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Effective surveillance for early classical swine fever virus detection will utilize both virus and antibody detection capabilities

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ABSTRACT

Early recognition and rapid elimination of infected animals is key to controlling incursions of classical swine fever virus (CSFV). In this study, the diagnostic characteristics of 10 CSFV assays were evaluated using individual serum (n = 601) and/or oral fluid (n = 1417) samples collected from −14 to 28 days post inoculation (DPI). Serum samples were assayed by virus isolation (VI), 2 commercial antigen-capture enzyme-linked immunosorbent assays (ELISA), virus neutralization (VN), and 3 antibody ELISAs. Both serum and oral fluid samples were tested with 3 commercial real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays. One or more serum samples was positive by VI from DPIs 3 to 21 and by antigen-capture ELISAs from DPIs 6 to 17. VN-positive serum samples were observed at DPIs ≥ 7 and by antibody ELISAs at DPIs ≥ 10. CSFV RNA was detected in serum samples from DPIs 2 to 28 and in oral fluid samples from DPIs 4 to 28. Significant differences in assay performance were detected, but most importantly, no single combination of sample and assay was able to dependably identify CSFV-inoculated pigs throughout the 4-week course of the study. The results show that effective surveillance for CSFV, especially low virulence strains, will require the use of PCR-based assays for the detection of early infections (< 14 days) and antibody-based assays, thereafter.

1. Introduction

Classical swine fever virus (CSFV) is an OIE-listed pathogen with significant economic consequences resulting from clinical disease, lost export markets for pigs and pig-derived products, and costs resulting from control and/or eradication programs (Fernández-Carrión et al., 2016). CSFV has been eliminated or excluded from domestic pig populations in North America, Australia, New Zealand, and Western Europe (Paton and Greiser-Wilke, 2003), but remains endemic in Asia (Paton et al., 2000), parts of South and Central America, Europe, and Africa (Kirkland et al., 2012; Aiki-Raji et al., 2014).

Feral pigs in many parts of the world are a reservoir of CSFV and present a perpetual risk for the introduction of the virus into local CSFV-free domestic swine populations (Müller et al., 2011). In addition, CSFV may reach free populations via movement of commercial live pigs and CSFV-contaminated semen, fomites, or pork products. The consequence of introducing CSFV into a free zone depends on the structure of the industry, the number of farms initially infected, the population size of infected farms, and the time interval from introduction to detection (Boender et al., 2014; Relun et al., 2016; Yadav et al., 2016). As reviewed by de Vos et al. (2004), new introductions often go unrecognized for weeks or months, thereby providing the opportunity for further viral spread and complicating control efforts.

Effective control of CSFV is based on an awareness of the capacity of the virus to quickly spread into free areas and ready access to diagnostic technologies compatible with rapid and accurate detection. CSFV-infected pigs shed virus in various secretions and excretions even before the onset of clinical signs, e.g., oropharyngeal fluids, saliva, and nasal discharge within 2 days of exposure, feces and urine within 4 days, and blood by 6 days (van Oirschot, 1999; Weesendorp et al., 2011; Kirkland et al., 2012). Detectable levels of CSFV-specific antibody appear 10–15 days after exposure (Moennig, 2000). Although individual animal specimens, e.g. tonsil and peripheral blood leucocytes, are excellent diagnostic specimens, a swift and decisive response to CSFV requires the use of samples that are more easily and rapidly collected in the field and are compatible with high-throughput testing, e.g., serum and oral

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fluids. In most countries, achieving the laboratory surge capacity sufficient to meet emergency testing demands will require the use of commercially manufactured diagnostic kits. In anticipation of this scenario, the objective of this study was to evaluate the diagnostic characteristics of a variety of traditional and commercial CSFV assays for serum and oral fluids in pigs exposed to CSFV under experimental conditions.

2. Materials and methods

2.1. Experimental design

Direct and indirect methods of CSFV detection were evaluated using serum (n = 601) and oral fluid (n = 1417) samples from ALD- or LOM-inoculated pigs from −14 to 28 days post inoculation (DPI). ALD is a reference strain used in CSFV neutralization assays performed at the Chulalongkorn University Veterinary Diagnostic Laboratory (CU-VDL). LOM is a modified-live vaccine commonly used in CSFV-endemic areas of South-East Asia (Lim et al., 2016). Serum samples were tested by (1) virus isolation (VI); (2) 3 commercial real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays, i.e., Qiagen® virotype CSFV RT-PCR (PCR1); LSI VetMax™ CSFV (PPC) (PCR2); and Tetracore® CSFV rRT-PCR (PCR3); (3) 2 commercial antigen-capture ELISAs, i.e., IDEXX CSFV Ag serum plus test (Ag1) and PrioCHEK® CSFV Antigen ELISA (Ag2); (4) virus neutralization (VN); and (5) 3 commercial antibody ELISAs, i.e., IDEXX CSFV Ab Test (Ab1), PrioCHEK® CSFV Antibody 2.0 ELISA (Ab2), and BioChek CSFV E2 Antibody ELISA (Ab3). Oral fluid samples were tested by PCR1, PCR2, and PCR3. All CSFV assays were performed according to the manufacturers' recommended procedures. Descriptive statistics were used to define the onset and duration of detection over the course of the study. Qualitative test responses (negative/positive) were analyzed for significant differences in the rate of detection using the LOGISTIC procedure with binary test results as the response and test types (VI, rRT-PCR, VN, antigen and antibody ELISA) as the explanatory variable for each DPI (SAS® 9.4; SAS® Institute Inc., Cary, NC, USA). This study was conducted under the approval (#1473003) of the Chulalongkorn University Institutional Animal Care and Use Committee.

2.2. Animals

Sixty unvaccinated, CSFV antibody-negative pigs (15–16 weeks of age, 35–40 kg) were obtained from a commercial farm free of both PRRSV and CSFV. Animals were placed in research facilities (Chulalongkorn University Laboratory Animal Center, CULAC; Bangkok, Thailand) one week prior to the initiation of the experiment (30 pigs/replicate, 2 replications). Within each replication, pigs were randomized to ALD (n = 15) or LOM (n = 15) inoculation groups and housed separately by room upon arrival. To facilitate individual pig oral fluid sampling, pigs were housed individually in pens in each room. Prior to inoculation, pigs were determined to be free of other pestivirus infections (BVDV and BDV) by antibody ELISA (IDEXX BVDV p80 Ab test; IDEXX Laboratories, Inc. Westbrook, ME), antigen-capture ELISA (IDEXX BVDV Ag/Serum Plus, IDEXX Laboratories, Inc.), and RT-PCR (BVDV RT-PCR kit; QIAGEN, Leipzig, Germany) testing of serum samples collected on DPIs −14, −7, and 0.

At DPI 0, pigs in the ALD-inoculated group were intranasally administered 2 ml of a solution containing ALD strain CSFV (Asian field strain, CUVDL210-52, CU-VDL, Bangkok, Thailand) at a concentration of 1×10^5 tissue culture infective dose 50 ($TCID_{50}$)/ml. Pigs in the LOM-inoculated group were intramuscularly administered 2 ml of a solution containing LOM strain CSFV (HC-VAC, Choong Ang Vaccine Laboratories Co., Ltd., Daejeon, Korea) at concentration of 1×10^3 $TCID_{50}$/ml. The inoculum was back-titrated after pig inoculation to confirm virus viability and to verify the virus concentration. Thereafter, clinical signs and rectal temperatures (mercury thermometer per rectum for 1 min) were observed and recorded on a daily basis through the end of the study (DPI 28).

2.3. Sample collection

Blood samples (n = 601) were collected on DPI -14, -7, 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17, 21, and 28 using a single use collection system (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ). To improve the estimates of assay performance during the initial stages of the infection yet minimize animal stress, subgroups of 5 pigs each were bled in rotation for the first 6 days. Subgroup 1 was bled on DPIs 1 and 4; subgroup 2 on DPIs 2 and 5; and subgroup 3 on DPIs 3 and 6. Thereafter, all pigs were bled on DPIs 7, 10, 14, 17, 21, and 28. Blood samples were centrifuged at 3000 × g for 5 min then serum was aliquoted into prelabeled (pig ID, date, random number) cryovial tubes (Corning® Inc., Corning, NY), and stored at -80°C until tested.

Oral fluid samples (n = 1417) were collected daily from DPI −14 to 28 by suspending a 100% cotton rope (0.5″, 1.27 cm) in each pen for 30 min prior to the morning feeding. Oral fluid was harvested by passing the rope (within a plastic bag) through a hand-made wringer. Liquid in the bottom of the bag was decanted into a tube and the volume recorded. Thereafter, oral fluid samples were aliquoted into 2-ml cryovial tubes (Corning® Inc., Corning, NY) and stored at -80°C. Personnel collecting oral fluid or working with animals were strictly assigned to each room to avoid cross-contamination. Oral fluid samples (n = 1417) collected on −14, −7, 0, and then from DPIs 1 through 28 were tested, as described below.

2.4. Virus detection

For virus detection, each serum sample (20 µl) was mixed with 180 µl minimum essential medium (MEM; Gibo® by Life Technology®) and then serially 10-fold diluted (10^{-1}–10^{-8}) in MEM. Thereafter, 100 µl of each dilution was transferred to 4 wells of a 96-well plate (Corning® 96-well clear flat bottom polystyrene microplate, Corning® Inc., Corning, NY). SK-6 cells (100 µl; 3×10^5 cells/ml) were added to each well and the plate incubated (37 °C, 5% CO_2) for 72 h. After discarding the media, cell monolayers were fixed with 0.4% formaldehyde in 0.5% PBST (50 µl) for 25 min and then washed 3 times with 0.5% PBST (200 µl). Virus was visualized by adding WH303 classical swine fever-specific monoclonal antibody (50 µl; RAE0826, APHA Scientific, Surrey, UK) diluted 1:1000 in 0.5% PBST with 1% BSA. Thereafter, plates were incubated at room temperature (RT) for 1 h and washed 3 times with 0.5% PBST (200 µl). Polyclonal rabbit anti-mouse IgG/HRP (50 µl; P0161, Dako Denmark A/S, Denmark) diluted 1:300 in 0.5% PBST with 1% BSA was then added and the plate incubated at RT for 1 h. After an additional washing step, 50 µl of substrate solution, i.e., 3-Amino-9-Ethyl Carbazole (1 ml; Sigma-Aldrich, USA), acetate buffer (19 ml; 0.1 M acetic acid + 0.1 M sodium acetate) and 30% H_2O_2 (20 µl; Sigma-Aldrich), was added and the plate incubated at RT for 1 h. The plate was then washed with deionized water, air dried, and examined for color development using light microscopy. The CSFV ALD strain (10^3 $TCID_{50}$/20 ul) was used as a positive control and MEM as a negative control. The virus titer was calculated using the Reed Müench method and expressed as the median tissue culture infectious dose ($TCID_{50}$) (Reed and Muench, 1938).

2.5. Real-time reverse transcription-polymerase chain reaction (rRT-PCR)

All rRT-PCR assays were performed using the procedures and the instruments (automated extractions and thermocyclers) recommended by the manufacturers.

PCR1 Serum (140 µl) was used without pre-treatment whereas oral fluid was centrifuged at 14,000 × g for 30 s and then 140 µl of the supernatant was used for RNA extraction. Serum and oral fluid RNA extractions were performed using the QIAamp® Viral RNA Mini kit