RESEARCH REPORT
SWINE HEALTH

**Title:** Working toward a CSFV oral fluid antibody ELISA: defining oral fluid antibody (IgA, IgG) kinetics against envelope glycoproteins (E2, Erns) – **NPB #15-176**

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**Industry summary:**

Classical swine fever (CSF) is a highly contagious disease causing significant economic losses to swine producers in many part of the world. Although the US has been recognized as a CSFV-free area, effective tools for disease surveillance and monitoring for an active preparedness of this disease is required. Diagnostic assays based on oral fluid have been shown to be capable of excellence diagnostic performance, are extremely compatible with efficient surveillance, and are well-accepted by producers and veterinarians. The potential use of swine oral fluid as a sample for detection of CSFV infection was evaluated in this study. The study aimed to define oral fluid antibody kinetics (IgA, IgG) against two viral glycoproteins (E2 and Erns) using ELISA technique and to initiate the foundation to the logical development of a CSFV oral fluid antibody ELISA. The results showed that IgA and IgG antibody presented at detectable levels in oral fluid of both infected pigs and vaccinated pigs. Oral fluid IgG antibody provided strong and consistent detection over the time course of infection and vaccination. Compared to IgG responses, oral fluid IgA antibody showed lower and varied in the rate of detection. The results from the present study suggest a potential use of oral fluid antibody-based assays for detecting classical swine fever infection. The development of a sensitive and highly specific oral fluid CSFV antibody ELISA will be a key tool for cost-effective, large scale screening for CSFV. A CSFV oral fluid antibody ELISA would greatly improve the preparedness of the US Pork Industry.

**Keywords:** classical swine fever virus (CSFV), ELISA, E2, Erns, IgG, IgA, oral fluid

**Scientific Abstract:**

Classical swine fever virus (CSFV) is a highly contagious disease that still need an effective tools for disease surveillance and monitoring for CSFV endemic and free-area. Diagnostic assays based on oral fluid have been shown to be capable of excellence diagnostic performance for diagnosis of many swine diseases. The aim of the study was to define oral fluid antibody (IgA, IgG) kinetics against two immunogenic envelope glycoproteins (E2, Erns) of the CSFV and initiate the foundation to the logical development of a

CSFV oral fluid antibody ELISA. In this study, a panel of well-characterized oral fluid samples (*n*=732) created from CSFV-inoculated and CSFV-vaccinated pigs were used to study the kinetics of specific-isotype antibody (IgA, IgG) against E2 and Erns proteins. Generally, IgG oral fluid antibody provided strong S/P responses and consistency in the detection than IgA oral fluid antibody in both inoculated and vaccinated animals. That is, E2 and Erns IgG antibodies could be detected in oral fluid of both inoculated and vaccinated pigs as early as DPI/DPV10 and became 100% positive by DPI 14 in infected pigs and by DPV 21 in vaccinated pigs. In contrast, E2 and Erns IgA antibody showed weaker and inconsistent in the responses. Oral fluid CSFV IgG antibody (E2 and Erns) ELISAs provided high sensitivity and specificity to detect antibody produced from inoculation and vaccination. The assays demonstrate the feasibility of using oral fluid for an active CSFV surveillance and monitoring program with ability of large scale screening and cost-effective testing.

**Introduction:**

Classical swine fever (CSF) caused by the classical swine fever virus (CSFV) is a highly contagious swine disease that results in serious financial losses. 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 areas 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 of 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).

The highly variable clinical presentation of CSF precludes a diagnosis on the basis of clinical signs and lesions alone. The list of differential diagnoses varies with the presentation, but includes African swine fever, salmonellosis, erysipelas, eperythrozoonosis, pasterurellosis, actinobacillosis, *Haemophilus parasuis* infection, as well as PRRSV and PCV2 infections. Thus, accurate and rapid laboratory methods are mandatory for the detection, control, and/or elimination of CSFV. CSFV diagnostic methods 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.

The CSFV genome contains a single open reading frame encoding a polyprotein of 3898 amino acids that undergoes co- and post-translation processing by cellular and viral proteases, giving rise to four structural proteins C, Erns, E1 and E2, and seven non-structural protein Npro, p7, NS2-3, NS4A, NS4B, NS5A and NS5B (Rümenapf et al., 1993; Meyers and Thiel, 1996; Tautz et al., 1997). Following CSFV infection, antibodies are developed against the structural proteins E2, Erns and the non-structural protein NS3. Neutralizing antibodies that confer protective immunity are induced by E2 and Erns (König et al., 1995). Therefore, the serological diagnosis of CSF is mainly based on the detection of E2- or Erns-specific

antibodies (Moser et al., 1996; Moormann et al., 2000; Clavijo et al., 2001). Erns-based ELISAs have been developed for the evaluation of CSF E2-based DIVA vaccines. Currently, commercially-available DIVA-ELISA kits include the Chekit CSF-Marker (IDEXX Laboratories, USA) and PrioCHECK CSFV Erns (Prionics, USA).

Immunogenic regions of E2 and Erns have been identified. The E2 protein possesses an immunogenic domain located in the N-terminal region of about 120 amino acids, while Erns contains an immunodominant region encompassing three overlapping antigenic regions that induce antibody responses during CSFV infection (Lin et al., 2000 & 2004). However, the antibody kinetics (IgA, IgG) in oral fluid against E2 and Erns immunogenic regions have not been described to date.

Corthier (1976) first described the presence of CSFV antibodies in swine oral fluids. Either intranasal or intramuscular inoculation with classical swine fever virus produced antibody response measurable in serum and oral fluid (Corthier and Aynaud, 1977). More broadly, a variety of antibodies, pathogens, and acute phase protein can be detected in oral fluids from swine and other animals (Prickett and Zimmerman 2010). Most major swine pathogens and/or antibodies against them have also been reported in porcine saliva and/or oral fluids.

Recent oral fluid test development has focused on PCR-based and antibody-based assay for the detection of swine infectious diseases e.g., PRRSV (Kittawornrat et al., 2012), influenza virus (Panyasing et al., 2013), as well as foreign animal diseases, e.g., classical swine fever (Grau et al., 2015), African swine fever virus (Grau et al., 2015), and foot-and-mouth-disease virus (Eblé et al., 2004; Grau et al., 2015). Cumulatively, the data support the view that oral fluid-based diagnostics have the potential for the routine detection of a broad range of infectious agents of swine, including CSFV.

**Objective:**

To establish the foundation to the logical development of a CSFV oral fluid antibody ELISA, the aim of the project was to define oral fluid antibody (IgA, IgG) kinetics against CSFV envelope glycoproteins (E2, Erns).

**Materials and methods:**

**Oral fluid samples** A total of 732 individual oral fluid samples collected from inoculated ($n$ = 298) and vaccinated ($n$ = 434) pigs at day post inoculation (DPI) or vaccination (DPV) -14, -7, 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17, 21, and 28 were used for optimization, tested, and quantify the performance of CSFV oral fluid antibody-based assay.

**CSFV E2 and Erns proteins** Selected immunogenic regions of CSFV E2 and Erns proteins were cloned, expressed and purified as recombinant polypeptides and used as antigen coated on ELISA plates. The CSFV E2 and Erns recombinant protein were expressed and purified in mammalian and boculovirus system respectively.

**Oral fluid E2 and Erns antibody isotype-specific (IgA, IgG) indirect ELISAs** The CSFV E2 and Erns recombinant protein concentration, secondary antibody dilution (IgA, and IgG), sample dilution, incubation time and temperature were optimized for an appropriate condition. The 96-well microtitration plates (Cat no. 446469, Thermo Scientific) were manually coated with E2 and Erns proteins at optimal concentration. E2 proteins (1mg/ml) were diluted with PBS at 1:500 dilution. Erns protein (0.64 mg/ml) was diluted with PBS at 1:250 dilution. After the antigen dilution was prepared, 100 µl of solution was added in each well and incubated at 4ºC overnight. Thereafter, plates were washed 5 times with washing buffers. Next, 300 µl of blocking buffer was added per well, and incubated at room temperature for 2 h.

After discarding the blocking buffer, then plates were dried at 37ºC for 3 h and stored at 4ºC in a seal bag with desiccant packs until used. Plate lot with a coefficient of variation ≥10% were excluded.

Horseradish peroxidase (HRP)-conjugated goat anti-pig IgA (A100-102P, Bethyl Laboratories Inc., Montgomery, TX), and $IgG_{FC}$ (A100-104P, Bethyl laboratories Inc.) were used as secondary antibody conjugates for oral fluid CSFV antibody-isotype specific indirect ELISAs. IgA and IgG were diluted at 1:3000 with conjugate buffer solution. Reagents used for ELISA including sample diluent, substrate, wash solution and stop solution were prepared in the laboratory.

Oral fluid samples were diluted 1:2 (100µl:100µl) with sample diluent and incubated at 37ºC for 1 h. In-house negative and positive controls were added in duplicate each plate. After sample incubation, plates were washed with 350 µl of washing buffer for 5 times. Next, 100 µl of HRP- IgA or IgG conjugates were added and then incubated at 37º C for 1 h. Washing step was repeated. Then 100 µl of TMB substrate was added, and incubated at room temperature for 5 min in dark. Thereafter, 100 µl of stop solution was added and the plates was read at a wavelength of 450 nm immediately using ELISA plate reader (Biotek® Instruments Inc.) operated with commercial software (GEN5$^{TM}$, Biotek® instruments Inc.).

**Statistical analysis** The CSFV inoculation and vaccination on antibody isotype-specific S/P ratios were analyzed by repeated measures analysis of variance (ANOVA). The optimum cut-off value, diagnostic sensitivity and specificity for each assay were determined using receiver operating characteristic (ROC) analysis (MedCalc® version 12.2.1.0, Mariakerke, Belgium).

## Results

### Oral fluid antibody (IgA, IgG) against E2 and Erns proteins in inoculated pigs

The results from the present study showed that CSFV antibody (IgA, IgG) against both E2 and Erns proteins were present in oral fluid at the detectable levels. In inoculated pigs, the S/P ratio of E2 and Erns IgG antibody responses increased significantly at day post inoculation (DPI) 14-28 ($p<0.01$). The S/P responses of E2 and Erns IgG antibody were not different over a time course of infection (DPI -14 to 28). In inoculate pigs, the S/P ratio of E2 and Erns IgA antibody responses increased significantly at DPI 14 to 21 ($p<0.01$), thereafter the S/P responses trended to decline. The Erns IgA antibody response insignificantly detected at higher level compared to E2 IgA antibody responses.

### Oral fluid antibody (IgA, IgG) against E2 and Erns proteins in vaccinated pigs

Similar to responses of CSFV-inoculated pigs, the E2 and Erns IgG responses of CSFV-vaccinated animals was significantly detected at DPI 14-28 ($p<0.01$). In vaccinated pigs, only the Erns IgA antibody was significantly detected at DPV 14-28 whereas the E2 IgA antibody responses significantly increased only at DPV 21.

**Evaluation of the test performance**

The diagnostic performances of oral fluid isotype-specific antibody (IgA, IgG) against CSFV E2 and Erns proteins were evaluated by receiver operating characteristic (ROC) curve analysis. The cut-off values for CSFV oral fluid antibody (IgA, IgG) E2 and Erns ELISAs were ≥0.3. The estimate of diagnostic sensitivity and specificity (E2 and Erns IgG) were 97% and 100% respectively. Diagnostic sensitivity and specificity of E2 IgA were 76.9% and 98.9% whereas Erns IgA were 95.5% and 97.1% respectively.

## I. Discussion:

Detection of CSFV antibody using ELISA techniques is fast, inexpensive and suitable for screening large numbers of samples. The results from the present study showed that oral fluid antibody (IgG, IgA) against both E2 and Erns proteins were present at detectable levels in both inoculated and vaccinated animals. Oral fluid antibody responses varied among target proteins (E2 vs. Erns) and isotype-specific antibodies (IgA vs. IgG). However, oral fluid IgG antibody (E2 and Erns) ELISAs provided high sensitivity and specificity to detect antibody produced from both inoculation and vaccination, with an early detection of DPI/DPV 10. This assay demonstrates the feasibility of using oral fluid for an active CSFV surveillance and monitoring program with ability of large scale screening and cost-effective testing.

**The results of this study are in preparation for publication**
Manuscripts will be submitted to Clinical and Vaccine Immunology.

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