RESEARCH REPORT
SWINE HEALTH

Title: Development of classical swine fever virus diagnostic assays for porcine oral fluid samples - **NPB #11-001**

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Industry Summary:

With classical swine fever virus (CSFV) commonplace in the world and barely outside the borders of North America, it is reasonable to expect that CSFV will eventually be reintroduced into North America. The primary objective of this project is to optimize and validate technology capable of rapidly identifying premises infected with CSFV following its introduction into North America or other CSFV-free areas using oral fluid samples. In the present study, twenty pigs at the age of 21 days were obtained from a free-CSFV farm and divided into 3 groups. Pigs in group A (n=8) were intramuscularly inoculated with a low virulence CSFV strain (ALD, 10^5TCID$_{50}$/pig) at day 0 and re-challenged with a virulence strain (Bangkok-1950, 10^3TCID$_{50}$/pig) intramuscularly at 14 days post inoculation (DPI). Pigs in group B (n=8) were intramuscularly vaccinated with a commercial modified live CSFV vaccine (LOM) at day 0 and challenged with the virulence strain similar to pigs in group A at 14 DPI. Group C (n=4) was a negative control group. This modified experimental protocol allowed us to successfully collect the oral fluid samples from subclinically infected pigs and might mimic the CSFV situation in CSFV-endemic countries using modified live CSFV vaccines. Based on the modified real-time RT-PCR results (sensitivity at $10^{1.5}$ TCID$_{50}$/ml), a few vaccinated pigs having viremia for a few days after vaccination and after re-challenged with a high virulence strain, a few viremic pigs were found in both vaccinated and low virulence CSFV challenged groups. However, all samples were tested negative using a routine RT-PCR method (sensitivity at 10^3 TCID$_{50}$/ml). In addition, none of the oral fluid samples from all groups were tested positive using either the modified real-time RT-PCR or the routine RT-PCR. Possible use of a commercial blocking ELISA of classical swine fever antibody was also evaluated by using oral fluid samples from a CSFV-free herd mixed with serially diluted known CSFV antibody titers as a pilot study and compared to the neutralizing peroxidase-linked assay (NPLA). Interestingly, the low levels of NPLA antibody titers ($\log_2$ 1- $\log_2$ 3) could evidently be detected in the oral fluid samples obtained from the *in vivo* experiment (lower than average serum NPLA titers of pigs in the same group, $\log_2$ 3- $\log_2$ 6) but none of the oral fluid samples from this present study were positive by a commercial ELISA (sensitivity at least $\log_2$ 4 based on the pilot study). In conclusion, the modified real time RT-PCR yielded satisfactory sensitivity than the routine RT-PCR, but the sensitivity was not good enough to detect low virus levels, particularly in the oral fluid samples. Extracting genetic material from the oral fluid samples may be hampered by the mucous component in the saliva or other organic matters. Achievement of this objective could be done by developing a better genetic material extraction from oral fluid samples.

However, detecting CSFV antibody in the oral fluid samples is another alternative method and when adjusting a commercial blocking ELISA protocol to increase its sensitivity for improved surveillance in CSFV-free areas, thereby would enhance elimination and control efforts. In addition, testing oral fluid samples using an improved sensitivity blocking ELISA may benefit the North American swine producers to rapidly identify premises infected with CSFV following its introduction into North America much faster than using the molecular diagnostic methods.

Keywords: classical swine fever virus (CSFV), diagnosis, oral fluid, RT-PCR, serology

Scientific Abstract:

The primary objective of this project is to optimize and validate technology capable of rapidly identifying premises infected with classical swine fever virus (CSFV) following its introduction into North America or other CSFV-free areas using oral fluid samples. The *in vivo* pilot experiment was previously conducted and found that a high virulence CSFV strain (Bangkok-1950) at the titer of 10^3TCID$_{50}$/pig given intramuscularly killed the piglets within 5 days and infected pigs were unable to chew the rope while a low virulence CSFV strain (ALD) showing mild clinical signs did not kill the inoculated animals. The protocol, therefore, was modified to answer the field questions on vaccinated animals or animals previously exposed to the low virulence CSFV strain being re-challenged with the high virulence CSFV strain acting as CSFV reservoir. Producing subclinically CSFV-infected pigs allowed us to successfully collect the oral fluid samples mimicking the situation of using modified live CSFV vaccines in CSFV-endemic countries. In the present study, 20 3-week-old pigs were obtained from a free-CSFV farm with sow vaccination and divided into 3 groups. Pigs in group A were intramuscularly inoculated with a low virulence CSFV strain (10^5TCID$_{50}$/pig) on day 0 and re-challenged with the virulence strain (10^3TCID$_{50}$/pig) intramuscular at 14 days post inoculation (DPI). Pigs in group B was intramuscularly vaccinated with a commercial modified live CSFV (LOM) vaccine on day 0 and challenged with the virulence strain similar to pigs in group A at 14 DPI. Group C was a negative control group. It should be noted that all vaccinated- and low virulence CSFV infected-pigs had no obvious clinical sighs showing a few hemorrhagic lesions when necropsied. All samples including serum and oral fluid samples were tested negative using a routine RT-PCR method (sensitivity at 10^3 TCID$_{50}$/ml). Based on the modified real-time RT-PCR results (sensitivity at $10^{1.5}$ TCID$_{50}$/ml), a few vaccinated pigs showed viremia for at least 2 weeks after vaccination. When re-challenged with a high virulence strain, a few vaccinated- and low virulence CSFV-infected pigs were sporadically tested positive and 1 of 8 vaccinated-pigs was tested positive at the end of the experiment (16 days post re-challenged). However, none of the oral fluid samples from all groups were tested positive using either the real-time RT-PCR or the routine RT-PCR. It could be that the sensitivity of the tests was not sensitive enough to detect the low CSFV levels in the oral fluid samples or it could be due to the difficulty of the genetic material extraction method hampered by mucous component of the saliva.

A potential use of a commercial blocking ELISA of classical swine fever antibody detection was also evaluated comparing to a neutralizing peroxidase-linked assay (NPLA) using oral fluid samples from a CSFV negative herd mixed with serially diluted known CSFV antibody tiers. Interestingly, the low levels of NPLA antibody titers ($\log_2$ 1- $\log_2$ 3) could evidently be detected in the oral fluid samples (lower than average serum NPLA titers of pigs in the same group, $\log_2$ 3- $\log_2$ 6) but none of the oral fluid samples from this present study were tested positive by a commercial ELISA (sensitivity at least $\log_2$ 4). It should be noted that NPLA titers detected in the pooled oral fluid samples in each group were equal to or less than $\log_2$ 3, resulting in all negative detection when tested by the ELISA test kit. However, the present study proved that CSFV antibody could be detected in the oral fluid samples using NPLA and ELISA methods. Validation of the commercial blocking ELISA is undergoing to improve the sensitivity and specificity of the detection when using oral fluid samples. This evaluation contributes not only in the facilitation of CSFV antibody

detection method, but nevertheless helps in the rapid diagnosis and controlling aspects of CSFV, particularly in the CSFV-free countries.

In conclusion, the modified real time RT-PCR yielded satisfactory sensitivity than the routine RT-PCR, but the sensitivity was not good enough to detect low CSFV levels, particularly in the oral fluid samples. Effectiveness of a genetic material extraction method from oral fluid samples might be hampered by the mucous component in the saliva or other organic matters. Achievement of this objective could be done by developing a better genetic material extraction method from oral fluid samples to enhance the sensitivity of the molecular diagnosis. However, detecting CSFV antibody in the oral fluid samples is another alternative method for improved surveillance in CSFV-free areas, thereby enhancing elimination and control efforts much faster than using molecular diagnostic methods.

Introduction:

Classical swine fever (CSF) is a highly contagious viral disease causing major losses in pig populations in the endemic countries. CSF virus (CSFV) is a member of the genus *Pestivirus* within family *Flaviviridae* and an OIE "trade-disrupting" (previously List A) disease of domestic and feral pigs. According to oral histories collected in 1887, CSFV first appeared in Indiana about 1830 and in Ohio about 1833 (Kernkamp, 1961; USDA, 1889). It was termed "hog cholera" at that time because epidemics of cholera (*Vibrio cholera*) infection in humans were occurring at the same time and very much on the public's mind. CSFV was a virus discovered in 1903 when Bureau of Animal Industry (USDA) scientists found a "filterable agent" causing the disease (USDA, 1981). With the exception of Argentina, Australia, Canada, Chile, New Zealand, United States, Uruguay, and Western Europe, CSFV is endemic in most regions of the world with domestic or feral swine populations. Although domestic swine in Western Europe are generally considered free of CSFV, the virus circulates in feral swine populations, from where it occasionally re-enters domestic swine populations. Re-emergence of CSFV into the eliminated areas is common and previously occurred in recent history in Central America, Cuba, England, Germany, Mexico, Netherlands, South Africa, Thailand, and elsewhere. Empirically, current surveillance programs are not capable to preventing the movement of CSFV across borders. Pig is the only natural host of CSFV and the primary mean of virus transmission is by direct contact between infected and susceptible pigs, particularly via virus-contaminated secretions and excretions from infected pigs (oral fluids, feces, blood, urine, and nasal discharge), but indirect transmission also occurs (Ribbens et al., 2004). The rate of transmission depends on several parameters, particularly the quantity of virus excreted. Importantly, pigs may shed the virus before the onset of disease and continue to do so during the entire course of the infection (Van Oirschot, 1999). Apart from direct pig-to-pig contact, other possible transmission modes of CSFV are from pig carcass and products to susceptible pigs (Edwards, 2000). Following the slaughter process, frozen carcass and pork products are served as reservoir of CSFV. Viability of CSFV appears longer in frozen or chilled products due to protein-rich environment (Dahle and Liess, 1992). CSFV remains viable in frozen pork for years, but only a few months in chilled fresh pork (Edwards, 2000). Variation of survivability of CSFV depends on different physical and chemical environmental conditions *i.e.* initial amount of virus, temperature, pH, humidity, presence of organic matter, exposure to various chemicals and properties of the virus strain. For example, half life of virus lasts for a few days at 5°C, and a few hours at 30°C in pig feces or urine (Weesendorp et al., 2008).

Clinical Signs and Diagnosis

There are no unique clinical signs associated with CSFV infection. Clinical signs and lesions produced by CSFV vary considerably, depending on the strain of virus and various host factors, e.g., pig age, nutrition and concurrent infections. Additionally, complication with similar disease caused by ruminant pestiviruses (BVD, BVDV) mandate the requirement for differential laboratory testing. The OIE (www.oie.int/) recommends detection of CSFV using PCR-based assays, virus isolation in cell culture, or direct immunofluorescence on tissue sections from affected pigs. The OIE-recommended assays for the detection of antibody include ELISA and fluorescent antibody virus neutralization.

Prevention, Control and Surveillance

Recent CSF epidemics in Europe have shown that early recognition of CSF and prompt elimination of CSFV-infected animals is the key to control the disease (Le Potier et al., 2006). Once the infection has established itself in the population, it is difficult and costly to regain the CSFV-negative status. Historically, recognition of clinical signs associated with CSFV formed the basis of early detection of CSFV. However, the emergence of low-virulence CSFV strains and other diseases e.g. porcine circovirus associated diseases (PCVAD), African swine fever, and highly pathogenic porcine reproductive and respiratory syndrome virus (HP PRRSV) having similar clinical presentation have rendered this approach untenable.

CSFV and Oral Fluid Diagnostics

Oral fluid is the liquid present in the oral cavity. Oral fluid is a mixture of saliva and "oral mucosal transudate." Saliva is produced by the salivary glands; oral mucosal transudate enters the mouth by crossing the buccal mucosa from the capillaries.

Oral fluids contain both pathogens and antibodies. As recently reviewed by Prickett and Zimmerman (2010), economically important viral pathogens of swine present in oral fluid samples include vesicular stomatitis virus (Stallknecht et al., 1999), foot-and-mouth disease virus (Eble et al., 2004), PRRSV, PCV2 (Prickett et al., 2008a, b), and influenza virus. Specifically relevant to this proposal, Weesendorp et al (2009) reported the detection of three strains of CSFV (Brescia strain from Italy - 1951, Paderborn strain from Germany - 1997, Zoelen strain Netherlands - 1979) in oral fluids by both virus isolation and real-time RT-PCR through 44 days post inoculation (DPI).

Although the antibody concentration in oral fluid is lower than serum, antibodies in oral fluid are still of sufficient concentration to be detectable by many immunoassays. For that reason, oral-fluid samples have become the sample of choice for a number of human pathogens, e.g., HIV, hepatitis A, hepatitis B virus, and rubella (Parry et al., 1987; Frerichs et al., 1992; Connell et al., 1993; Emmons et al., 1995). Coincidentally, the first report of the detection of antibodies in oral fluids from pigs was with CSFV. Specifically, Corthier et al. (1976) reported that intranasal vaccination of pigs with the Thiverval strain of CSFV resulted in detectable antibody in pharyngeal secretions. They subsequently inoculated pigs via intranasal and intramuscular routes and measured the antibody response in serum and oral fluid (Corthier and Aynaud, 1977).

Objectives

The primary objective of this project is to optimize and validate technology capable of rapidly identifying premises infected with classical swine fever virus (CSFV) following its introduction into North America or other CSFV-free areas using oral fluid samples. Achievement of this objective will also provide technology for improved surveillance in CSFV endemic areas, thereby enhancing elimination and control efforts.

The specific objectives are focused on demonstrating the feasibility of detecting CSFV in oral fluids by modified PCR-based methods.

Year One: Develop, optimize, and validate an assay for the detection of CSFV and RT-PCR test for oral fluid samples.

1. Optimize and validate a modified RT-PCR assay for CSFV detection in oral fluid samples (comparing to the TVMDL optimized protocols)
2. Determine the RT-PCR assay detection limits (analytical sensitivity) and diagnostic sensitivity before *in vivo* experiment
3. Evaluate the earliest RT-PCR or the modified real time RT-PCR detection and document longitudinal detection in CSFV-challenged pigs

Materials and Methods

Viruses

A low virulence classical swine fever virus ALD strain kindly provided by the National Institute of Animal Health (NIAH), Thailand and a high virulent local strain (Bangkok-1950) provided by Dr. Pariwat Poolperm, Kasetsart University, Thailand were used for the routine RT-PCR and the modified real time RT-PCR validation protocols and for the challenge experiments. The stock viruses were propagated by inoculated into 4-week-old CSFV-free piglets and collected the whole blood at 7 days post inoculation (DPI). The virus titer was measured using PK-15 cell line, determined by an immunoperoxidase monolayer assay (IPMA) and kept frozen at a temperature below -70^{o}C prior use in the experiments. A local modified live CSFV vaccine (LOM strain) was also used.

Experimental design

Based on the *in vivo* pilot study results, the high virulence CSFV strain (Bangkok 1950)-inoculated pigs died within 5 days without chewing the rope while the low virulent CSFV strain (ALD)-inoculated pigs had moderate fever and mild clinical signs. The low virulence-infected pigs were able to chew on the rope and recovered within 10 DPI. However, both inoculated pigs had mark leucopenia demonstrating typical CSFV clinical signs. In order to keep the animal alive for oral fluid collection, the protocol was modified.

In the *in vivo* experiment, twenty pigs at the age of 21 days were divided into 3 groups as shown in Table 1. Pigs in group A were intramuscularly inoculated with the ALD strain (10^5TCID$_{50}$/pig) at day 0 and re-challenged with the Bangkok-1950 strain (10^3TCID$_{50}$/pig) intramuscularly at 14 days post inoculation (DPI). Pigs in group B were intramuscularly vaccinated with a commercial modified live CSFV vaccine at day 0 and challenged with the Bangkok-1950 strain similar to pigs in group A at 14 DPI. Group C was a negative control group. All pigs were observed clinical signs daily and collected clinical samples for 30 days as shown in Table 2.

Since the in vivo experimental design was modified and allowed us to successfully collect the oral fluid samples. The modified protocol might possibly mimic the CSFV situation when using modified live CSFV vaccines in CSFV-endemic countries. We expected the vaccinated- and the low virulence CSFV-infected animals would have low levels of viremia and shed virus for a few days or longer via oral fluid. After re-challenged with the high virulence strain (Bangkok-1950), a few pigs in both groups might shed the high virulence virus more or less in oral fluid or serum samples. Since we had at least 30 days of the experiment, the serum samples collected from those pigs would be useful for CSFV antibody evaluation in the future. It should be noted that pigs in the negative control group were terminated at 14 DPI due to the limitation of the facility for other projects.

Table 1: *In vivo* experimental design

Protocol	**Low virulent & high virulence challenged group (Group A: n = 8)**	**Vaccinated & high virulence challenged group (Group B: n = 8)**	**Negative control group (Group C: n = 4)**
Inoculation on the first day (IM)	ALD	LOM strain	Mock media
Inoculation after 14 days (IM)	Bangkok-1950	Bangkok-1950	ND
Oral fluid collection	0-30 DPI	0-30 DPI	0-14 DPI
Serum collection	0, 3, 7, 14, 17, 21, 30 DPI	0, 3, 7, 14, 17, 21, 30 DPI	0, 3, 7, 14 DPI
Necropsy	30 DPI	30 DPI	14 DPI

Note: Vaccine = LOM strain, Low virulence strain = ALD strain, High virulence strain = Bangkok-1950 strain; DPI = days after inoculation; ND = not done

Table 2: Clinical observation and sample collection protocol in the *in vivo* challenge experiment

Sample collection	Days post inoculation																															
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Oral fluid	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Serum	+				+				+			+				+			+				+			+			+			+
Whole blood	+				+				+			+				+			+				+			+			+			+
Clinical signs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
necropsy																+																+

Oral fluid collection and processing

- Oral fluid samples were collected from a CSFV-free herd and mixed with serially diluted ALD stock virus (known titers) *in vitro* for determination of the assay sensitivity of CSFV RNA detection by a routine RT-PCR and the modified real-time RT-PCR.
- Oral fluid samples from the challenge experiment in the animal facility were collected by cotton rope suspending in a location accessible to the pigs for at least 20 minutes before collection (Prickett et al., 2008).

The part of wet ropes were cut and squeezed to extract the oral fluids from the ropes. About 5 – 20 ml oral fluid were collected, centrifuged, aliquated and stored at - 20 ^{o}C until tested.

Serum neutralization test and ELISA

Serum neutralizing (SN) antibody titers against CSFV (ALD) were determined by a neutralizing peroxidase linked assay (NPLA) as described previously (Suradhat et al., 2001) and a commercial blocking ELISA (Idexx CSFV Ab Test) as suggested by a manufacturer. SN titer was the reciprocal of the highest dilution of the serum that completely inhibited viral infection. Both NPLA and ELISA tests were done on collected serum and oral fluid samples.

CSFV RT-PCR

To demonstrate the sensitivity of a routine RT-PCR performed in the laboratory, serially diluted known CSFV titers were used to standardize the measurement of CSFV titers in the oral fluid samples comparing with a modified real-time RT-PCR test. All experimental samples were transported to the laboratory and stored at -20^{o}C before RNA extraction.

RNA extraction: All samples were processed as soon as possible to maximize the chance of obtaining good quality viral RNA using the known virus titer (10^5 $TCID_{50}$/ml) of CSFV (ALD) stock. Total RNA was extracted by QIAamp® viral RNA mini kit (Qiagen Holden, Germany) and Invitek according to the manufacturer's instructions. The amount of total RNA extracted was compared and tested with the modified real time RT-PCR.

A routine reverse transcriptase RT-PCR protocol: In this study, a one-step RT-PCR was used to amplify the specific genome of CSFV from the oral fluid-based samples and was performed with a Master-mix kit' instruction of Veterinary Diagnostic Laboratory, Faculty of Veterinary Sciences, Chulalongkorn University. The CSFV ALD strain was mixed with the negative control swine oral fluid samples to make a virus titer of 10^4TCID_{50}/ml. Serial 10 fold dilution was done to 10^0TCID_{50}/ml. The mixtures were extracted by QIAamp® viral RNA mini kit (Qiagen) as a manufacturer's instruction. The extracted viral RNAs were tested and detected by the RT- PCR as the following protocol.

Primers for E2 gene detection

Forward primer 5' AGR CCA GAC TGG CCN TAY GA 3' (2228-2250)
Reverse primer 5' TTY ACC ACT TCT GTT CTCA 3' (2898-2880)
PCR product size is 650 bp.

One step RT-PCR reaction Mix (25 µl reaction)

Reaction component	(µl)
Access Quick™2X (Promega, USA.)	12.5
AMV Reverse transcription enzyme (Promega, USA.) 5U/ µl	0.5
Forward Primer (20 pM final)	1
Reverse Primer (20 pM final)	1
RNA template	5
Nuclease-free water	5
Total Volume	25

Thermal cycling conditions

Instrument	Step	Temp (^{o}C)	Duration (min.)	Cycles
Thermo Hybaid PxE0.2	cDNA synthesis	50	30	1
	Pre denaturation	95	15	1
	Denature	94	1	40
	Annealing	55	1	
	Extension	72	1	
	Final extension	72	10	1

Agarose gel electrophoresis: The amplicon was mixed with loading buffer and the mixture was analyzed on 1% agarose gel run in TBE buffer using electrophoresis chamber (100 volt) for 60 min. The gel was visualized by the UV illuminator to detect 650 bp PCR products.

A modified CSFV real time RT-PCR protocol: To optimize the modified real time RT-PCR protocol, the CSFV ALD strain was again used and RNA extraction was performed similarly to the routine RT-PCR before subjected to the modified real time RT-PCR as the following protocol.

Primers for 5'UTR genomic RNA detection:
CSF_PIA_DC_R: GGC CTC TGC AGC GCC CTA T
CSF_PIA_DC_F: TGC CCA AGA CAC ACC TTA AC

A modified real time RT-PCR reaction Mix

Reaction component	1-Well Plate (µl)
RT Enzyme Mix (125x)	0.16
*RT-PCR Mix (2x)	10
Forward Primer (200 nM final)	0.8
Reverse Primer (200 nM final)	0.8
RNA template	5
Nuclease-free water	3.24
Total Volume	20

*Power SYBR Green RNA-to-C_T 1-Step (Applied Biosystems, CA, USA)

Thermal cycling conditions

Instrument	Step	Temp (^{o}C)	Duration	Cycles
StepOnePlus7500 (Applied Biosystems)	Holding	48	30 min	Hold
	Enzyme Activation	95	10 min	Hold
	Denature	95	15 sec	40
	Anneal/Extend	55	1 min	

Results

Twenty 3-week-old CSFV-free pigs from a sow CSFV vaccination herd were obtained to the animal facility, injected with DRAXXIN® (*tulathromycin*) Injectable Solution (Pfizer Animal Health, USA) and acclimatized for 3 days. During the acclimatization period, pigs were previously trained to chew on the rope. Numbers of pigs were randomly divided and treated as mentioned in Table 1. Pigs were vaccinated and inoculated with the low virulence strain (ALD) as assigned, respectively. No obvious clinical signs or significant elevated body temperature (Figure 1) were observed in all pigs. The control pigs were necropsied at 14 DPI and found no CSFV positive results in serum samples or other samples. High virulence CSFV strain (Bangkok-1950) was given intramuscularly to all pigs of both remaining groups (Table 1) and again, no obvious clinical signs or fever were observed in the vaccinated pigs but elevated body temperature (103-105 F) was observed only in the ALD-inoculated pigs after re-challenged with the high virulence strain for 3 days. All remaining pigs looked clinically normal and chewed on the rope as shown in Figure 2. However, pigs (4 of 8 pigs) from both groups were found positive for CSFV in the serum tested by virus isolation in SK6 cell line at 3 days after re-challenged with the high virulence strain (data not shown). The results demonstrated that clinically normal vaccinated- or low virulence-infected pigs had mild degree depression and anorexia when re-challenged with the high virulence strain but recovered very quickly within a few days. No differences in elevated body temperature were found among pigs in all 3 groups (Figure 1).

Mild leucopenia was seen after vaccination, challenged and re-challenged for a few days (Figure 3). Macroscopic lesions including mild petechial hemorrhages in major organs such as kidney, liver, lung, lymph nodes, stomach and urinary bladder are shown in Table 3. CSFV antibody was detected from an individual serum and pooled oral fluid samples in each group using a commercial blocking ELISA compared to the results of averaged neutralizing peroxidase-linked assay (NPLA) of pigs in the same group. High levels of serum NPLA titers, possibly from maternally-derived antibodies, were detected at 3 DPI in all groups. As expected, the levels of serum NPLA titer increased in the ALD challenged group at 10 DPI and the vaccination group at 14 DPI and again after re-challenged with the high virulence strain in both groups. Interestingly, the levels of NPLA antibody titers could evidently be detected in the oral fluid samples. Using an ELISA protocol as suggested by the manufacturer compared to the conventional NPLA method demonstrated the sensitivity of the ELISA when NPLA titer was over $\log_2$ 4. It should be noted that NPLA titers detected in the pooled oral fluid samples in each group were equal to or less than $\log_2$ 3, resulting in negative detection of all oral fluid samples when tested by the blocking ELISA (Figure 3).

All samples (oral fluid = 154 samples and serum = 180 samples) were also collected from the experimental pigs as suggested by the modified protocol (Table 2) and submitted to FADDL (Plum Island) for nucleic acid purification and detection by TVMDL optimized protocols.

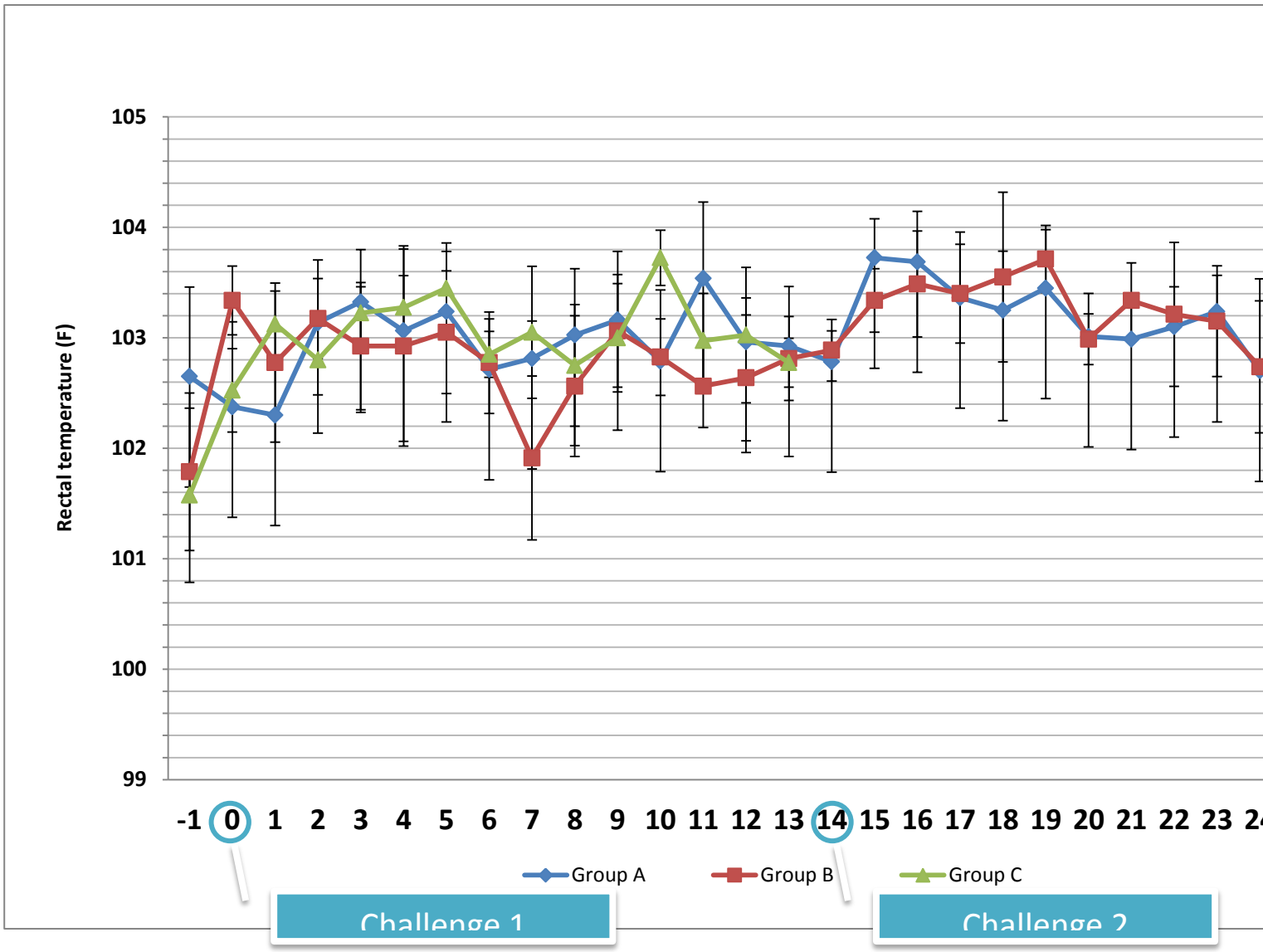

Figure 1: Body temperature of experimental pigs during the experiment

Figure 2: Clinically normal pigs in both groups (A and B) after re-challenged with the high virulence CSFV

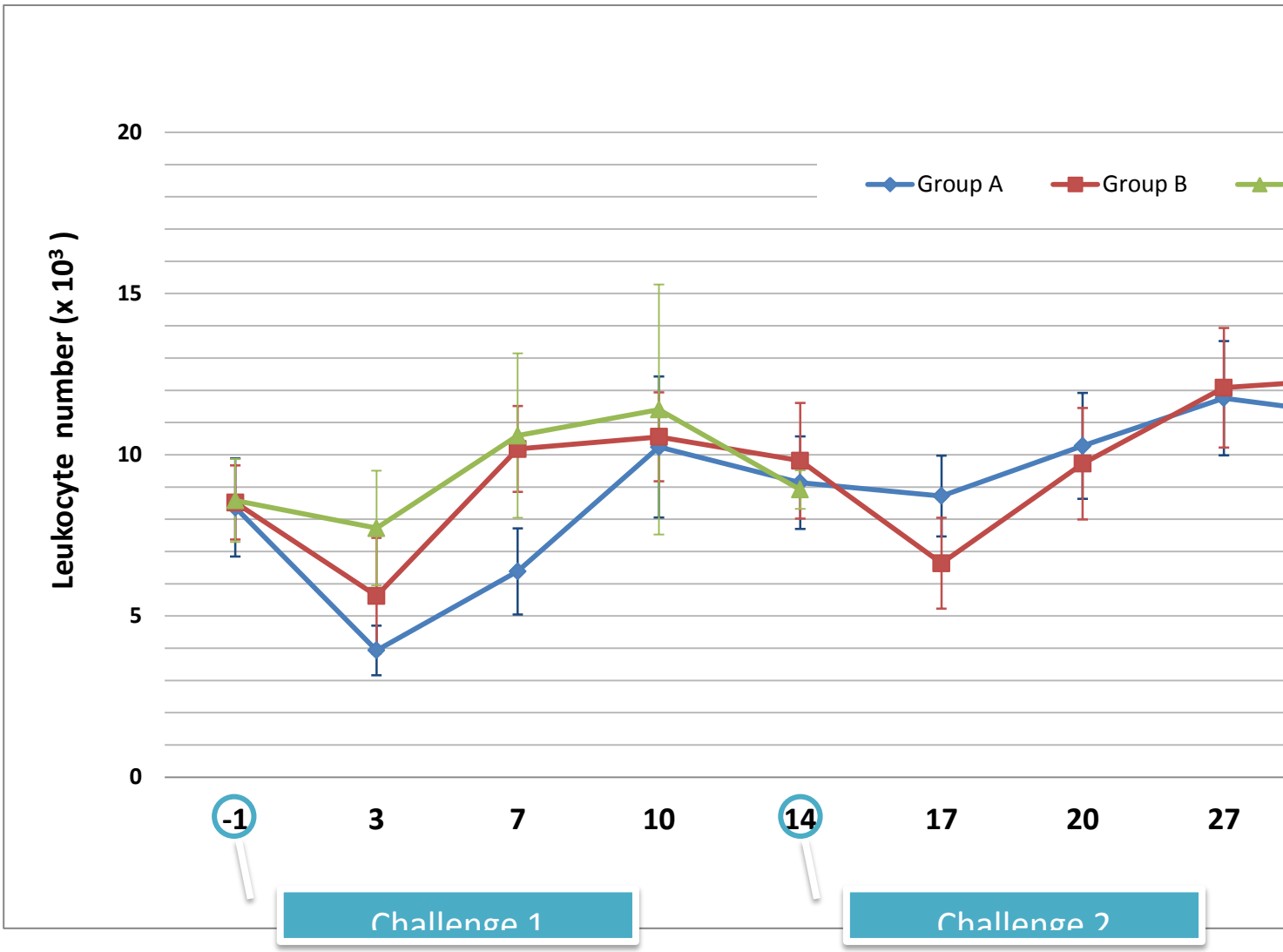

Figure 3: Leukocyte count (x 10^3 cells/dl) during the experiment

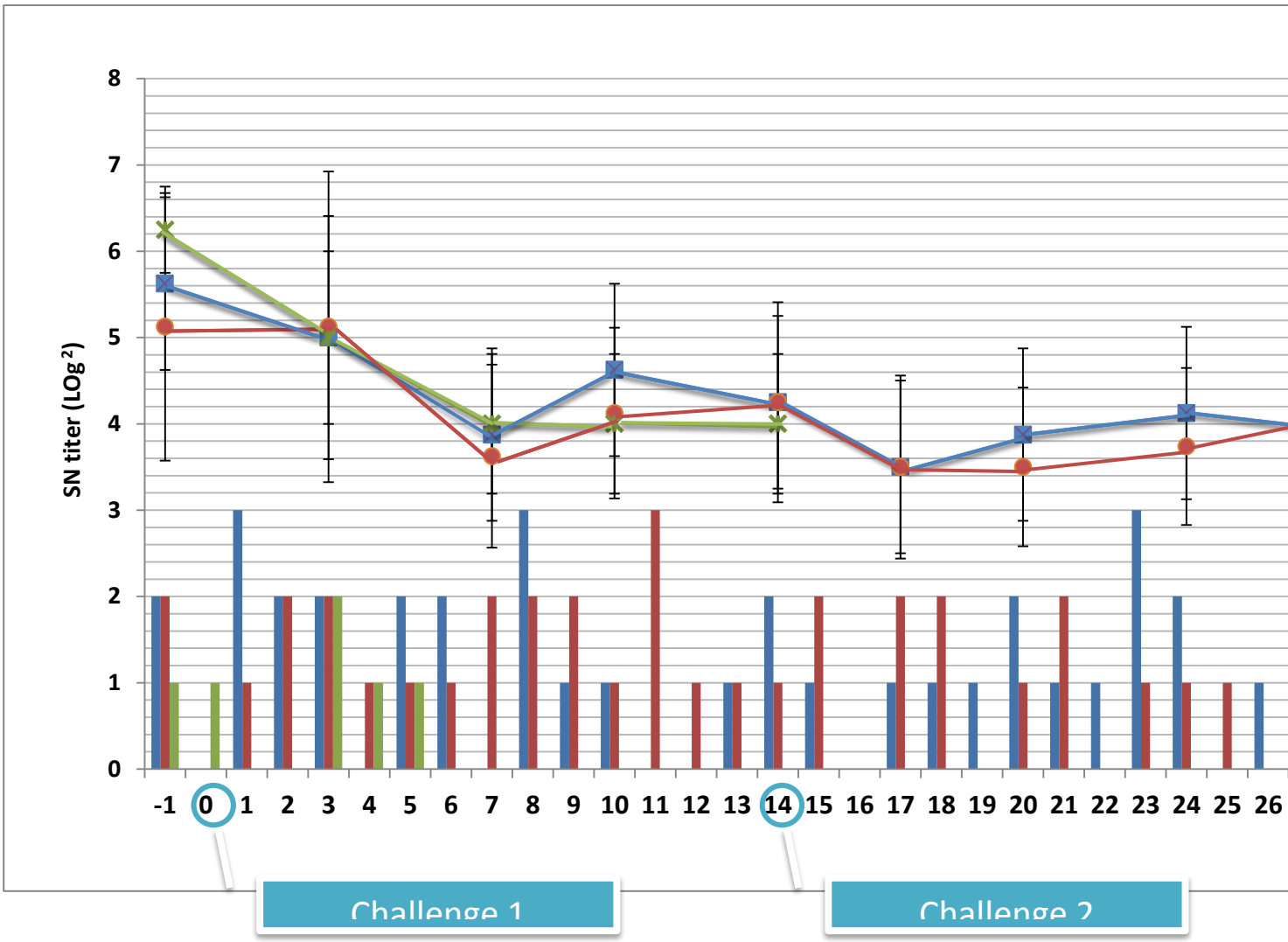

Figure 4: Serum neutralizing antibody titers in serum and oral fluid samples in each experimental group

Table 3: Macroscopic lesions when necropsied at 14 DPI in control pigs (Group C) and at 30 DPI in both infected groups (Group A and B).

	D14	**D30**	
	Group C *(n=4)*	**Group A** *(n=8)*	**Group B** *(n=8)*
Splenic infarction	0	4	2
Peripheral hemorrhage of the lymph node	0	1	0
Petechial hemorrhage of the kidney	0	2	1
Subcapsular hemorrhage of the kidney	0	0	2
Cortico-medullary congestion	0	2	0
Hemorrhage of the lung	0	3	2
Hemorrhage of the heart	0	0	2
Hemorrhage of the stomach	0	2	3
Hemorrhage of the liver	0	2	1
Hemorrhage of the urinary bladder	0	1	2
Brain congestion	1	3	5
Average lung score (0-3)	0	0.875	0.25

The modified TVMDL RT-PCR protocol sensitivity was compared with the routine protocol used in the laboratory. The results showed that the modified protocol was able to detect all tested CSFV strains including the ALD strain, vaccine strain, and local field strains while the routine protocol could not detect the vaccine strain (Figure 5). The modified protocol was optimized and then evaluated the sensitivity using the ALD strain. The results showed that the modified protocol was able to detect the ALD strain at the titer of $10^{1}TCID_{50}$/ml. while the routine protocol was able to detect the virus at the titer of $10^{3}TCID_{50}$/ml (Figure 6). The routine RT-PCR method used in the laboratory could not detect CSFV in all serum and oral fluid samples from the *in vivo* experiment.

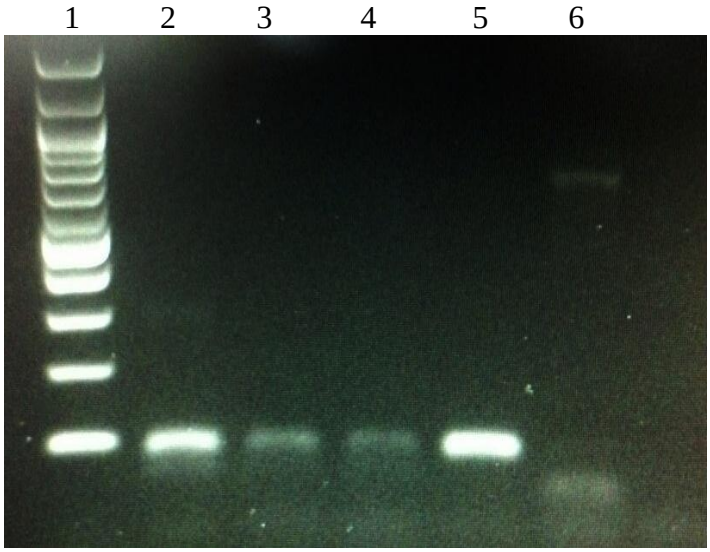

Figure 5: The modified TVMDL RT-PCR tested on various strains of CSFV. Lane 1=DNA marker 100 bp., Lane 2= ALD strain, Lane 3=vaccine strain1, Lane 4=vaccine strain 2, Lane 5=local filed strain and Lane 6= negative control

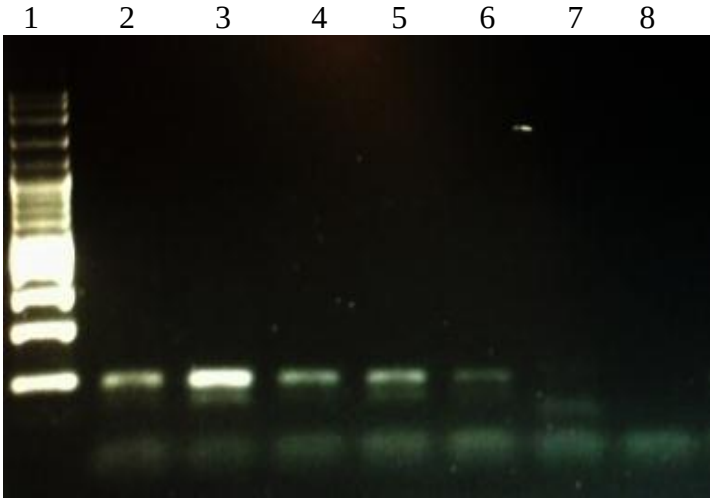

Figure 6: Sensitivity test of the modified TVMDL CSFV RT-PCR. Lane 1= DNA marker 100 bp, Lane 2-7 showed serial 10 fold dilution of CSFV ALD strain from $10^{5.25}$ to 10^{0} $TCID_{50}$/ml, Lane 8=negative control

Figure 7 showed that QIAamp® viral RNA mini kit yielded the real time RT-PCR results much faster than using Invitek. The cycle threshold (C_t) of the modified TVMDL real time RT-PCR showed that this method could detect the CSFV RNA in swine oral fluid samples at the minimal of $10^{1.5}TCID_{50}$ in the reaction mixture. The samples that gave C_t value below 33.3 with the melting temperature (T_m) of the PCR product around 81.5±0.3 were considered positive for CSFV (Table 4). Unfortunately, CSFV could not be detected in all oral fluid samples by the modified TVMDL real time RT-PCR but a few serum samples were detected positive by the modified TVMDL real time RT-PCR (Table 5).

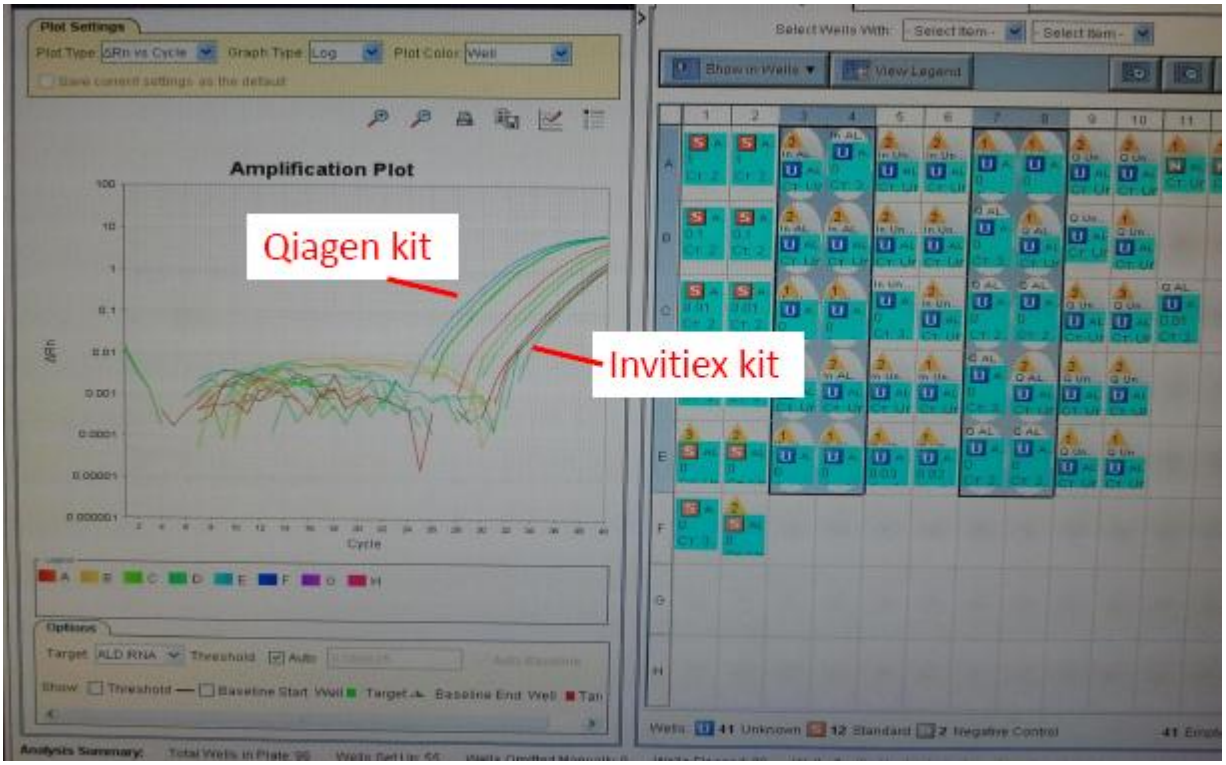

Figure 7: Sensitivity of the modified real time RT-PCR when using differences RNA extraction kits

Table 4: C_t and T_m of standard virus (CSFV ALD strain) in oral fluid samples detected by the modified real time RT-PCR

Virus titer (log $TCID_{50}$)	C_t	T_m
4.5	20.16	81.53
3.5	23.27	81.45
2.5	28.03	81.42
1.5	32.17	81.42

Based on the results from Table 5, only 1 of 8 pigs from group A after re-challenged with the high virulence virus was tested positive in the serum sample while a few vaccinated pigs were sporadically tested positive in sera at 7 DPI (2 of 8 pigs) and 14 DPI (1 of 8 pigs) and again after re-challenged with the high virulence virus 7 days (2 of 8 pigs) and 16 days (1 of 8 pigs). The results indicated that virus from the modified live virus vaccine could spread in blood circulation for at least 14 days after vaccination.

Table 5 CSFV positive in serum detected by a modified real time RT-PCR

Group	Day post inoculation									
	-1	3	7	10	14	17	21	24	27	30
A	-	-	-	-	-	-	-	+ (1/8)	-	-
B	-	-	+ (2/8)	-	+ (1/8)	-	+ (2/8)	-	-	+ (1/8)
C	-	-	-	-	-	-	-	-	-	-

Discussion

After vaccinated or challenged with the low virulence CSFV virus, all pigs had no obvious clinical signs but only mild leucopenia was observed. This scenario represents the endemic CSFV-areas having pigs with maternal-derived antibody exposed to the low virulence virus. Interestingly when re-challenged with the high virulence CSFV virus, a few pigs in both groups were tested positive for CSFV in the serum samples using the modified real time RT-PCR. The mimic situation demonstrated that subclinically CSFV-infected pigs were able to carry the virus for a few weeks without showing obvious clinical signs. When necropsied, those pigs had subclinical gross lesions including mild petechial hemorrhages in major organs. It should be noted that those reservoir animals might shed the virus sporadically without being noticed by the producers.

The modified real time RT-PCR was able to detect CSFV in serum samples of a few pigs after vaccination and after re-challenged with the high virulence virus. It should be noted that the virus isolation test from serum samples at 3 DPI of the low virulence infected group demonstrating 4 of 8 pigs having viremia (data not shown) while the modified real time RT-PCR had no positive samples. Accordingly, the virus isolation test is more sensitive than the molecular diagnosis but time consuming and inconvenience. Unfortunately, all oral fluid samples were tested negative for CSFV either by the routine RT-PCR or the modified real time RT-PCR. From this present study, RNA extraction from oral fluid samples appeared to be troublesome since high content of mucopolysaccharides in saliva and other organic matters in oral fluid samples did have the negative impact on the yielded RNA (Prickett and Zimmerman, 2010). It could also be that low CSFV levels in the oral fluid samples might exacerbate the

sensitivity of the modified real time RT-PCR. It should also be noted that during the *in vitro* pilot study for sensitivity test using the oral fluid samples from a CSFV negative herd mixed with serial dilution of known virus titers demonstrated that the sensitivity of the modified real time RT-PCR was at least $10^{1-1.5}$ $TCID_{50}$/ml. Those mentioned interfering factors would have some negative impacts when tested the samples from the *in vivo* experiment.

In addition, attempting to try another alternative diagnosis such as testing a commercial blocking ELISA using the oral fluid samples demonstrated that the manufacturer's protocol was unable to detect the CSFV antibody lower than $\log_2$ 4 of NPLA titer. Adjusting the ELISA protocol might help enhancing the sensitivity of the blocking ELISA when using oral fluid samples.

In conclusion, the modified real time RT-PCR yielded satisfactory sensitivity than the routine RT-PCR, but the sensitivity is not good enough to detect low virus levels, particularly in the oral fluid samples. Effectiveness of a genetic material extraction method from oral fluid samples might be hampered by the mucous component in the saliva or other organic matters. Achievement of this objective could be done by developing a better genetic material extraction method from oral fluid samples to enhance the sensitivity of the molecular diagnosis. However, detecting CSFV antibody in the oral fluid samples is another alternative method for improved surveillance in CSFV-free areas, thereby enhancing elimination and control efforts much faster than using the molecular diagnostic methods.

Publications and Proceedings yielded from this study.

1. Yonlayong Woonwong, Panchan Sitthicharoenchai, Korakrit Poonsook, Jirapat Arunorat, Porjit Choojai, Rachod Tantilertcharoen, and Roongroje Thanawongnuwech. CSFV neutralizing antibody detection in oral fluid samples. The 38th International Conference on Veterinary Sciences 2013, Bangkok, Thailand, January 16-18, 2013. P. 314-315.
2. Panchan Sitthicharoenchai, Yonlayong Woonwong, Korakit Poonsuk, Jirapat Arunorat, Chonnatcha Muangpaisarn, Kanokwan Samatiwat, Worapatch Konthong, Wannaporn Sattathara, Jeff Zimmerman and Roongroje Thanawongnuwech. Evaluation of a commercial ELISA test kit on classical swine fever antibody detection using oral fluid samples. The 5th ESPHM 2013, Edinburgh, United Kingdom, May 22-24, 2013 (Accepted)

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