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Short communication

E2 and E^{rns} isotype-specific antibody responses in serum and oral fluid after infection with classical swine fever virus (CSFV)

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ABSTRACT

Oral fluid sampling for the detection of classical swine fever virus infection provides a relatively inexpensive method for conducting active CSF surveillance. The purpose of this study was to detect CSFV nucleic acid and antibody in serum and oral fluid samples in a group of 10 pigs infected with the moderate CSFV strain, Paderborn. Based on clinical signs, outcome, and other results, pigs were placed into one of three disease outcome groups; Acute, Chronic and Recovered. Oral fluid and serum samples were analyzed for the presence of CSFV nucleic acid along with E2 and E^{rns} surface protein-specific IgM, IgG and IgA responses. The results were summarized into a timeline of detection events beginning with the appearance of E2-IgM in serum (3 DPI) followed by CSFV nucleic acid in serum (6 DPI), CSFV nucleic acid in oral fluid (8 DPI), E2-IgG in serum (20 DPI), and E2-IgG in oral fluid (24 DPI). The results show that a combination of molecular and serological analyses of oral fluid can be incorporated into CSF surveillance.

1. Introduction

The causative agent of classical swine fever, CSF virus (CSFV), is a positive sense, 12.4 kb single-stranded RNA virus in the genus *Pestivirus*, family *Flaviviridae*. The principal clinical outcome associated with CSFV infection is acute disease that results in high mortality. However, several recent strains are associated with a more chronic or persistent infection, which has made it more difficult to detect disease outbreaks (Depner et al., 1996). For example, an outbreak of CSF in 1997 in the Netherlands spread relatively unnoticed for 5–7 weeks (Stegeman et al., 2000). One aspect of the delay in recognition of a problem was the reduced virulence of the virus. As a result of the Dutch experience and other outbreaks caused by moderately virulent strains, there is growing need to develop and deploy low-cost active surveillance strategies as a means for early detection. One approach is the incorporation of oral fluid sampling, which has been applied successfully for the detection of porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), swine influenza virus (SIV) and CSFV (Prickett et al., 2008, 2011; Detmer et al., 2011; Dietze et al., 2017; Grau et al., 2015). CSFV nucleic acid and antibodies are readily detected in oral fluid from pigs infected with a variety of CSFV isolates (Petrini et al., 2017; Panyasing et al., 2013; Panyasing et al., 2018a, b).

The purpose of this study was to extend our knowledge of CSFV detection through the analysis of CSFV nucleic acid and antibody responses in serum and oral fluid in pigs experimentally infected with the moderately virulent genotype 2.1 strain, Paderborn (Uttenthal et al., 2003). In addition, serological responses were analyzed according to IgM, IgG and IgA isotype specificity against the major CSFV antigens, E2 and E^{rns}.

2. Materials and methods

2.1. Virus and animals

All experiments involving animals were according to Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations. Pigs were humanely euthanized by intravenous pentobarbital injection according to American Veterinary Medical Association guidelines on euthanasia. Experiments were approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee and Institutional

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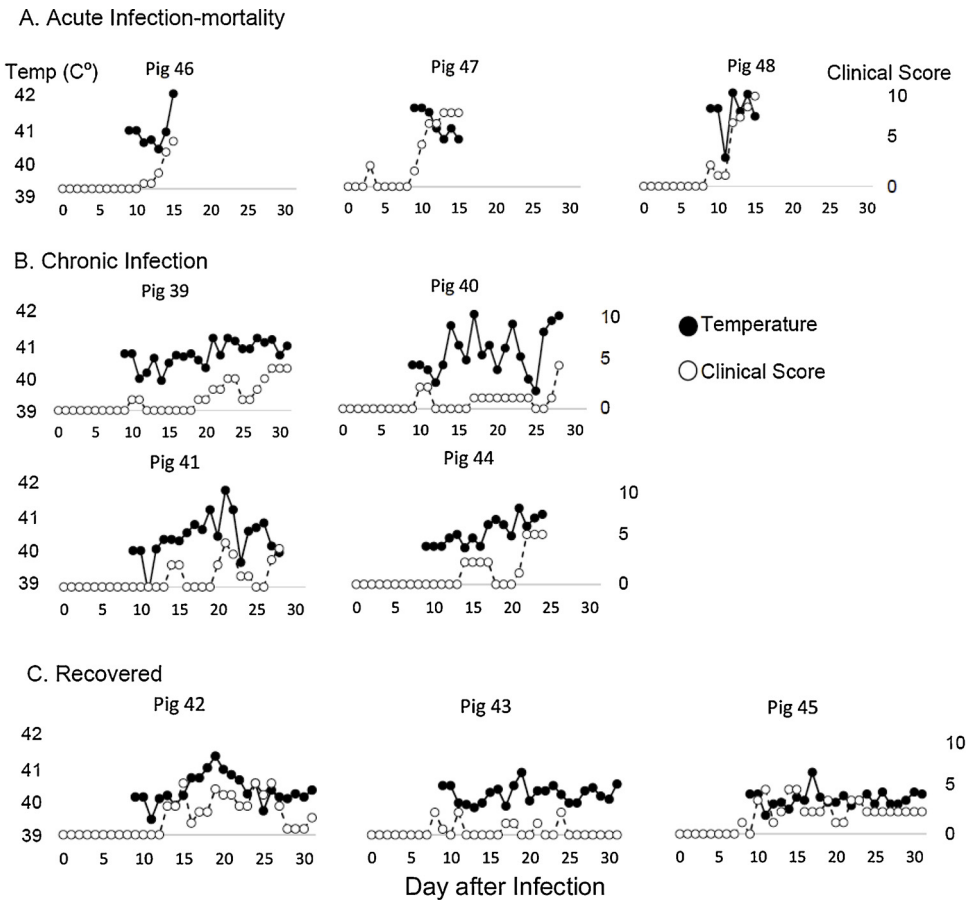

Fig. 1. Temperature and clinical score measurements for Paderborn-infected pigs.

Biosafety Committee.

The CSFV Paderborn strain was propagated on PK15 cells maintained in MEM (Life Technologies) and supplemented with 7% fetal bovine serum, L-glutamine, Penicillin/Streptomycin, and Amphotericin-B (Gibco) at 37° and 5% CO_2. At 3 days after inoculation, the media was removed and virus concentration determined by titration on PK15 cells. The tissue culture infectious dose 50% ($TCID_{50}$) was calculated according to the method of Reed and Muench (1938).

Ten White x Landrace pigs were housed in the KSU Biosecurity Research Institute (BRI) BSL-3Ag facility. After 5 days of acclimation, pigs were infected intranasally with 8×10^3 $TCID_{50}$ CSFV Paderborn in 2 ml of medium. Clinical signs were assessed daily according to Mittelholzer et al. (2000). Oral fluid samples were collected daily from the pen of pigs using the rope method (Prickett et al., 2008). Blood was collected in serum separation tubes from each pig at 0, 3, 6, 10, 14, 20, 28, and 31 days post-infection (DPI). All samples were stored at −80 °C.

2.2. CSFV nucleic acid detection

Viral RNA was measured in 50 ul of serum oral oral fluid using a MagMax-96 viral RNA extraction kit (ThermoFisher Scientific). RT-PCR, including primers used for PCR, was performed according to Risatti et al. (2003). The assay included 5 ul of total RNA and an Ag-Path-ID One-Step RT-PCR kit (Thermo Fisher) on a BioRad CFX96 Touch Real-Time PCR instrument. Log $TCID_{50}$ equivalents for serum were estimated using a standard curve constructed by assaying dilutions of tissue culture-derived virus.

2.3. Measurement of CSFV-specific antibody

For virus neutralization, heat-inactivated serum was serially diluted 1:2 in 100 ul of cell culture media on a 96-well plate. CSFV Paderborn, 20 $TCID_{50}$ in 100 ul of media, was added to each well and incubated for 1 h at 37 °C in 5% CO_2. Samples were transferred to a 96-well plate of 60–70% confluent PK-15 cells. After 1 h, wells were replaced with fresh media and returned to the incubator for three days. The plate was fixed and stained. The virus neutralization (VN) titer was reported as the last dilution negative for the presence of virus.

The recombinant E2 and E^{rns} proteins used for ELISA were based on the ALD CSFV strain, which share 89.9 and 90.2% identity, respectively, with the Paderborn peptide sequence. Cloning, expression and purification of the polypeptides are described in Panyasing et al. (2018b). E2 or E^{rns} polypeptides, diluted in PBS, were added at a final concentration of 190 ng or 250 ng, respectively, to a 96 well plate and incubated at 4 °C for 16 h. Plates were washed with PBS containing 0.05% Tween-20 (PBST) and then blocked with PBS containing 1% BSA. After 2 h at room temp, excess blocking buffer was removed and the plates were dried at 37 °C for 3 h and stored in a sealed bag with desiccant at 4 °C. Serum or oral fluid was diluted 1:50 or 1:2, respectively, in sample buffer (PBST containing 1% BSA). Samples, 100 ul, were added to each well and incubated at 37 °C for 1 h and washed with PBST. Horseradish peroxidase (HRP) -labeled secondary antibodies (100 ul/well) were added and incubation continued at 37 °C for 1 h. Secondary antibodies were prepared by conjugating isotype-specific goat anti-porcine antibodies with HRP, as described in Panyasing et al. (2018b). HRP-labeled anti-IgG was diluted 1:1000. HRP-labeled anti-IgM and IgA were diluted 1:3000. After washing, 100 µl of TMB substrate (T0440, Sigma-Aldrich) was added to each well and incubation continued at room temperature for 5 min in the dark. Stop solution (100 ul of S5814, Sigma-Aldrich) was added to each well and the plate was read at 450 nm using an ELISA plate reader (Biotek® Instruments Inc.). Results were reported as optical density (OD).

Table 1
Measurement of CSFV nucleic acid in serum[a].

		Day after Infection					
Pig	3	6	10	14	20	28	31
Acute							
46	> 45	> 45	35.4 +/− 0.7(4.6)	28.7 +/− 0.4(6.7)			
47	> 45	> 45	32.0 +/− 0.6(5.6)	25.1 +/− 0.4(7.8)			
48	> 45	37.3 +/0.7(4.0)	26.9 +/− 0.1(7.2)	25.0 +/− 0.2(8.6)			
Chronic							
39	> 45	> 45	> 45	> 45	29.4 +/− 0.2(6.4)	23.2 +/− 1(8.4)	25.6 +/− 0.2(7.6)
40	> 45	> 45	> 45	> 45	29.8 +/− 0.2(6.3)	25.0 +/− 0.2(7.8)	
41	> 45	> 45	> 45	> 45	27.5 +/− 0.1(7.0)	20.3 +/− 0.2(9.3)	
44	> 45	> 45	> 45	> 45	29.8 +/− 0.2(6.3)		
Recovered							
42	> 45	> 45	> 45	> 45	39.4 +/− 0.8(3.3)	33.0 +/− 0.1(5.3)	> 45
43	> 45	> 45	> 45	> 45	> 45	36.4 +/− 0.9(4.3)	> 45
45	> 45	> 45	33.9 +/− 0.5(5.0)	35.2 +/0.4(4.6)	> 45	37.9 +/− 0.3(3.8)	> 45

[a] The results are presented as mean CT and standard deviation for three replicate measurements of each sample. The corresponding $logTCID_{50}$ CSFV equivalents are in parentheses. The gray areas identify days that samples were not available because pigs were euthanized prior to serum collection.

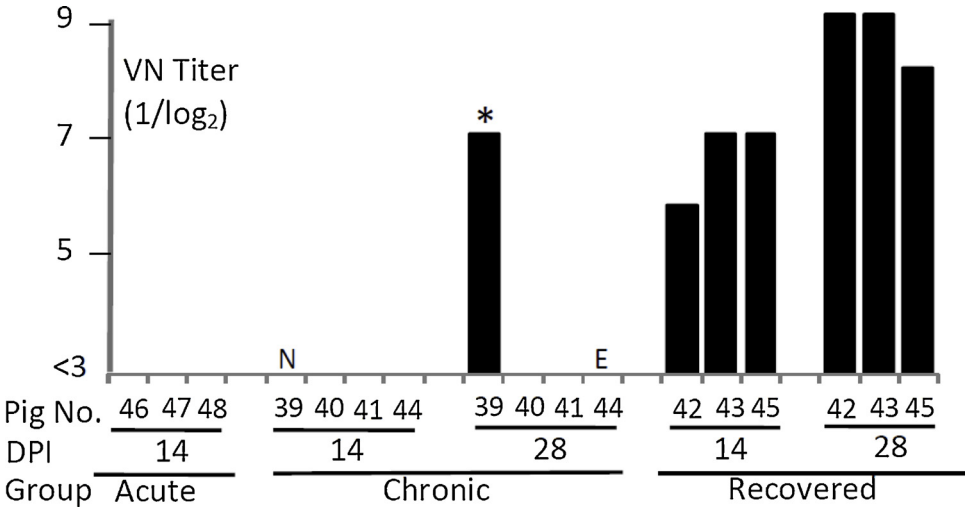

Fig. 2. Virus neutralization (VN) titers in serum. N, not done; E, no sample (pig was euthanized). *Sample collected at 31 DPI.

3. Results and discussion

Based on clinical signs and outcome, the CSFV-infected pigs could be placed into three distinct groups (see Fig. 1). In the first clinical group, pigs 46, 47, and 48 developed acute clinical signs with temperatures greater than 41 °C. At the gross anatomic level, the pigs showed a diffuse hemorrhagic congestion in lymph nodes and multifocal to diffuse ecchymoses and petechiae on the lungs and kidneys. All three pigs were euthanized by day 15 of the study and were placed in the "Acute Group" (see Fig. 1A). Pigs 39, 40, 41 and 44, exhibited early clinical signs, including pyrexia (> 40 °C), diarrhea and anorexia, but partially recovered only to have the clinical signs reappear. Three of the four pigs were euthanized at 31 days post-infection (DPI). Anatomical pathology was similar to the acute pigs. These four pigs were placed in the "Chronic Group" (see Fig. 1B). The last three pigs, 42, 43 and 45 developed pyrexia and clinical signs, but recovered without treatment. However, anatomic pathology related to CSFV infection was apparent. These last three pigs were placed in the "Recovered Group" (see Fig. 1C).

As summarized in Table 1, viral nucleic acid followed a pattern consistent with the clinical outcomes. For example, by 10 DPI all pigs in the Acute Group were positive for CSFV nucleic acid (Ct < 45) with Ct values between 25.0 and 28.7 at 14 DPI. In the Chronic Group, detectable quantities of virus appeared later, at 20 DPI. Even though Pig 45 showed detectable amounts of virus at 10 DPI, nucleic acid quantities for the Recovered Group were relatively low with peak Ct values ranging between 33.0 and 39.4.

The three outcome groups were also evaluated for virus neutralizing activity in serum and oral fluid (see Fig.2). At 14 DPI, pigs in the Acute and Chronic groups were negative for VN activity; whereas, all pigs in the Recovered Group were VN-positive, with titers between 32 and 64. At 28 DPI, the VN titers increased. Only Pig 39 in the Chronic Group was VN-positive at 31 DPI. NA activity was not detected in oral fluid (data not shown). When taken together, clinical signs, viral nucleic acid and neutralizing antibody responses were consistent with the three outcomes.

One goal of this study was to determine the ability of IgM, IgG and IgA present in serum and oral fluid to detect E2 and E^{rns} antigens. Since it was apparent that the virus and immunological responses of the Acute, Chronic and Recovered groups were different, the serum antibody results were reported for the different groups. As shown in Fig. 3, the E2 and E^{rns} IgM serum responses appeared early following infection and remained elevated throughout the study. IgM was the only CSFV-specific Ig detected in the Acute Group. At 3 DPI, IgM OD values for E2 and E^{rns} were at least 2 standard deviations above the mean of the 0 DPI values. E2-specific IgG was first detected for the recovered group at 20 DPI. E2-specific IgA reached detectable levels at 28 DPI for the Chronic and Recovered groups of pigs. E^{rns}-specific IgA OD values remained near background levels for all groups during the study period. In general, antibody responses appeared earlier and achieved higher levels in

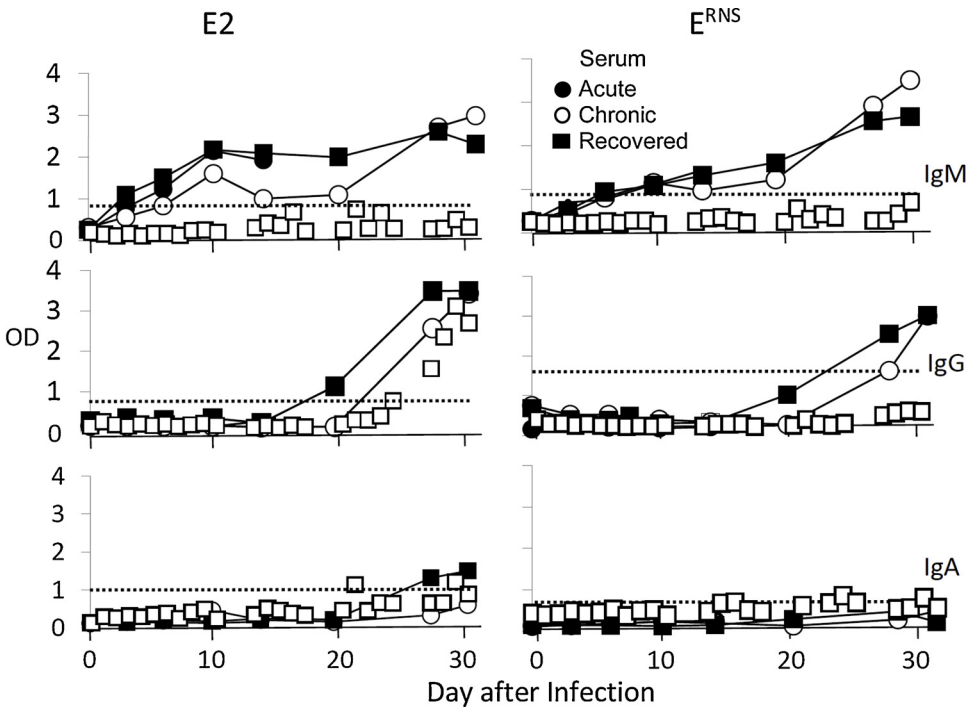

Fig. 3. CSFV antigen-specific antibody responses in serum and oral fluid. Results for serum are shown as the mean optical density (OD) for each clinical outcome group. The dotted line represents two standard deviations above the mean for the negative samples. The open squares represent the OD values for the oral fluid obtained from the group of pigs.

Table 2
CSFV RT-PCR in oral fluid samples[a].

Day	N	Ct
0-7	10	> 45
8	10	34.9 +/1.0
9	10	33.2 +/− 0.8
10	10	30.5 +/− 0.3
14	10	24.2 +/− 0.1
20	7	27.9 +/− 1.4
28	6	23.2 +/− 0.2
31	4	20.1 +/− 0.3

[a] Results expressed as mean +/1 standard deviation for three replicates.

the Recovered Group.

For oral fluid, CSFV nucleic acid was first detected at 8 DPI (see Table 2), which corresponds to the first appearance of clinical signs in the acute pigs (see Fig. 1A). CSFV nucleic acid levels in oral fluid increased from a Ct of 34.9 on 8 DPI to a Ct of 24.2 by 14 DPI. At 20 DPI, the Ct value increased to 27.9. Thereafter, the amount of virus in the remaining oral fluid samples showed decrease in Ct values until the end of the study.

The results for antibody reactivity in the oral fluid samples are shown in Fig. 3. Except for E2-specific IgG, the OD values were relatively low when compared to serum. E2-specific IgG showed an increase above background at 21 DPI. The other isotype responses were near background. The E^{rns}-specific antibody isotype responses were low and highly variable.

A timeline summarizing the results from this study is presented in Fig. 4. This timeline provides insights into how combinations of molecular and serological evaluation of serum and oral fluid can be used for detection and surveillance strategies. In general, serum is a better sample for early detection. The exception is the presence in oral fluid detectable amounts of nucleic acid at 8 DPI. This study confirms that IgG is the preferred isotype for the diagnosis of pig viral infections in both serum and oral fluid (Ramirez et al., 2012; Rotolo et al., 2017). The early appearance of IgM at three DPI is important, but is in contrast to other studies describing the first appearance of IgM-specific E2 antibody activity at two weeks after infection (Li et al., 2013). The results from this study support the notion that the detection of CSFV-specific antibody in serum or oral fluid is dependent on the strain of CSFV used for infection, the method used for antibody detection, along with the antibody isotype being detected. The results provide further support for the use of a combination of assay and sample matrices for early detection and continuous monitoring of CSF.

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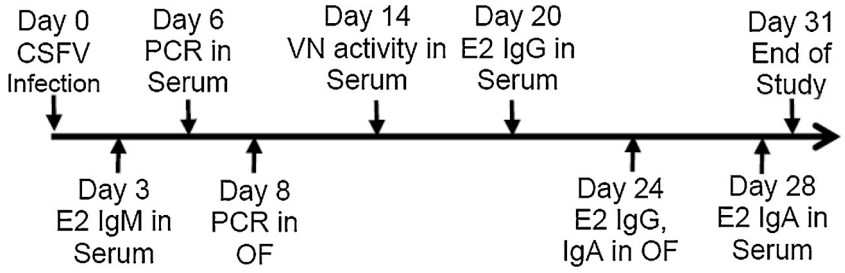

Fig. 4. Timeline for detection of CSFV nucleic acid and antibody in serum and oral fluid samples in a population of Paderborn-infected pigs. For serum and OF, the number identifies the DPI of the first positive result.

and editing the manuscript.

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