RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of diagnostic performance characteristics of commercially-available CSFV tests – **NPB #14-087**

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Industry summary:

Classical swine fever virus (CSFV) is endemic and circulates in many regions of the world; therefore, the potential re-emergence of CSFV is a continual risk. It is in the pork producers' best interests to develop an effective CSFV detection-and-response strategy, recognizing that an effective response must be based on reliable technology capable of quickly identifying and eliminating foci of infection.

The goal of this research was to evaluate the diagnostic performance characteristics of commercially-available CSFV tests. Samples used in this study were collected from pigs (n=30) intranasally inoculated with CSFV and from pigs (n=30) vaccinated with CSFV modified live vaccine. Following the CSFV inoculation, serum samples were collected on days post inoculation 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17, 21, and 28. Serum (n=602) were tested by commercially-available CSFV assays including rRT-PCR (3 commercial assays), antigen-capture ELISAs (2 commercial assays), and antibody ELISAs (3 commercial assays). In addition, virus isolation (VI) and serum neutralization (SN) tests were also performed for comparison.

The results from the present study indicated that each CSFV assay had its limitation(s), in large part depending on the test target (virus, antigen, nucleic acid, or antibody). Notably, commercial CSFV rRT-PCRs were more sensitive for early detection, whereas antibody assays were more sensitive in later stages. Therefore, it is important to perform the assay(s) most appropriate to the stage of infection (acute vs. chronic) and intended purpose (screening vs. confirmatory). Overall, commercial rRT-PCR and antibody ELISAs are suitable for large scale screening whereas virus isolation and serum neutralization should be used exclusively as confirmatory assays.

Keywords: classical swine fever virus (CSFV), real-time RT-PCR, virus isolation, antigen- capture ELISA, antibody ELISA, serum neutralization

Scientific Abstract:

Classical swine fever virus (CSFV) is endemic and circulates in many regions of the world; therefore, the potential re-emergence of CSFV is a continual risk. It is in the pork producers' best interests to develop an effective CSFV detection-and-response strategy, recognizing that an effective response must be based on a technology and an organization capable of quickly identifying and eliminating foci of infection. The goal of this research was to evaluate the diagnostic performance characteristics of commercially-available CSFV tests.

In this study, 602 serum and 1,411 oral fluid samples were collected from inoculated (ALD strain) or vaccinated pigs (LOM strain) from -14 to 28 days post inoculation (DPI) or vaccination (DPV). Virus detection was attempted by virus isolation (VI), 3 commercial real time, reverse-transcription polymerase chain reaction (rRT-PCR) assays, and 2 commercial antigen-capture ELISAs. Antibody detection was evaluated using serum neutralization (SN) and 3 commercial antibody ELISAs.

Following inoculation with the CSFV ALD strain, clinical signs observed in infected pigs included fever (>104^{o}F), lethargy, anorexia, diarrhea, constipation, and skin hemorrhage. CSFV was isolated from serum as early as DPI 3 (20%) and as late as DPI 21(13%); by rRT-PCR as early as DPI 2 (10%) and as late as DPI 28 (27%); and by antigen-capture ELISAs as early as DPI 6 (60%) and as late as DPI 17 (21%). Neutralizing antibody was detected as early as DPI 7 (21%), with detection by commercial antibody ELISAs early as DPI 10 (15-22%). By DPI 17, there was no difference in the detection between SN and antibody ELISAs (95% vs. 84-90%).

CSFV-vaccinated pigs were positive by rRT-PCR as early as DPI 5 (20-50%) and as late as DPI 10 (28-31%). Only a few samples were positive by antigen ELISAs. CSFV antibody was detected in vaccinates as early as DPV 10 by SN (86%) and antibody ELISAs (3-14%). By DPV 21, antibody detection in vaccinated pigs was not statistically significantly different between the SN (100%) and antibody ELISAs (86.2-89.7%).

The results from the present study indicated that each CSFV assay had its limitation(s) in detection, in large part depending on the test target (virus, antigen, nucleic acid, or antibody). Notably, commercial CSFV rRT-PCRs were more sensitive for early detection, whereas antibody assays were more sensitive in later stages. Therefore, it is important to perform the assay(s) most appropriate to the stage of infection (acute vs. chronic) and intended purpose (screening vs. confirmatory). Overall, commercial rRT-PCR and antibody ELISAs are suitable for large scale screening whereas virus isolation and serum neutralization should be used exclusively as confirmatory assays.

Introduction:

Classical swine fever virus (CSFV) is a member of the genus *Pestivirus* in the family *Flaviviridae*, along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV). CSFV is an OIE-listed agent because of direct losses, losses in trade on pigs and derived products, and the substantial costs of maintaining immunization or eradication program. The potential of CSFV to spread over long distances to area previously free of CSFV is well-documented, e.g., Israel (David et al., 2011), and South Africa (Sandvik et al., 2005). This testifies to the fact that current surveillance programs are not capable to preventing all movement of CSFV across borders and justifies preparations for dealing with incursions of CSFV.

CSFV is endemic in Asia (Paton et al., 2000), many parts of South and Central America, the Caribbean, and parts of Europe and Africa (Kirkland et al., 2012). CSFV has been eliminated or excluded from domestic pig populations in the North America, Australia, New Zealand, and Western Europe (Paton and Greiser-Wilke, 2003). CSFV is transmitted between infected pigs and susceptible pigs by direct contact, or by fomites. Virus-contaminated secretions and excretion from infected pigs include oral fluid, blood, nasal discharge and urine. Pigs may shed the virus before the onset of disease (Van Oirschot, 1999). Importantly, pigs that develop

chronic forms of the disease continue to shed virus continuously or intermittently throughout their lifetime (Moennig 2000). Contaminated pork and pork products are a potential source of CSFV because the virus remains viable in frozen pork for years and for months in chilled fresh pork (Edwards, 2000).

Attenuated virus vaccines are widely used to control CSFV in endemic areas and/or eradicate CSFV using a "test and slaughter" program. Wild boars in Europe and other areas are a reservoir for CSFV and present a continual source of reinfection for domestic swine. Early recognition of CSFV and rapid elimination of CSFV-infected animals is the key to controlling the disease, as demonstrated in the recent CSFV epidemics in European countries (Kirkland et al., 2012).

The highly variable presentation of CSF precludes a diagnosis on the basis of clinical signs and lesions alone. In acute forms, the pathological picture is often hemorrhagic. The list of differential diagnoses varies with the presentation, but includes salmonellosis, erysipelas, eperythrozoonosis, pasterurellosis, actinobacillosis, *Haemophilus parasuis* infection, as well as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) infections. Therefore, accurate and rapid laboratory methods are mandatory for the detection, control, and/or elimination of CSFV. Diagnostic methods for CSFV detection include immunohistochemical and fluorescent antibody techniques for the direct detection of CSFV antigen in tissues, antigen-capture ELISAs, virus isolation in cell culture, and reverse transcription-polymerase chain reaction (RT-PCR). Serum antibody assays include the fluorescent antibody virus-neutralization test (VNT), the neutralizing peroxidase-linked assay (NPLA), and antibody ELISAs.

Objective:

The objective of the project was to guide the selection of CSFV assay(s) for use in the event of the introduction of CSFV into North America or other CSFV-free zones. This objective was achieved by evaluating the diagnostic characteristics of commercially-available CSFV tests (PCR-, antibody-, and antigen capture-based assays) using sequential samples collected from inoculated or vaccinated pigs under experimental conditions over a period of -14 to 28 day post inoculation (DPI) or vaccination (DPV).

Material and Methods:

Animals – 60 unvaccinated, CSFV antibody-negative pigs (35-40 kg) were placed in research facilities (Chulalongkorn University, Thailand). All animals were confirmed free of CSFV and other *pestiviruses* (BVDV and BDV) by antibody and PCR testing 3 times prior to CSFV inoculation (DPI -14, -7, 0). At DPI 0, pigs were intranasal inoculated with CSFV $1x10^5$ $TCID_{50}$/ml (ALD strain) or vaccinated with CSFV modified live vaccine (LOM strain).

Serum sampling – Serum samples collected prior to inoculation (DPI -14, -7, 0) were used to create a panel of known negative samples. To improve the estimates of assay performance during initial stages of the infection, subgroups of pigs were bled in rotation for the first 6 days. Subgroup 1 were bled on DPI 1 and 4; subgroup 2 on DPI 2 and 5; and subgroup 3 on DPI 3 and 6. Thereafter, all pigs were bled on DPIs 7, 10, 14, 17, 21, and 28 (See Fig 1). Serum samples were centrifuged at 1,800 *g* for 5 min, aliquoted, and stored at -80˚C until tested. Serum samples were assayed by virus isolation, CSFV real-time RT-PCR, antigen-capture ELISAs, serum neutralization and antibody ELISAs.

Oral fluid sampling – Individual oral fluid samples were collected daily from DPI -14 to 28 using unbleached 100% cotton ropes (1/2-inch) suspending for about 30 minutes prior feeding in the morning. Oral fluid samples were aliquoted in pre-randomized cryovial tubes, without centrifugation, and stored at -80˚C until tested.

Virus isolation (VI) – CSFV in serum was isolated in a SK-6 cell line and cultured at 37˚C in 5% CO_2 for 4 days. Since the virus does not cause a cytopathic effect (CPE), the presence of the virus and viral titer were determined using a peroxidase linked virus titration assay described previously (Suradhat et al., 2001).

CSFV real time RT-PCR - Serum samples were tested by three commercial rRT-PCR including (1). Qiagen® virotype CSFV RT-PCR (Labor Diagnostik GmbH Leipzig (LDL), Leipzig Germany), assigned as PCR1; (2). LSI VetMax™ CSFV (PPC) (Life Technologies, Le Bois Dieu, Lissieu, France), assigned as PCR2; and (3). Tetracore® CSFV rRT-PCR (Tetracore, Inc., Rockville, MD USA), assigned as PCR3. Testing were performed according to the manufacturers' recommended procedures.

CSFV antigen-capture ELISAs - Serum samples were tested by two commercial antigen- capture test kits including (1). IDEXX CSFV Ag Serum Plus (IDEXX Laboratories, Inc., Westbrook, ME USA), assigned as Ag1; and (2). PrioCHEK® CSFV Antigen ELISA (Prionics Lelystad, Lelystad, Netherlands) assigned as Ag2. Testing were performed according to the manufacturers' recommended procedures.

CSFV antibody ELISAs – Serum samples were assayed by three commercial antibody ELISAs including (1). IDEXX CSFV Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, ME USA), assigned as Ab1; (2). PrioCHEK® CSFV Ab2.0 (Prionics Lelystad, Lelystad, Netherlands), assigned as Ab2; and (3). BioChek CSFV-E2 Antibody Test Kit (BioChek, Scarborough, ME USA), assigned as Ab3. Samples were tested as recommended by the manufacturers.

Serum neutralization (SN) – Serum neutralizing antibody titers were determined using the neutralizing peroxidase linked assay (NPLA) as described previously (Suradhat et al., 2001). The SN titers were calculated as the reciprocal of the highest dilution of the serum that completely inhibit viral infection.

Analysis of data – Descriptive statistics were used to describe onset of detection, diagnostic sensitivity and diagnostic specificity over the course of acute infection. Qualitative responses of

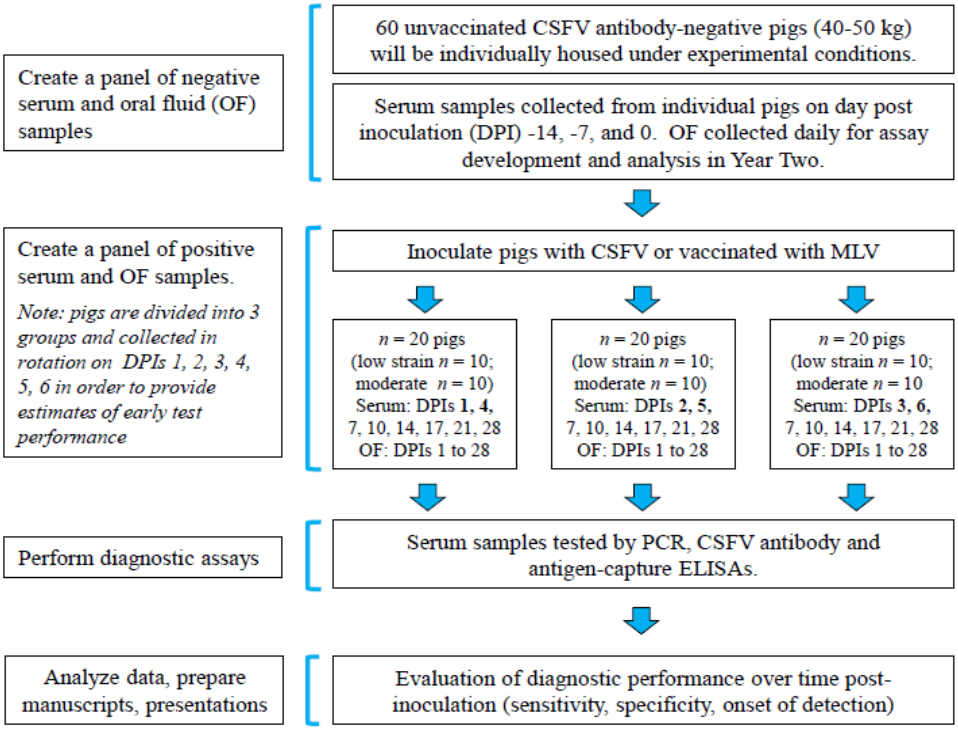

Figure 1. Experimental design

the CSFV tests (virus isolation, rRT-PCR, serum neutralization, antigen-capture and antibody ELISAs) were analyzed for significant differences among assays for each sampling time point (Cochran's Q) using a commercial software (MedCalc Software, Mariakerke, Belgium).

Results

Inoculated and vaccinated animals

After inoculation, all pigs developed various clinical signs including fever (>104°F), lethargy, anorexia, diarrhea, constipation, and skin hemorrhage (cyanosis, at tails, hind legs, and ears) between 3 and 25 days post inoculation (DPI) (Fig 2 and 3). Onset of clinical signs were observed at DPI 3 and 4. Fever was the first and only clinical sign initially showed at this period. Vaccinated animals developed only fever for 1-3 days (DPV 3 to 5) after vaccination, and remained clinically normal through the end of the study (DPV 28). At necropsy, inoculated pigs showed hemorrhagic lesions in various organs, i.e., lung, kidney, spleen, lymph nodes, urinary bladder and heart (Fig 4 and 5).

Figure 2. Inoculated pigs showed severe diarrhea at DPI 4

Figure 3. Inoculated pigs had skin hemorrhage, cyanosis at tail and hind legs

Figure 4. Multifocal infarction and hemorrhage of the spleen

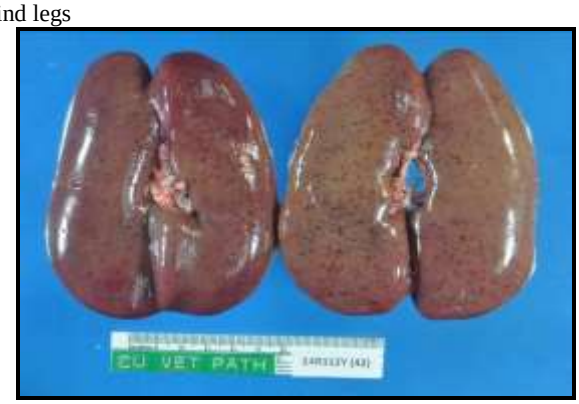

Figure 5. Petechial hemorrhage of the kidneys

Sample collection

A panel of negative and positive serum and oral fluid samples were successfully created in this study. A total of 602 serum and 1,411 oral fluid samples were collected from individual pigs from -14 to 28 day post inoculation (DPI) and vaccination (DPV). The samples were aliquoted and stored at -80ºC.

CSFV rRT-PCR

In inoculated pigs, CSFV was detected as early as DPI 2 and 3 by PCR2 (10%), and PCR1 (10%) and PCR3 (10%) respectively. The CSFV was detectable through DPI 17 (31.6%), 21 (13.3%), and 28 (26.7%) by PCR1, PCR3, and PCR2, respectively. Serum samples from inoculated pigs were 100% positive by all 3 rRT-PCR at DPI 5-7. In vaccinated pigs, modified live viruses were briefly detected in serum at DPV 5 (20%) to DPI 10

(3.4%) by virus isolation. CSFV was detected from vaccinated pigs as early as DPV 5 by PCR1 (50%), PCR3 (20%); and DPV 6 by PCR2 (20%) respectively. The CSFV remained detectable through DPV 10 by PCR1 (27.4%), PCR2 (3.4%); and DPV 14 (6.9%) by PCR3, respectively. At DPV 10, the highest percent positive were detected from PCR1 (76.7%), PCR2 (36.7%), and PCR3 (83.3%). No false positives were observed in any of the 3 commercial rRT-PCR assays.

CSFV isolation

CSFV was isolated from serum of inoculated pigs as early as DPI 3 (20%), peaked at DPI 6 (100%) and remained detectable through DPI 21 (13.3%) (Fig 6). The highest virus titer detected was at DPI 7 and DPI 10 (up to 10^5 $TCID_{50}$). The attenuated viruses were isolated from

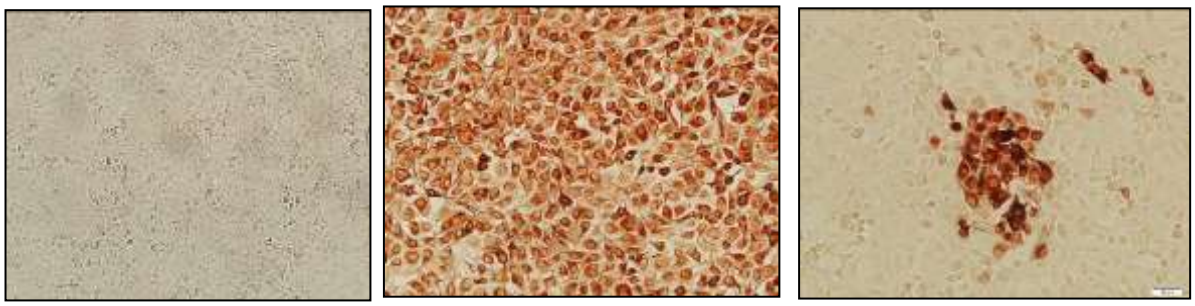

Figure 6. Example of CSFV isolation from serum using NPLA technique.

vaccinated pigs as early as DPV 5 (20%), peaked at DPV 7 (56.7%) and remained detectable through DPV 10 (3.4%). The maximum virus titer detected in vaccinated animals was 10^2 $TCID_{50}$ at DPV7 and DPV 10.

Antigen-capture ELISAs

In inoculated pigs, CSFV antigens were detected at DPI 6 (60%) to DPI 17 (21.1%) and DPI 10 (3.7%) to DPI 17 (15.8%) by Ag1 and Ag2 respectively. Ag2 gave two false positive results (out of 90) in serum of inoculated pigs. In vaccinated animals, Ag1 detected CSFV antigens only one sample at DPV 14 and gave no false positive results. Ag2 detected CSFV antigens between DPV 1 (10%) and DPV 7 (3.3%) and gave two false positive results (out of 90).

Serum neutralization (SN)

Neutralizing antibodies produced in inoculated pigs were detected as early as DPI 7 (21.4%) and became 100% positive by DPI 21. In vaccinated animals, neutralizing antibodies were detected as early as DPI 10 (86.2%) and became 100% positive at DPV 17.

CSFV antibody ELISAs

In inoculated pigs, CSFV antibody was detected as early as DPI 10 (22.2%, Ab 1 and 2) and DPI 7 (3.6%, Ab3) respectively. By DPI 21, 100% of inoculated pigs were positive by all three antibody ELISAs. In vaccinated pigs, CSFV antibodies were detected as early as DPI 10 by Ab1 (3.4%), Ab2 (6.9%); and as early as DPV 7 by Ab3 (3.3%). By DPV 28, 96.6% of samples were positive by all commercial antibody ELISAs. Ab3 gave two false positive results from serum of inoculated pigs (out of 90) and one false positive result from vaccinated pigs (out of 90).

Discussion:

The results from the present study indicated that each CSFV assay had its limitation(s) in detection, in large part depending on the test target (virus, antigen, nucleic acid, or antibody). Notably, commercial CSFV rRT-PCRs

were more sensitive for early detection, whereas antibody assays were more sensitive in later stages. Therefore, it is important to perform the assay(s) most appropriate to the stage of infection (acute vs. chronic) and intended purpose (screening vs. confirmatory). Overall, commercial rRT-PCR and antibody ELISAs are suitable for large scale screening whereas virus isolation and serum neutralization should be used exclusively as confirmatory assays.

The results of this study are in preparation for publication and presentation in the following venues:

- Manuscripts will be submitted to Veterinary Microbiology.
- 47th annual meeting of the American Association of Swine Veterinarians (AASV).
- 2015 North American PRRS Symposium (NA-PRRS) Emerging and Foreign Animal Diseases conference.

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