The Use of ELISA in Classical Swine Fever Virus Antibody Monitoring in Taiwan


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ABSTRACT The aim of this study was to establish a quantitative classical swine fever virus (CSFV) antibody monitoring system in Taiwan. First, we used a set of serum samples from vaccinated pigs to correlate antibody titer against CSFV between a virus neutralization (VN) test and a blocking enzyme-linked immunosorbent assay (ELISA). A good correlation (r = 0.892) was found between the VN and the ELISA results. To establish a reference serological profile for groups with good vaccination efficacy, 300 serum samples from 14 pig farms that displayed good immune responses with lapinized Chinese C (LPC) vaccination were used in this assay. The mean ELISA antibody inhibition value (%) was 104.6% in these pigs. To evaluate the interference of maternal antibody, three- and six-week-old pigs with different levels of maternal antibody were vaccinated with LPC vaccine, and the antibody response was assayed six weeks later. Seroconversion of the piglets displayed a quadratic regression correlation against maternal antibody levels. Serum samples of LPC-vaccinated pigs and of pigs infected with the CSFV could be differentiated by the blocking ELISA kit if the serum samples were diluted 20-fold. In conclusion, the commercially available CSFV ELISA kit results have a high correlation with the VN test results and can be used in the quantitative assessment of levels of CSFV antibody in Taiwan. [Sheu YT, Chien MS, Wang CS, Lin CC, Liao JW, Liu CI, *Lee WC. The use of ELISA on classical swine fever virus antibody monitoring in Taiwan. Taiwan Vet J 32 (4): 248-257, 2006. *Corresponding author TEL.: 886-4-2284 0894, FAX: 886-4-2286 2073, E-mail: wcleee@dragon.nchu.edu.tw]

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious disease of pigs caused by infection with a Pestivirus, classical swine fever virus (CSFV). In acute CSF, pigs develop high fever, leukopenia and extensive hemorrhagic lesions of skin and vital organs, while chronic infection is less specific and clinical signs are often complicated with secondary infections [3, 18]. CSF has become an endemic disease in most infected regions. For years, lapinized Chinese C (LPC) strain vaccine has been used to prevent the disease in endemic areas. Although the epidemic situation can be effectively controlled by vaccination, it is difficult to eradicate it by live vaccines due to multiple factors such as proper implementation of vaccination regimens, interference of maternal antibody with vaccination and virus persistence [1, 5, 8, 9]. In CSF vaccinated pig farms, CSFV infection is often found in a chronic form with decreased incidence of CSFV pat-
hognomonic lesions [10]. Therefore, it is difficult to monitor the chronic infection by pathological diagnosis. Similarly in live virus-vaccinated areas, it is difficult to monitor for CSFV infection by means of antibody tests, although the prevalence of CSFV antibody can reflect herd immunity in a vaccinated herd.

The most common and gold standard method used to detect CSFV antibodies is the virus neutralization (VN) antibody test [7, 17], but it may not be suitable for mass monitoring because it is expensive, time-consuming and labor-intensive. An enzyme-linked immunosorbent assay (ELISA) based on CSFV E2 antigen has been developed in recent years and has proven to be highly sensitive and specific for CSFV infection [13, 20]. These tests can be used to screen pigs for CSF exposure and are taken as the reference for elimination of CSFV in non-vaccinated areas [2, 6]. However in areas where CSF is endemic and live-virus vaccines are used to control the infection, it is difficult to differentiate vaccinated from infected pigs [11]. Therefore, the aim of this study was to establish a quantitative monitoring system in areas using attenuated vaccine. This included establishing a reference serological profile for vaccinated animals, determining the effect of interference by maternal antibody, and discriminating CSFV infection from vaccination.

**MATERIALS AND METHODS**

**Serum samples and their sources**

1. To evaluate the correlation between ELISA and VN antibody tests, 19 six-week-old pigs were intramuscularly injected with 2 mL CSFV E2 subunit marker-vaccine containing 32 μg glycoprotein E2 (Bayovac®, Batch no: E2-99-EP-001) and boosted four weeks later. Serum samples were collected at different times before and after vaccination. Ten serum samples from specific-pathogen-free (SPF) pigs were collected as negative controls, and serum samples from 10 vaccinated pigs that had been experimentally infected and had recovered from CSFV infection and showed high VN titers were used as positive controls.

2. To establish a reference serological profile after LPC vaccination, 300 serum samples from finisher pigs were collected from 14 volunteer pig farms showing a good immune response after LPC vaccination (antibody prevalence >80% on the farm) as representative field samples.

3. To evaluate the interference of maternal antibodies with vaccination using attenuated virus, maternal antibody was first assayed by ELISA to establish a decay curve from 20 pigs born to sows with regular LPC vaccination on a pig farm with no history of CSFV infection. In addition 20 piglets were vaccinated with LPC at three weeks of age in the presence of high maternal antibody titers and 43 others were vaccinated at six weeks of age when less maternal antibody was present. Blood samples were collected before, and six weeks after, vaccination to determine the development of antibody.

4. To discriminate possible CSFV infection using a blocking ELISA test with diluted serum samples, serum samples from LPC-vaccinated pigs (N=10) and pigs experimentally infected and recovered from CSFV infection (N=10), both with ELISA antibody values ≥130% were used in this study. Other serum samples from a CSFV infected farm with an incomplete vaccination regime, single LPC vaccination at week 5, were also included to verify detection of CSFV infection by the dilution of samples.

**CSFV neutralization test** The CSFV VN antibody test used exaltation of Newcastle disease virus cytopathic effect (END) method as described previously [7]. Briefly, the serum samples were serially diluted in a 96-well culture plate and incubated with 100 TCID₅₀ of strain A76 CSFV for one hour. Primary pig testis cells were added to each well (2 × 10⁴/well). Four days later, 1 U hemagglutination titer of Newcastle disease virus was added to each well. Cytopathic effects were observed by staining the cell cultures with 0.5% crystal violet solution three days later. The maximum dilution that could inhibit CPE formation was regarded as the neutralizing antibody titer.

**CSFV antibody ELISA kit** The commercial CHEKIT® CSF Sero ELISA kit (Dr. Bommeli AG) with a sensitivity of 97.4% and specificity of 99.0% is a blocking ELISA where plates are coated with CSFV E2 protein, the sample is first put on the plate.
which coated with CSFV E2 protein, and then the remaining free E2 on the plate is detected with an anti-E2 reagent. The assay followed the manufacturer’s recommended procedures. The ELISA value was expressed as percentage inhibition (%) and calculated as: Value (%) = \( \frac{(OD_{neg} - OD_{Sample})}{(OD_{neg} - OD_{pos})} \) x 100%. The sample was considered negative if the value was <40%, positive if the sample was >60%, and ambiguous if the value was between 40% and 60%.

**Statistical analysis**  Linear regression was used to analyze the correlation between VN antibody titers and ELISA antibody value. Quadratic regression was used to analyze the correlation between maternal ELISA antibody value and seroconversion after LPC vaccination. To discriminate between possible CSFV infection and LPC vaccination, the Student’s t-test was used to compare ELISA values of the positive control group and the LPC vaccination group after serial dilution.

**RESULTS**

**Correlation of ELISA and virus neutralization antibody tests**  One hundred and sixteen CSFV E2 subunit marker vaccinated serum samples, 10 SPF-negative and 10 vaccinated and infected control serum samples were examined. All serum samples were assayed by the VN antibody test and the ELISA test. Correlation was carried out using endpoint VN titers and ELISA antibody values. A simple linear regression with a high correlation (r = 0.892) between ELISA inhibition value and VN titers was obtained (Fig. 1). Using the fitted straight line (Fig. 1), the positive cutoff value for the ELISA assay, 60% inhibition, was equivalent to a VN antibody titer of 1:12.

**CSFV antibody profile after LPC vaccination**  To monitor the immune response after vaccination in a field situation, 300 serum samples from 14 volunteer pig farms showing a good antibody response after LPC vaccination (antibody prevalence >80%) were used to obtain the ELISA profile of finisher pigs. The mean ELISA antibody value obtained from these pigs was 104.5%, the coefficient of variation (CV) was 31.0%, and the mode of the ELISA antibody value was in the 101-120% subgroup (Fig. 2).

**Decay of maternal antibody**  Test sera were collected from 20 pigs born to sows with regular twice yearly LPC vaccinations. Piglets at three weeks of age were 100% positive for CSFV antibody, with values

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**Fig. 1** The correlation between virus neutralizing (VN) antibody titers and ELISA values. Serum samples were collected from 19 piglets vaccinated with CSFV E2 subunit marker-vaccine. 10 SPF-negative control and 10 CSFV-infected pigs as strong positive controls. All sera were measured by both neutralizing antibody and ELISA tests. The CSFV ELISA antibody inhibition value (%) was plotted against VN titers. The coefficient of correlation (r) and the line equation determined by a simple linear regression test are indicated in the panel.

**Fig. 2** A representative serological profile of finisher pigs with good LPC vaccination efficacy. Serum samples of 300 finisher pigs from 14 pig farms using LPC vaccination (serum prevalence >80%) had CSFV antibody ELISA test values determined and expressed as inhibition value (%).
ranging between 73% and 149% inhibition. The prevalence of CSFV antibody gradually decreased to 35% and 10% positive piglets at six and nine weeks of age respectively. Until twelve weeks of age, there was no positive sample detected. Samples were subgrouped based on the levels of maternal antibody at week three, then linear regression correlations of maternal antibody decay in different subgroups were obtained as shown in Fig. 3.

**Evaluation of the interference of maternal antibodies to LPC vaccination** To evaluate the interference of maternal antibodies to LPC vaccination, piglets were vaccinated once with LPC vaccine at three or six weeks of age in the presence of different levels of anti-CSFV maternal antibody. Before vaccination, the pigs were categorized into four subgroups according to maternal antibody ELISA value: ≤30%, 31-60%, 61-90%, and ≥90%. Six weeks after LPC vaccination, the seroconversion rates in the different groups were 100%, 84.6%, 77.7% and 0%, respectively, and displayed a quadratic regression correlation between maternal antibody and seroconversion rate (Fig. 4). There was no 91-110% subgroup in the samples assayed, but according to the quadratic regression analysis, the predicted seroconversion rate of this subgroup was 31.7%.

**Monitoring possible CSFV infection by ELISA** VN titers from CSFV-infected and recovered piglets were divided into four subgroups based on maternal antibody ELISA value.

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**Fig. 3** Decay of anti-CSFV maternal antibody. Piglets (N=20) were born from sows which were vaccinated with LPC twice yearly. Blood samples were taken every three weeks and assayed by the ELISA method. The CSFV ELISA antibody level was expressed as the inhibition value ( ). Positive samples were determined by a cutoff of ≥ 60%. Piglets were split into subgroups depending on the levels of maternal antibody at three weeks of age as indicated on the top of each panel. The coefficient of correlation (r) and the linear equation of decreasing antibody with age are indicated in each panel.
Fig. 4 Interference with LPC vaccination by anti-CSFV maternal antibody. Single CSFV vaccination was conducted on three- or six-week-old pigs (N=63). Piglets were subgrouped depending on the level of maternal antibodies before vaccination as indicated on top of each panel (A-D). CSFV antibody was assayed by the ELISA method and seroconversion was determined at six weeks after LPC vaccination, as indicated in each panel. A quadratic regression correlation of % of animals to seroconvert to starting maternal antibody ELISA value is shown in panel E.

pigs are much higher than those from LPC-vaccinated pigs [21] although both give high values on the commercial blocking ELISA (146.5% ±9.45% vs. 143.6% ±13.16%; P = 0.5858) and cannot be differentiated using the standard ELISA assay (Fig. 5). Therefore, an ELISA method using diluted serum was tested to see if it distinguished between CSFV infection and LPC vaccination. When the samples were diluted five-fold, the ELISA antibody values after CSFV infection were significantly different from the LPC controls (131.3% ±8.30% vs. 108.8% ±13.40%, respectively; P = 0.0003). When the samples were further diluted to 20-fold, the difference in ELISA antibody values between the CSFV-positive group and the
LPC-vaccinated group was amplified further (105.0% ±13.60% vs. 48.4% ±13.22%, respectively; \( P = 0.0000002 \)). If a cutoff based on the mean value plus two standard deviations of the LPC-vaccinated group was used with 20-fold diluted serum, an ELISA inhibition value of over 75% would indicate a sample taken after CSFV infection rather than LPC vaccination.

To test this serum dilution method as an indicator of CSFV infection, 20 serum samples from finishers collected from a CSFV-infected farm (confirmed by virus isolation), were assayed by VN antibody and ELISA tests. Both methods had similar antibody profiles showing poor vaccination response and a bimodal distribution after an incomplete CSFV vaccination. In the VN test, the distribution of antibody in most (13/20) of the finisher pigs fell in the low antibody titer category with a VN titer less than 1:16, but the VN titers of 4 finisher pigs were more than 1:1024 (Fig. 6A). In the ELISA antibody test, 60% (12/20) of the samples remained ELISA-antibody negative after vaccination with the mean titer being 56.3% and the CV being 90.5% (Fig. 6B). Four samples with ELISA antibody values >130% were diluted 20-fold and retested, in which 3 of these samples remained above the 75% inhibition value (Fig. 6C) indicating infection by CSFV.

Fig. 6 Identification of CSFV-infected pigs by high antibody titers at a pig farm with CSFV infection. Sera were collected from finisher pigs (\( N = 20 \)) on a pig farm with CSFV infection. The sera were assayed by VN antibody test (A) and ELISA (B) test, respectively. Sera with high ELISA inhibition values (≥130%) in panel B were diluted 20-fold with buffer solution and then assayed again using the standard procedures of the ELISA kit (C). A cutoff of inhibition value 75% (based on the cutoff derived in Fig. 5) was used to identify possible CSFV infected animals. 0x is undiluted serum and 20x is 20 fold diluted serum in panel C.
DISCUSSION

In most CSFV-endemic areas, live-virus vaccines have been used to prevent CSF disease. Therefore, the quantitative differences in the antibody response to infection versus vaccination need to be exploited to develop meaningful monitoring assays as qualitative assays (e.g., positive versus negative ELISA tests) are of little value. To develop an ELISA based monitoring assay for CSFV-vaccinated areas, not only should the sensitivity and specificity of the ELISA be considered but also the correlation of ELISA assay results with VN antibody results, the 'gold standard' test. We have shown here that the results from a commercially available ELISA test for anti-CSFV antibody showed a simple linear regression with a high correlation coefficient (r = 0.89) with VN antibody titer values. The cutoff of ELISA inhibition value of 60% for a positive test was approximately equivalent to a VN titer of 1:12. This is close to the previously determined cutoff for positive neutralizing antibody titers [16, 17]. Therefore, the CHEKIT* CSF Sero ELISA kit is suitable for quantitative and qualitative CSFV antibody monitoring. In this study we also tested other commercial ELISA kits which showed different levels of correlation with the VN test (data not shown). Therefore, if using a different ELISA kit, workers will need to establish their own reference data for quantitative monitoring.

Intensity, uniformity and prevalence of antibody are used as measures of vaccination potency. As a herd antibody prevalence over 80% can effectively prevent disease outbreaks, this parameter is regarded as an index of good vaccination efficacy under field conditions. On this premise, the sera of finisher pigs from 14 farms with good vaccination histories were analyzed and showed a mean ELISA antibody value of 104.5 ± 32.4%, which, using Fig. 1, equaled a VN antibody titer of 1:78. In these 14 farms, the CV fell in the range 7.3%-47.2% (data not shown). If these data were analyzed using 95% confidence intervals, mean ELISA inhibition values above 95% and CVs within 40% could be regarded as the minimal target value for farms to show good LPC vaccination efficacy. On those farms where ELISA values of finisher pigs were lower than the target value or the antibody results were widely scattered, factors that may influence vaccination potency should be evaluated to improve the immune response. Although the antibody response is being used as an indicator of protection from infection, it has been shown experimentally that after vaccination, pigs are protected before the development of detectable antibody, and that protection is correlated to the presence of CSFV-specific interferon-gamma (IFN-γ) secreting cells [14, 15]. It may suggest that establishment of the CSFV memory system after LPC vaccination in pigs is very important for protection against CSFV infection.

Among the factors that influence vaccination potency, interference by maternal antibody is of particular concern [8, 16, 19]. After vaccination, piglets with different intakes of maternal antibody and at different ages experience different levels of maternal antibody interference [9, 15]. In a preliminary study, piglets with low maternal antibody levels developed good antibody responses after LPC vaccination, with no obvious difference between one and two vaccinations (data not shown). It takes six weeks to develop high antibody levels after LPC vaccination. Therefore, to evaluate the effect of different levels of maternal antibody, a six-week interval after vaccination was required to fully measure the antibody response in the piglets. In this experiment, maternal antibody interference with CSFV vaccination was evaluated using the ELISA method, and a quadratic correlation was found between the starting level of maternal antibody and seroconversion rate to vaccination. For ELISA antibody values ≤ 30% before vaccination, the seroconversion rate was 100%. The seroconversion rate declined as starting maternal antibody levels increased. Among 10 animals with high maternal ELISA antibody values (≥111%), the ELISA value of three pigs after LPC vaccination decreased, but remained at weak antibody positive levels (Fig. 4D). From the rate of maternal antibody decay (Fig. 3), the ELISA may still be measuring residual maternal antibody at week nine. Therefore, these three pigs were considered to have shown no seroconversion. Similarly, the results of previous experiments showed that no seroconversion occurred after LPC vaccination in pigs with maternal VN antibody titers ≥ 1:48, while 74-90% seroconversion was found after vaccination in the pigs.
with maternal VN antibody titers ≤1:24 [4]. Using our quadratic regression line, if a VN titer of 1:32, corresponding to an ELISA inhibition value about 83%, is set as the value for primary effective LPC vaccination, the predicted conversion rate would be about 58.9%. This predicted seroconversion rate is close to that previously reported using the VN test [4]. Although there is no significant difference in immune response after one or two vaccinations when piglets with low levels of maternal antibodies are used, in field situations piglets will experience different levels of maternal antibody interference when vaccinated. Therefore, two vaccinations with live CSFV vaccine are still required to enforce herd immunity against CSFV infection. To evaluate the potential application of our findings in predicting seroconversion after vaccination, experiments were carried out on 5 pig farms after a single LPC vaccination (data not shown). The predicted seroconversion rates on two farms were very close to the prevalence rates of finisher pigs. However, there was a discrepancy of 11% to 13% on other farms, which may be related to slight discrepancies in blood sampling time and variability of maternal antibody levels on those farms. The reference data produced here for seroconversion rates and rates of decay of maternal antibody may be applied to determine an appropriate time for vaccination of a piglet, but more sample evaluation is needed to confirm the practicality of this approach.

Although antibodies to field CSFV infection cannot be distinguished from antibodies to vaccine virus [18], previous research has shown that pigs develop high VN antibody titers after recovery from CSFV infection, usually above 1:256 and even over ≥1:1024. This is different from the serological profile of LPC-vaccinated pigs that usually develop VN antibody under 1:256 [5, 21]. In this experiment, we also noted that the mean ELISA value after LPC vaccination was 104.5%, which was about VN antibody titer 1:78, which is lower than pigs which have recovered from CSFV. Therefore, by detecting subgroups of pigs with high antibody titers by an ELISA method, potential infection by field virus on pig farms might be identified. There was a linear correlation in the samples with a VN antibody titer <1:256 to the ELISA value. However in samples with a VN antibody titer ≥1:256, this linear correlation was lost and the line began to plateau. When these samples were tested by the ELISA, the resulting values ranged between 106% and 162%, with the majority above 130%. Therefore, subgroups with an ELISA value >130% were regarded as a subgroup with high ELISA antibody levels, and were used further to differentiate samples from natural CSFV-infected and LPC-vaccinated subgroups. The results showed a significant difference between CSFV-infected and LPC-vaccinated groups using diluted serum. An ELISA antibody value of 75% with 20-fold diluted serum samples was used as a cutoff for presumed CSFV infection, based on the mean value of the LPC group plus two standard derivations. This application had been further confirmed in several field cases (data not shown), including one case reported here. However, after LPC vaccination, we cannot exclude that excessively high neutralizing antibody titers may occur in a minority of pigs for unknown reasons. Therefore, RT-PCR techniques or virus isolation are still required to identify CSFV infection on possible infected premises.

In conclusion, the results from the CHEKIT® CSF Sero ELISA kit were highly correlated with VN antibody test results and so the ELISA could be used to quantitatively monitor CSFV antibody levels. The ELISA can be used to evaluate vaccination potency and determine a proper vaccination program with minimal interference from maternal antibodies. Moreover, it can be modified and if used extensively, could be applied to monitor natural cases of CSFV infection. In the future, it might replace VN antibody tests and be applied in mass serum screening for CSFV antibody in areas where live-virus vaccine is used.

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在臺灣地區應用酵素鍵結免疫吸附法對豬瘟抗體之監控

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摘要 本實驗之目的是建立臺灣地區豬瘟抗體之定量監控系統。首先，我們以免疫後篩選血清，進行豬瘟中和及抗體力値及商用阻斷型酵素鍵結免疫吸附法（blocking enzyme-linked Immunosorbent assay, ELISA）測試豬瘟抗體強度（抑制值；%）之相關性比較；結果顯示，中和抗體力値與 ELISA 抑制值呈現高度之線性相關（相關係數 r = 0.892）。為建立豬瘟疫苗免疫後之參考血清相，從 14 個豬瘟疫苗反應良好之豬場共計 300 個血清樣品進行分析比較，顯示其 ELISA 平均抗體抑制值為 104.6%。為評估移行抗體對免疫干擾之影響，取 3 週及 6 週齡不同移行 ELISA 抗體抑制值之篩選血清樣品，並於免疫後 6 週進行血清檢測轉陽率，結果顯示血清轉陽率與移行抗體 ELISA 抑制值呈現二次線性迴歸相關。此外，如將血清樣品先行 20 倍稀釋後再進行檢測，來自免疫豬瘟疫苗免疫豬之 ELISA 檢測抑制值可以與豬瘟病毒感染恢復豬隻血清區別。由此些結果推論，商用酵素鍵結 ELISA 抗體強度與血清中和抗體力値具有高度相關性，因此在台灣可應用於豬瘟抗體之定量分析與監控。[許岳蒼、簡茂盛、王金順、林正忠、劉正義、李維誠。應用酵素鍵結免疫吸附法建立臺灣地區豬瘟抗體之監控。台灣獸醫誌 32(4): 248-257, 2006。* 聯絡人 TEL: 04-2284 0894，FAX: 04-2286 2073，E-mail: wclee@dragon.nchu.edu.tw]

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