Short Communication

Evaluation of a commercial ELISA test kit on classical swine fever antibody detection using oral fluid samples

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Abstract

Classical swine fever virus (CSFV) contributes to economic loss of swine production in endemic countries. Serum neutralization and enzyme-linked immunosorbent assay (ELISA) are serological tests commonly used for monitoring CSFV antibody status with serum samples. This experiment evaluated the detection of CSFV antibody in oral fluid samples in commercial ELISA test kit by *in vitro* study of negative oral fluid mixed with serum of known CSFV serum neutralizing (SN) titer and *in vivo* oral fluid samples obtained from experimental animals. Correlations of SN titer and S/P ratio of ELISA were observed with the *in vitro* oral fluid samples, indicating the stability of the antibody in the oral fluid and the potential of oral fluid as an alternative specimen for CSFV diagnosis. Diagnostic sensitivity of the oral fluid detection using *in vitro* samples was as high as 95.83% when the ELISA procedures were modified (i.e. 12 h incubation at 4°C and addition of 100 µl antibody conjugate). The *in vivo* experiment used oral fluid and blood samples obtained from 20 piglets (20 days old) which were divided into 3 experimental groups: challenged with ALD strain, low virulence CSFV (A) (n=8); vaccinated (B) (n=8); and negative control (C) (n=4). The animals were vaccinated with CSFV LOM strain modified live vaccine at 0 day post inoculation (dpi) and re-challenged (A&B) with high virulence CSFV on 14 dpi and later euthanized at 30 dpi. Results from the *in vivo* oral fluid samples demonstrated low detectable antibody titers (SN titer 0-4) using neutralizing peroxidase-linked assay (NPLA) and all samples were negative to CSFV indirect ELISA performed at the optimal condition obtained from the *in vitro* protocol.

Keywords: classical swine fever, enzyme-linked immunosorbent assay, neutralizing peroxidase-linked assay, oral fluid, swine

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Introduction

Classical swine fever is a devastating disease, causing severe loss of swine production in endemic countries. The use of diagnostic tests is essential for the detection and monitoring of herd health status in endemic areas and surveillance in disease-free areas. In order to detect classical swine fever virus (CSFV) antibody, the gold standard method is through serum neutralization (SN), i.e. neutralizing peroxidase-linked assay (NPLA). This technique, however, is time-consuming and labor-intensive. Moreover, it involves animal restraint, many pieces of equipment and meticulous process of viral propagation. Alternatively, many commercial tests have been developed and used in the detection of both CSFV antigens and antibodies such as enzyme-linked immunosorbent assay (ELISA). The advantages of ELISA method over NPLA for CSFV antibody detection include more rapid detection that is useful for disease surveillance and exclusion of harboring live CSFV. Commercially available ELISAs for detection of CSFV antibody, both indirect and competitive techniques, have been implemented with limited use in the field in order to evaluate the status of the disease. Recently, oral fluid samples have been used in antibody detection in many major swine diseases including porcine circovirus-2 (PCV-2), porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine epidemic diarrhea virus (PEDV), swine vesicular disease virus (SVDV) and *Actinobacillus pleuropneumoniae* (Prickett et al., 2007; Prickett et al., 2008a; Prickett et al., 2008b; Prickett et al., 2011; Kittawornrat et al., 2010; Kittawornrat et al., 2012, Panyasing et al., 2014; Bjustrom-Kraft et al., 2016; González et al., 2017; Senthilkumaran et al., 2017). The oral fluid collection method is less stressful, simple and practical for both farmers and veterinarians. This study evaluated the detection of antibody against CSFV from *in vitro* and *in vivo* oral fluid samples using commercial ELISA test kits and modified the amount of conjugate, sample incubation time and temperature to better enhance the sensitivity and diagnostic specificity of the antibody detection.

Materials and Methods

In vitro study on the sensitivity of ELISA: Negative oral fluid samples collected from a CSF-free herd and porcine serum of known CSF SN titer obtained from Chulalongkorn University-Veterinary Diagnostic Laboratory (CU-VDL) were used for testing the potential use of oral fluid on an indirect ELISA test kit (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands). Prior to testing, all of the negative oral fluid samples were confirmed to be free of CSFV antibody using NPLA. Total of six high SN titer sera were selected and retested to confirm the antibody titer level. Two-fold serial dilution with PBS was performed on the serum samples. Pooled negative oral fluid sample and PBS diluted sera were mixed together at the ratio of 1:1 to obtain antibody titers equivalent to 64, 32, 16, 8, 4, 2 and <2. These *in vitro* samples were tested with the indirect ELISA at different conditions to evaluate the test performance. Condition and cut-off that provided the most appropriate diagnostic sensitivity and specificity were re-evaluated using oral fluid samples from the animal experiment.

Animal experiment and sample collection: Twenty 20-day-old PRRSV-free piglets were randomly assigned to 3 experimental groups: challenged with low virulence CSFV ALD strain (A) (n=8), modified live CSFV LOM strain vaccination (B) (n=8) and negative control (C) (n=4). At 0 day post inoculation (dpi)/dpv, group A was challenged with CSFV ALD strain (10^5 $TCID_{50}$/ml) and the vaccination (B) group was immunized with a modified live vaccine LOM strain (HC-VAC) and later euthanized on 30 dpi. The experiment was approved by the Faculty of Veterinary Science, Chulalongkorn University Animal Care and Use Committee (approval number 13310019). Pen-based oral fluid samples were collected daily from -1 dpi/dpv to 30 dpi/dpv using a method described elsewhere (Kittawornrat et al., 2010; Prickett et al., 2010; Kittawornrat et al., 2012). Serum samples were collected from all animals on -1, 3, 7, 10, 14, 17, 21, 24, 27 and 30 dpi/dpv.

Enzyme-linked immunosorbent assay (ELISA) and neutralizing peroxidase-linked assay (NPLA): A commercial classical swine fever indirect ELISA test kit was used in the detection of CSF antibody in oral fluid (BioChekCSFV Antibody Test Kit, Reeuwijk, The Netherlands). The i*n vitro* oral fluid samples and *in vivo* oral fluid samples were diluted with diluent at 1:30 ratio before transferring into a 96-well CSF-E2 ELISA coated plate (100 µl/well). Negative oral fluid mixed with SN titer negative serum (1:1), and pure negative oral fluid were used as negative controls for *in vitro* and *in vivo* oral fluid ELISA testing, respectively. Both *in vitro* and *in vivo* oral fluid samples were tested using the protocol suggested by the manufacturer (base condition) by adding 100 µl/well of pre-diluted anti-swine IgG antibody conjugated with horseradish peroxidase enzyme (conjugate), incubating samples for 1 h at room temperature (RT; 23-27°C). Positive and negative cut-off S/P ratios were determined from the 2-fold serial diluted *in vitro* samples with this base condition. Samples with NPLA titer of ≥ 8 were determined as positive and < 8 as negative. Incubating conditions were modified as follows: volume of conjugate (150 µl/well vs 200 µl/well), sample incubation temperature (4°C vs 37°C), and time (6 h vs 12 h). The incubation at 6 h and 12 h were held at 4°C due to high bacterial contamination in the oral fluid samples, which might degrade the coated antigen on the ELISA plates and antibody in the specimen. Subsequently, TMB color substrate was added (100 µl/well) and incubated for 15 min at RT, followed by the $MgSO_4$ stop solution (100 µl/well). Optical density (OD) of each well was evaluated at a wavelength of 405 nm. S/P ratios were calculated by dividing the subtracted background OD value of the samples with the subtracted background OD value of the positive control (OD sample-OD negative control/OD positive-OD negative control).

The NPLA test was carried out using a modified OIE Terrestrial Manual protocol (OIE, 2014), with the virus concentration of 300 $TCID_{50}$/ml (ALD strain) grown on SK-6 cell line. Two-fold serial dilution of the samples (serum and *in vivo* oral fluid) was

performed in the 96-well plate (50 µl /well) and incubated with 50 µl of viral suspension (300 $TCID_{50}$/well). The plates were incubated for 60 min at 37°C in 5% CO_2 incubator followed by addition of 100 µl of the 2 × 10^5 cell/ml SK-6 cell suspension (100 µl) and incubation at 37°C in 5% CO_2 incubator for 48 h. The plates were fixed and stained with immunoperoxidase monolayer assay (IPMA) to detect viral-infected cell with a method described elsewhere (OIE, 2014).

Statistical analysis and data analysis: Analysis of variance (ANOVA) was used to assess differences between each variable factor based on the S/P ratio results in the *in vitro* oral fluid samples. Post hoc multiple comparison tests were used to evaluate significant differences between different ELISA protocols. Significant differences were found when $p < 0.05$ and very significant differences were found when $p < 0.001$.

Results and Discussion

CSF detection in oral fluid samples: The use of oral fluid in swine disease detection has spread widely as an alternative tool in disease diagnosis and surveillance (Ramirez et al., 2012; Rotolo et al., 2017). Most tests that have been developed for oral fluid disease detection were antigen based tests such as PCR, viral sequencing and viral isolation (Loftager et al., 1993; Loftager et al., 1995; Stallknecht et al., 1999; Prickett et al., 2008b; Kittawornrat et al., 2010; Irwin et al., 2010; Prickett et al., 2011; Romagosa et al., 2012). Many veterinary diagnostic laboratories in the United States provide routine services for PCR detection of oral fluid samples for swine diseases including SIV, PCV-2, PEDV, transmissible gastroenteritis coronavirus and PRRSV. This type of specimen has been validated for CSFV antigen detection in wild boars (Mouchantat et al., 2014) and in domestic swine (Dietze et al., 2016; Panyasing et al., 2018). At present, the PRRSV antibody ELISA oral fluid test kit is the only commercially available test kit for swine disease detection. Previous serological based works focused on swine oral fluid samples for detecting antibody for influenza A virus (Panyasing et al., 2014), surveillance of the African Swine Fever virus (Mur et al., 2013) and more recent implement with bacterial disease including detection of *Actinobacillus pleuropneumoniae* ApxIV toxin antibody (González et al., 2017), PEDV (Bjustrom-Kraft et al., 2016) and SVDV (Senthilkumaran et al., 2017). Detection of CSF antibody in oral fluid samples have been tested via NPLA method (Giménez-Lirola et al., 2016; Petrini et al., 2017). To the extent of our knowledge, no commercial indirect ELISA has yet been validated with CSF antibody detection from oral fluid specimens. This research validated the detection of antibody against CSFV in oral fluid samples using commercial CSFV indirect ELISA demonstrating the potential of the specimen to be used for CSFV diagnosis.

In vitro study on the sensitivity of commercial ELISA: A model representing oral fluid with known CSFV antibody levels was established by using the negative oral fluid with the addition of serum of known SN titer. The ELISA S/P ratio representing the antibody level in the *in vitro* oral fluid correlated with the levels of SN titers, indicating the stability of the antibody in the oral fluid sample (Table 1). Factors that may interfere with the stability and detection of immunoglobulins in oral fluid sample include endogenous substances in oral fluid (e.g. electrolytes, immunoglobulins, proteins, enzymes, urea and ammonia) (Humphrey and Williamson, 2001; Prickett and Zimmerman, 2010; Chiappin et al., 2007), storage temperature and sample processing (Nurkka et al., 2003; Morris et al., 2002; Pinsky et al., 2003).

Table 1 Comparison of average S/P ratio of *in vitro* oral fluid samples with modification of volume of secondary conjugate, sample incubation temperature and incubation time

NPLA level	Base condition	Variation of conjugate volume		Variation of incubation temperature		Variation of incubation time	
	100 µl RT, 1 h (n=18)	150 µl RT, 1 h (n=6)	200 µl RT, 1 h (n=6)	4°C 100 µl, 1 h (n=6)	37°C 100 µl, 1 h (n=6)	6 h 100 µl, 4°C (n=6)	12 h 100 µl, 4°C (n=6)
64	2.648±0.78 [a]	2.605±0.67	2.688±0.67	1.290±0.60[b]	2.477±0.76 [a]	1.124±0.51[b]	4.289±0.64[c]
32	1.438±0.55 [a]	1.452±0.54	1.517±0.42	0.646±0.32[b]	1.208±0.44[ab]	0.299±0.30[b]	2.931±0.65[c]
16	0.696±0.43 [a]	0.661±0.25	0.670±0.24	0.242±0.14[b]	0.630±0.26[ab]	0.000[b]	1.599±0.52[c]
8	0.196±0.16 [a]	0.281±0.15	0.235±0.09	0.037±0.03	0.165±0.09	0.000[b]	0.566±0.33[c]
4	0.137±0.064[*]	N/A	N/A	N/A	N/A	N/A	N/A
2	0.044±0.012[**]	N/A	N/A	N/A	N/A	N/A	N/A
<2	0.034±0.023[**]	N/A	N/A	N/A	N/A	N/A	N/A

RT, room temperature; h, hour(s);
[a,b,c] Significant differences between each condition compared with manufacturer's protocol ($p<0.05$); [*]n=4; [**]n=2;
[****] Calculation of sensitivity and specificity with SN titer ≥ 8 determined as positive samples

The positive S/P ratio titer of the *in vitro* oral fluid sample was established at the base protocol (100 µl/well conjugate volume, 1 h at RT) at ≥ 0.3 with sensitivity of 79.17% and specificity of 100% (Table 2). The modification of commercial ELISA protocol in conjugation volume, time and temperature of sample incubation observed had significant increase in S/P ratio (Table 1) and the level of sensitivity of detection was highest at 100 µl/well, 12 h at 4°C, respectively (sensitivity of 95.83%) (Table 2). The decrease in S/P

ratio was noted when incubating at 6 h (Table 1); this is possibly due to the low temperature of incubating condition (4°C) that was performed with long incubation period to prevent bacterial overgrowth. The increase in temperature and the conjugate volume did not display significant changes in the S/P ratio (Table 1), although slight increase in sensitivity was observed with higher conjugate volume (83.33%) (Table 2). Increasing the amount of antibody conjugate would enhance the detection signal in an indirect ELISA by increasing the number of attached conjugate. In this study, the antibody conjugate was pre-diluted by the manufacturer, applying higher volume of the conjugate was suspected by being insufficient to increase the detection signal. Several other types of ELISAs have been tested for CSF antibody detection including direct ELISA, blocking ELISA (Have, 1984) and complex-trapping blocking ELISA (Wensvoort et al., 1988; Colijn et al., 1997) and some of which have become commercially available and could potentially be used to detect antibody in oral fluid samples. Due to the amount limitation of ELISA test kit, low SN titer dilutions were not performed with time and temperature modification protocols and the specificity was not evaluated. This study demonstrated the potential of oral fluid specimen to be used as an alternative specimen in CSFV diagnosis.

Table 2 Sensitivity and specificity of ELISA assay on *in vitro* oral fluid samples with modification of volume of secondary conjugate, sample incubation temperature and incubation time at different cut-off S/P ratio. Calculation of sensitivity and specificity is based on samples with SN titer ≥ 8 determined as positive.

Cut-off S/P ratio	Sensitivity (%)							Specificity (%)*		
	Base condition	Variation of conjugate volume		Variation of incubation temperature		Variation of incubation time		Base condition	Variation of conjugate volume	
	100 µl RT, 1 h (n=80)	150 µl RT, 1 h (n=27)	200 µl RT, 1 h (n=27)	4°C 100 µl, 1 h (n=24)	37°C 100 µl, 1 h (n=24)	6h 100 µl, 4°C (n=24)	12h 100 µl, 4°C (n=24)	100 µl RT, 1 h (n=80)	150 µl RT, 1 h (n=27)	200 µl RT, 1 h (n=27)
0.1	88.89	95.83	95.83	66.67	91.67	37.50	95.83	62.5	100	66.67
0.2	86.11	95.83	95.83	66.67	74.5	37.50	95.83	100	100	100
0.3	79.17	83.33	83.33	54.17	70.83	37.50	95.83	100	100	100
0.4	70.82	75.00	70.83	45.83	70.83	37.50	91.67	100	100	100
0.8	55.56	54.17	58.33	20.83	54.17	12.50	79.17	100	100	100

RT, room temperature; h, hour(s);
*Specificity of temperature and incubation time variation conditions were not evaluated.

Detection of antibody in oral fluid samples obtained from experimental animals using commercial indirect ELISA: Neutralizing antibody in oral fluid samples was detected using NPLA method although it did not demonstrate high antibody level as the serum at the same time point. This result is in contrast with previously reported CSF antibody detection in oral fluid where neutralizing antibodies were not detected in seropositive pigs (Petrini et al., 2017). In our study, the serum samples which contained average SN titers ranged from $2^{3.5}$-$2^{6.25}$. In the same group of pigs and on the same day of serum collection, the neutralizing titers detected in the oral fluid ranged from 2^0-2^2 (Figure 1). Due to the low volume of oral fluid samples and the very low level of antibody titers tested using NPLA, the samples obtained at 25-30 dpi/dpv were excluded from the ELISA study. *In vivo* oral fluid samples tested with the optimal condition of ELISA protocol determined from the *in vitro* study (conjugate volume of 100 µl/well, sample incubation time of 12 h at 4°C) all had S/P ratio less than the cut-off positive value of 0.3. CSF vaccination and viral inoculation in the animal experiment did not appear to have antibody response due to the high level of maternal antibody in this set of piglets resulting in low amount of antibody secretion in the oral fluid. Impact from the method of oral fluid collection, specimen handling protocol and oral fluid sample processing might affect the test performance. Time suggested for oral fluid collection is 20-30 minutes and the ropes should be processed immediately and not be left to dry. Contamination of bacteria in the oral fluid can affect the NPLA method as well as potentially degrade the immunoglobulins in the sample. To prevent these problems, the oral fluid samples were re-suspended by centrifugation to clean up the debris, filtrated and antibiotic was added in the cell culture media to prevent bacterial growth.

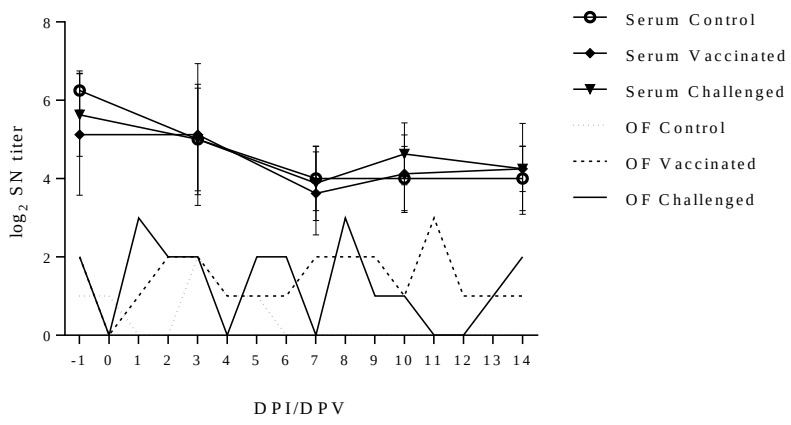

Figure 1 Neutralizing peroxidase-linked assay (NPLA) titers from the vaccinated, challenged and control groups of experimental animals measured from serum and oral fluid (OF) samples

In conclusion, this study has proven that the CSFV antibody could be detected in the oral fluid using NPLA and ELISA assay and the modification of the ELISA was made to enhance the sensitivity of the antibody detection. Higher amount of antibody in the oral fluid and longer incubation time could promote the ability of oral fluid detection with ELISA assay. Specimen processing and storage could also crucially affect the outcome of antibody detection in oral fluid samples.

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บทคัดย่อ

การประเมินการใช้ชุดทดสอบอีไลซาในการตรวจหาแอนติบอดี ของโรคอหิวาต์สุกรจากตัวอย่างน้ำลาย

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โรคอหิวาต์สุกรเป็นโรคที่ก่อให้เกิดความเสียหายต่อการผลิตสุกร มาตรการในการเฝ้าระวังและตรวจสอบสถานะของโรคจัดเป็นปัจจัยหนึ่งที่มีความสำคัญต่อการควบคุมและป้องกันโรคเป็นอย่างยิ่ง การตรวจวินิจฉัยหรือการศึกษาสถานะการติดเชื้อของโรคอหิวาต์สุกรโดยมากนิยมใช้วิธีการตรวจหาแอนติบอดีที่จำเพาะต่อเชื้อไวรัสอหิวาต์สุกรจากตัวอย่างซีรัมด้วยวิธี นิวทรัลไลซิงเปอร์ออกซิเดสลิงค์แอสเซ (NPLA) และอีไลซา (ELISA) การศึกษาครั้งนี้ได้ทดสอบประสิทธิภาพการตรวจวัดแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรด้วยวิธีอีไลซาโดยใช้ตัวอย่างน้ำลายสุกร โดยทดสอบจากตัวอย่างที่เตรียมในห้องทดลองจากการผสมน้ำลายสุกรที่เก็บมาจากฝูงสุกรที่ปราศจากแอนติบอดีต่อโรคอหิวาต์สุกรกับซีรัมของสุกรที่ทราบระดับแอนติบอดีที่แน่นอน และตัวอย่างน้ำลายจริงจากลูกสุกรทดลองที่แบ่งเป็น 3 กลุ่ม ได้แก่ 1) สุกรกลุ่มที่ได้รับไวรัสอหิวาต์สุกรสายพันธุ์เอแอลดี (ALD) ซึ่งมีความรุนแรงในการก่อโรคต่ำ (กลุ่ม A) (n = 8) 2) สุกรกลุ่มที่ได้รับวัคซีนต่อโรคอหิวาต์สุกร (กลุ่ม B) (n = 8) และ 3) สุกรกลุ่มควบคุมลบ (กลุ่ม C) (n = 4) โดยที่สุกรกลุ่ม A และ B ซึ่งได้รับไวรัสหรือวัคซีน ตามลำดับ ในวันที่ 0 และได้รับเชื้อพิษซ้ำที่เป็นไวรัสอหิวาต์สุกรสายพันธุ์ที่มีความรุนแรงในการก่อโรคสูง (สายพันธุ์ Bangkok 1950) ในวันที่ 14 ทำการเก็บเลือดและน้ำลายจากสุกรทั้ง 3 กลุ่ม และทำการการุณยฆาตสุกรทั้งหมดในวันที่ 30 การทดสอบตัวอย่างน้ำลายจากห้องปฏิบัติการพบว่า ชุดตรวจสอบอีไลซาสามารถตรวจพบแอนติบอดีที่จำเพาะต่อไวรัสอหิวาต์สุกรจากตัวอย่างน้ำลายที่เตรียมในห้องทดลองได้ และความไวในการตรวจสอบสามารถสูงได้ถึง 95.83% โดยการบ่มตัวอย่างน้ำลายที่เตรียมในห้องทดลองในชุดตรวจสอบอีไลซาที่ 12 ชั่วโมงในอุณหภูมิ 4°C และเติมสารคอนจูเกต (conjugate) ปริมาตร 100 µl/หลุม ส่วนการตรวจระดับแอนติบอดีในตัวอย่างน้ำลายจริงด้วยวิธี NPLA นั้น พบว่าตรวจพบได้ในระดับต่ำ และตัวอย่างน้ำลายจริงให้ผลลบจากการทดสอบในอีไลซาด้วยการบ่มตัวอย่างน้ำลายจริงที่ 12 ชั่วโมงในอุณหภูมิ 4°C และเติมสารคอนจูเกตปริมาตร 100 µl/หลุม

คำสำคัญ: โรคอหิวาต์สุกร อีไลซา นิวทรัลไลซิงเปอร์ออกซิเดสลิงค์แอสเซ (NPLA) น้ำลาย สุกร

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