaccination at a herd basis (Wang *et al*., 2020b).

Generally, ELISA is a semi-quantitative assay. The S/P ratio from ELISA refers to the unit of the absorbance value that reflects positive or negative status. In this study, only one ELISA test was evaluated; thus, predicted SN titer should not be used inclusively for other CSFV ELISAs. Positive samples with upper-high antibody titer may eventually have limited maximum binding capacity to the capture antigen coated in the well. This limitation is a restraint for commercial ELISA to evaluate or estimate the specific level of antibodies. However, direct quantitation of antibody level by the observed S/P values can be performed by initially determining the baseline concentration using serial dilution and measuring the optical density (OD) from each dilution. The results of the OD value were then plotted against the concentration of each dilution to create the standard curve. Then, the equation generated from the standard curve was used to determine the antibody concentration of unknown samples (Żak *et al*., 2021).

Antibodies against classical swine fever virus exhibit cross-protectivity among different strains, including the moderately virulent CSFV ALD strain. This study described the CSFV ALD strain (subgroup 2.2) for serum neutralization assay. Phylogenetic analysis has identified subgroups 1.1, 2.1, and 2.2 as the predominant CSFV genogroups circulating in Thailand (Parchariyanon *et al.,* 2012). While CSFV antibodies demonstrate better neutralizing activity against homologous viruses, they can also neutralize heterologous strains (Chen *et al*., 2023). ELISA results have shown no significant differences in IgG binding abilities between homologous and heterologous CSFV E2 proteins (Chen *et al.,* 2023). Regarding vaccine-induced cross-protection, live-attenuated C-strain (subgroup 1.1) vaccination has fully protected pigs against challenges with subgroup 2.2 strains (Damrongwatanapokin *et al*., 2002).

In conclusion, the CSFV S/P values obtained from Erns antibody ELISA were highly correlated with the level of neutralizing antibody titers. Therefore, the results from CSFV ELISA could provide a useful estimation of protective antibody levels for herd health monitoring.

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