Detection of viral pathogens of swine using oral fluid specimens

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Detection of viral pathogens of swine using oral fluid specimens

by

John Rodger Prickett

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology (Preventive Medicine)

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Ames, Iowa
2009

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Dedication

I dedicate this dissertation first and foremost to my cherished family. To my parents, who have continually challenged me while providing unconditional love. To my brother, the creature with whom I share the most blood, respect, and joy in this world.

Secondly, I dedicate this dissertation to my mentor, major professor, and friend Jeff Zimmerman. I thank him for the innumerable hours of professional and private mentorship, for sharing his wisdom and standards as a scientist and citizen, and for the dedication and respect with which he treats his “student colleagues.”
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ABSTRACT

The use of oral fluid for the assessment of health and diagnosis of disease in humans and animals has a surprisingly long history. Early investigators attempted to evaluate metabolic diseases in humans by testing oral fluid for a variety of analytes, e.g., glycogen, crystal salts, and acid salts. This early work led to the conclusion that the "principals" present in serum were also present in saliva. As early as 1909, sensitive and specific agglutination of "Micrococcus melitensis" (Brucella melitensis) by oral fluid from patients diagnosed with Malta Fever was reported, thereby, indirectly demonstrating the presence of antibody in saliva. After these early reports, developments in oral fluid diagnostics were generally overshadowed by technical improvements in the detection of analytes in blood or serum. This began to change following a report of the detection of antibodies against human immunodeficiency virus (HIV) in oral fluid from patients with acquired immunodeficiency syndrome (AIDS). Because of this report and/or because of concurrent advances in diagnostic technology, oral fluid-based assays for a wide range of infectious and non-infectious diseases, drugs, hormones, and disease markers have been developed and implemented in the last two decades.

The objective of the first study was to determine whether PRRSV and/or anti-PRRSV antibodies were present in oral fluids at diagnostic levels. The level and duration of PRRSV and anti-PRRSV antibodies in serum and oral fluids was evaluated in three age groups of pigs (4, 8, or 12 weeks of age) inoculated with a type 2 (North American) PRRSV isolate. Serum, buccal swabs, and pen-based oral fluid samples were collected for 63 days following inoculation. Specimens were assayed for PRRSV by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and for anti-PRRSV antibodies by enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA). PRRSV was detected by real-time qRT-PCR in serum for approximately 5 weeks and in oral fluids for approximately 4 weeks postinoculation. Pig age at the time of inoculation had no effect on the quantity or duration of virus in oral fluid samples. Low levels of anti-PRRSV antibody were detected in oral fluid samples by ELISA and IFA. Although the approach
remains to be validated in the field, the results of this experiment suggest that pen-based oral fluid sampling could be an efficient, cost-effective approach to PRRSV surveillance in swine populations.

The objective of the second study was to validate the use of oral fluids to detect infections with porcine respiratory and reproductive syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) in three commercial swine herds. Oral-fluid and serum samples were collected from one barn on each of three PRRSV-infected finishing sites. Six pens per barn (20 to 30 pigs per pen) were sampled repeatedly, beginning when the pigs entered the facilities (3 weeks of age), and then at 5, 8, 12, and 16 weeks of age. Serum samples were tested using a commercial PRRS ELISA. Both serum and oral-fluid samples were tested for PRRSV by quantitative reverse-transcriptase polymerase chain reaction (PCR), and oral fluids were tested for PCV2 by qRT-PCR. Site One pigs seroconverted to PRRSV at 12 weeks of age, and Site Two and Three pigs at 5 to 8 weeks of age. All individual serum samples tested PCR-negative for PRRSV in pigs 3 to 5 weeks old, while >1 sample tested positive in pigs 8, 12, and 16 weeks old, with 77% agreement between oral-fluid and serum pen-level results. At all sites, >1 oral-fluid sample tested PCR-positive for PCV2 beginning when pigs were 8 weeks old. Oral-fluid samples may be used to monitor PRRSV and PCV2 infections in commercial production systems. PRRSV virus is detectable in oral fluids for 3 to 8 weeks, and PCV2 may be detectable for >8 weeks. Sampling at 2- to 4-week intervals is recommended for surveillance of PRRSV and PCV2.

The objective of the third study was to evaluate the stability of porcine reproductive and respiratory syndrome virus (PRRSV) and anti-PRRSV antibodies in oral fluid as a function of time and temperature. A 4-liter pool of swine oral fluid was collected from 16-week old finisher pigs. To ensure uniform, quantifiable levels of virus and antibody over time, the pool was “spiked” with 4 ml of PRRSV isolate ISU-P containing $1 \times 10^{12}$ RNA copies per ml and 10 ml of concentrated hyper-immune anti-PRRSV antibodies. The pool was divided into 3 equal portions: (1) no treatment; (2) chlorhexidine digluconate at 0.01% by volume; (3) isothiazolinone at 3 parts per million. Each treatment was run in triplicate at each of five temperatures (-20°C, 4°C, 10°C, 20°C, 30°C). Samples were removed at specific intervals (0
hr, 12 hr, 24 hr, 48 hr, 72 hr, 144 hr, 216 hr, and 288 hr), stored at -80°C, and then assayed for: (1) PRRSV RNA; (2) IgM, IgA, and IgG; (3) ELISA-detectable PRRSV-specific antibody; (4) culturable bacteria per ml. The results showed that the stability of anti-PRRSV antibody and PCR-detectable PRRSV was highly temperature-dependent, with antimicrobial treatment providing no improvements in stability at lower temperatures. In particular, both virus and antibody were stable at $\leq 10^\circ$C over 12 days of storage. Conventional serum storage protocols (freezing or refrigeration at 4°C) will preserve PRRSV and anti-PRRSV antibody in oral fluid diagnostic samples.

The objective of the fourth study was to evaluate the onset, level, and duration of quantitative polymerase chain reaction-detectable PCV2 and anti-PCV2 antibody in oral fluid was evaluated using samples collected from experimentally-inoculated pigs for 98 days post inoculation (DPI). Pigs ($n = 24$) were obtained at 3 weeks of age and randomly allocated to 4 treatment pens of 6 pigs each: (1) negative control group; (2) inoculated with PCV2a (strain ISU 40895) on DPI 0; (3) inoculated with PCV2a (strain ISU-40895) on DPI 0 and re-challenged at DPls 35 and 70; (4) inoculated with PCV2a (ISU-40895), PCV2b (PVG4072), and PCV2a (ISU-4838) on DPls 0, 35, and 70, respectively. Serum was collected from each animal and one oral fluid sample was collected from each pen (group) every other day from DPI 2 through DPI 14 and weekly through 98 DPI. Oral fluid samples were assayed for the presence of PCV2 by PCR, anti-PCV2 IgG antibody by enzyme linked immunosorbent assay (ELISA), and anti-PCV2 antibody isotypes (IgA, and IgM) by ELISA. Serum was assayed for anti-PCV2 IgG by ELISA. Anti-PCV2 antibodies (IgG, IgM, and IgA) were detected in oral fluid from experimentally inoculated pigs from 14 to 98 DPI. PCV2 was detected by PCR in oral fluid samples from all pens of inoculated pigs at 2 DPI. Thereafter, PCV2 was detected in oral fluid throughout DPI 98. Overall, the data indicated that PCV2 infection in swine populations can be efficiently monitored using oral fluid specimens.
DISSERTATION ORGANIZATION

This dissertation consists of five chapters. Chapter 1 is the general introduction and review of the literature, “The development of oral fluid-based diagnostics and applications in veterinary medicine”. Chapter 2, “Detection of porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions” is published in the Journal of Veterinary Diagnostic Investigation. Chapter 3, “Surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections using oral fluid samples” is published in the Journal of Swine Health and Production. Chapter 4, Stability of PCR-detectable PRRSV and anti-PRRSV antibody in swine oral fluid spiked with PRRSV and anti-PRRSV antibody” is submitted for publication in the Journal of Swine Health and Production. Chapter 5, “Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation” is in preparation for submission to Veterinary Research. References, tables, and figures for each research manuscript follow the discussion section of each. The last chapter contains the general conclusions of the dissertation research.
CHAPTER 1. THE DEVELOPMENT OF ORAL FLUID-BASED DIAGNOSTICS 
AND APPLICATIONS IN VETERINARY MEDICINE

For submission to Animal Health Research Reviews

John Prickett

"Saliva is not one of the popular bodily fluids. It lacks the drama of blood, the sincerity of sweat and the emotional appeal of tears." Irwin D. Mandel (1990)

Introduction

The purpose of this review is to summarize the history of the development and implementation of oral fluid diagnostics for infectious diseases of human and domestic animals. The use of oral fluid for the assessment of health and diagnosis of disease in humans and animals has a surprisingly long history. Early investigators attempted to evaluate metabolic diseases in humans by testing oral fluid for a variety of analytes, e.g., glycogen, crystal salts, and acid salts (Michaels, 1901). This early work led to the conclusion that the "principals" present in serum were also present in saliva. As early as 1909, Pollaci and Ceraulo reported sensitive and specific agglutination of "Micrococcus melitensis" (Brucella melitensis) by oral fluid from patients diagnosed with Malta Fever, thereby, indirectly demonstrating the presence of antibody in saliva. After these early reports, developments in oral fluid diagnostics were generally overshadowed by technical improvements in the detection of analytes in blood or serum. This began to change following a report of the detection of antibodies against human immunodeficiency virus (HIV) in oral fluid from patients with acquired immunodeficiency syndrome (AIDS) (Archibald et al., 1986). Because of this report and/or because of concurrent advances in diagnostic technology, oral fluid-based assays for a wide range of infectious and non-infectious diseases, drugs, hormones, and disease markers have been developed and implemented in the
last two decades (Mandel, 1993; Tabak, 2007).

**Basic concepts**

The fluid in the oral cavity consists of saliva and transudates originating from the circulatory system. The major salivary glands of mammals vary in number, structure, location, and size (Shackleford and Wilborn, 1968), but humans and the domestic animals (cat, dog, pig, ruminants, horse) all possess at least three major salivary glands, as well as numerous minor salivary glands. Humans possess parotid, submandibular, and sublingual glands (Navazesh, 1993); equines and swine have parotid, mandibular, and sublingual glands (Sisson, 1975a, 1975b); ruminants have four glands, i.e., the parotid, mandibular, monostomatic sublingual, and polystomatic sublingual glands (Habel, 1975); the dog and cat have parotid, mandibular, sublingual, and zygomatic, glands, with the cat also possessing two well-developed molar salivary glands (Wiggs and Lobrime, 1997).

The composition of saliva varies among salivary glands, but regardless of species, saliva is mostly water in which are suspended a myriad of molecules with important biological functions, e.g., mucin, amylase, lysozyme, lipase, and proline-rich glycoproteins. Within the oral cavity, saliva and its constituents perform important functions related to lubrication, antimicrobial activity, cleansing, bolus formation, mastication, digestion, taste, tooth remineralization, and phonation (Llena-Puy, 2006).

In addition to saliva, the fluid in the oral cavity contains serum transudate that crosses the oral mucosa (oral mucosal transudate) and gingiva (gingival crevicular fluid) from capillaries located in the oral mucosa and the gingival tissues (Cameron and Carman, 2005; Delima and Van Dyke, 2003). The process of passive transudation was first demonstrated by intravenously injecting fluorescein dye into the hind leg of dogs (n = 6) and recording fluorescence on filter paper strips collected within and at the gingival crevice (Brill and Krasse, 1958). In these experiments, the dye appeared at the gingival crevice within 30 seconds after injection, but within 60 minutes had not appeared in the eye, hard palate, floor of the mouth, buccal, or labial mucosal, tongue, alveolar mucosa, nasal cavity, rectum, or vagina. The authors proposed that antibodies reached the oral cavity from serum via the
gingival crevice and served a protective role in the mouth. This experiment was followed by similar work in which humans ingested fluorescein dye capsules (Brill and Björn, 1959). Similar results were reported, with the addition of fluorescein dye appearing in more tissues than was seen in dogs, i.e., nasal mucous membranes. Passive transfer of virus from serum to oral fluid was demonstrated with Coxsackie B-1 virus. Rabbits were intravenously injected with Coxsackie B-1 virus (Madonia et al., 1966) and the virus was detected in oral fluid samples 2 minutes post-injection at concentrations of $1 \times 10^4$ median tissue culture infectious dose ($\text{TCID}_{50}$) per 0.1 ml or greater. As a result of these experiments, the connection between the circulatory system and the oral cavity was identified. Subsequent work established that the fluid in the oral cavity also reflected the concentration of hormones, drugs, antibodies, viruses, and other components in serum (Mandel, 1993).

In the context of this review of diagnostic applications, it is important to be aware that the composition of fluid from the oral cavity is markedly affected by the method of collection (stimulated vs. unstimulated), collection device (Chang et al., 2009), and/or the site of sample collection within the oral cavity (Atkinson et al., 1993). Atkinson et al. (1993) define "oral fluid" as the fluid in the oral cavity collected by use of an absorptive device. This describes the process and the method most commonly used to collect diagnostic samples. Therefore, "oral fluid" will be used in this sense for the remainder of this review.

**Historical developments leading to oral fluid diagnostics**

Beginning with the work of Pollaci and Ceraulo in 1909, evidence for the presence of antibodies in oral fluid accumulated slowly over the first half of the 20th century. Antibodies against *Treponema pallidum* were demonstrated in oral fluid in 1940 (Kanter and Appleton, 1940) and again in 1953 (Coleman and Appleman, 1953). Citing the work of Pollaci and Ceraulo (1909) and Coleman and Appleman (1953), Wheatcroft (1957) analyzed matched serum and oral fluid samples from persons infected with *Brucella melitensis* and demonstrated a correlation ($r = 0.674$) between serum antibody complement fixation titers and oral fluid bacterial agglutination titers.

The presence of serum proteins in human oral fluid was demonstrated in 1960 (Ellison et al.,
Kraus and Konno (1963) showed that antibody was only present in oral fluid when it was present in the serum of the same individual, concluding that this was evidence of a "selective and individually regulated transfer of serum proteins into saliva." This would later prove to be partially true, but would not fully account for all of the antibody in oral fluid.

By 1963 (Kraus and Konno, 1963), independent research had demonstrated the presence of antibody and/or antigen in human oral fluid from patients infected with a variety of pathogens. Investigators had repeatedly commented on the consistently lower concentration of antibody in oral fluid when compared to matched serum samples and had expressed concern about the impact of lower antibody concentration on the diagnostic sensitivity of antibody-based assays. Three concepts were common to the research literature of the time: (1) the absence of oral fluid reactions in seronegative subjects; (2) lower antibody titers in oral fluid vs. matched serum samples; and (3) the inconsistent results of oral fluid samples from seropositive subjects.

By 1964, five antibody isotypes (IgM, IgG, IgA, IgD, IgE) had been identified in humans (Martin 1969) and it had became evident that IgA was the predominant isotype present in oral fluid (Tomasi and Ziegelbaum, 1963). The identification of a secretory component in conjunction with IgA (South et al., 1966) and IgM (Brandtzaeg, 1975) provided evidence for local production of antibody.

**Sources of oral fluid antibodies**

Definitive evidence of the passage of serum antibody (IgG, IgM, IgA) from the circulatory system into the oral cavity was reported by Challacombe et al. in 1978 using rhesus monkeys. Specifically, intravenously injected radio-labeled IgG, IgM, and IgA was detected in oral fluid 30 minutes later (first sampling point). This simple experiment demonstrated the transfer of serum-derived antibody into oral fluid.

Local production of antibody by serum-derived plasma cells in salivary glands and duct-associated lymphoid tissue (DALT) was also described during this period (Beckenkamp, 1985; Brandtzaeg, 1981, 1989; Crawford et al., 1975; Mestecky 1987, 1993; Morrier and
Barsotti, 1990; Nair and Schoeder, 1986). These cells secreted IgA into saliva in conjunction with ductal and actinar epithelial cells expressing receptors specific for IgA. In humans, minor salivary glands (MSG) were also demonstrated to play a substantial role in IgA production, contributing 30 to 35% of total IgA in response to local antigenic stimuli (Crawford et al., 1975). IgM and IgG were also found to be locally secreted, but at lower concentrations than IgA (Challacombe et al., 1995). Corroborative data from other species supported these initial observations. For example, immune responses in the local tissues of the nasal and oral cavities were examined in mice orally immunized with biodegradable microparticles and IgA-secreting cells were demonstrated in salivary glands and nasal-associated lymphoid tissue (NALT) by ELISPOT assays and the secreted antibody was demonstrated in oral fluid by ELISA at all sampling points post-inoculation (Challacombe et al., 1997).

**Oral fluid diagnostics in human medicine**

The conclusive demonstration of systemic and locally produced antibody (IgA, IgM, IgG) in oral fluid suggested its possible use as a diagnostic specimen for infectious diseases, but the report that precipitated the development of oral fluid diagnostics was the isolation of human t-cell leukemia virus type III (HTLV-III, later renamed human immunodeficiency virus) from oral fluid collected from people with AIDS (Groopman et al., 1984). By 1986, antibodies against HTLV-III were demonstrated in patients with AIDS and/or their sexual partners (Archibald et al., 1986). This pivotal publication suggested that detection of clinically healthy individuals infected with HTLV-III could be achieved using oral fluid specimens instead of serum. This concept was quickly evaluated and pre-clinical trials on 600 individuals reported that oral fluid and serum results were equivalent (Schaefer, 1990). In 1990, the Food and Drug Administration (FDA) approved clinical trials for the comparison of a commercial oral fluid collection device at five sites within the U.S. (Schaefer, 1990) and in 1994, the FDA approved the first oral fluid collection kit for the detection HIV antibody (Nightingale, 1995).

Development of HIV detection technology continued speedily and in the period 2002 to 2006
the FDA approved four rapid HIV assays designed to detect antibodies in oral fluid specimens (Branson, 2007). Based on the data submitted for FDA approval, the first approved test (OraQuick® Rapid HIV-1 Antibody Test; OraSure Technologies, Inc., Bethlehem, PA) was estimated to have a diagnostic sensitivity of 99.6% (95% CI = 98.5%, 99.9%) and specificity of 100% (95% CI = 99.7%, 100%). An evaluation of the second generation assay (OraQuick® Advance Rapid HIV-1/2 Antibody Test; OraSure Technologies, Inc., Bethlehem, PA) reported a remarkably low false-positive rate of 0.27%, based on 166,058 tests performed between March 2005 and May 2008 at 10 New York City Sexually Transmitted Diseases (STD) clinics (Rothman and Kalish, 2009). False-positive results were determined by performing Western Blot analysis on serum samples from individuals with positive oral fluid screening results.

In human diagnostic medicine, a plethora of publications describing the development and application of oral fluid assays for HIV detection may be found in the literature, but the application of oral fluid diagnostics for pathogens and/or antibody have been described for several other infectious diseases (Table 1). Oral fluid offers some distinct advantages over serum-based testing. In particular, specimens are easily collected by personnel with minimal technical training, or even by patients in their homes, and samples are easily processed and stored (Chiappin et al., 2007). As a result, oral fluid-based testing has facilitated the collection of large amounts of epidemiological data on significant infectious agents, e.g., HIV in Africa (Connolly et al., 2004; Fylkesnes and Kasumba, 1998) and Thailand (Frerichs et al., 1994) and measles in Europe (Ramsay et al., 1997), Ethiopia (Nigatu et al., 2008), Brazil (de Azevedo Neto et al., 1995; Oliveira et al., 1998), and Africa (Ohuma et al., 2009).

**Oral fluid diagnostics in veterinary medicine**

The literature describing the detection of antibodies and pathogens in oral fluid of domesticated mammals describes core findings similar to those reported in human research. For simplicity, the veterinary research section of the review is organized by species.
Feline

**Detection of antibodies in oral fluid**  Harley et al., (1998) developed ELISA methods to quantify feline immunoglobulins in oral fluid and characterized the levels of IgG, IgM, and IgA in stimulated and non-stimulated oral fluid collected from healthy cats in 12 hour intervals for 4 days. They reported that oral fluid was most rich in IgA, stimulated oral fluid contained a lower concentration of immunoglobulin, and the concentration of immunoglobulin in oral fluid was generally consistent across multiple sampling points.

**Detection of pathogens in oral fluid**  The detection of feline leukemia virus (FeLV) antigen in feline oral fluids was first reported in the late 1980's (Lewis et al., 1987; Lutz and Jarrett, 1987) and oral fluid antigen tests are commercially available and commonly used to screen for FeLV infection. Recent publications have reported the detection of FeLV RNA in oral fluid by PCR compared to the antigen ELISA routinely used in veterinary clinics (Gomes-Keller et al., 2006a,b). They reported excellent diagnostic performance and close agreement between PCR and antigen capture ELISA results.

Feline immunodeficiency virus (FIV) was first demonstrated in oral fluid collected from inoculated cats in 1988 (Yamamoto et al., 1988). Subsequently, Poli et al., (1992) demonstrated detectable anti-FIV IgG in oral fluid samples collected from 15 of 16 seropositive cats and 0 of 16 seronegative cats. Anti-FIV IgA was also detected in oral fluid from 13 of 16 seropositive cats and 0 of 16 seronegative cats.

In addition to feline retroviruses, PCR detection of *Candidatus Mycoplasma turicensis’* (CMt) in oral fluid from cats was reported by Willi et al. (2006). Dean et al. (2007) corroborated the original report and also detected *Mycoplasma haemofelis* (Mhf) and *Candidatus Mycoplasma haemominutum* (CMhm) in oral fluid.

Canine

**Detection of antibodies in oral fluid**  Immunoglobulins in canine oral fluid have been described by Heddle and Rowley (1975) and in greater detail by German et al., 1998. As in other species, canines produce local IgA and IgM, but oral fluid also contains serum-derived IgG and albumin. German et al. (1998) found little variation in total immunoglobulin
concentration between multiple oral fluid samples collected from the same animal over time. In dogs experimentally challenged with *Taenia pisiformis*, specific IgG and IgA was detectable for 12 weeks post-inoculation, the end of the study (Kinder et al., 1992). The authors suggested oral fluid specimens could serve as an alternate method of detecting intestinal helminth infection and replace the traditional, but relatively diagnostically insensitive, process of detecting proglottides or eggs in fecal samples.

**Detection of pathogens in oral fluid** Of public health interest, some zoonotic pathogens are detectable in canine oral fluid. Rabies virus is commonly transmitted via animal bites and has been demonstrated in canine oral fluid (Côrtes et al., 1979; Fekadu et al., 1982; Kasempimolporn et al., 2000). Several strains of *Bartonella* spp. have been detected in canine saliva by PCR (Duncan et al., 2007).

**Equine and Ovine** In equines, local production of immunoglobulins in parotid and submandibular salivary glands has been demonstrated (Genco et al., 1969; Hurlimann and Darling, 1971). In sheep, IgA is the major immunoglobulin in oral fluid (Smith et al., 1975). Experiments using radio-labeled iodine demonstrated that the majority of IgA in sheep oral fluid is of local origin, with IgM selectively transported into respiratory secretions and saliva (Scicchitano et al., 1986). Diagnostically, equine oral fluid has been used to test for the presence of performance altering chemicals in race horses (Homer, 1976; Morgan and Gellhorn, 1947), but no reports of the use of oral fluid for the detection of infectious diseases were found for equines or ovines.

**Bovine** While oral fluid samples have been used to isolate and culture bacterial pathogens (*E. coli* and *Salmonella*) from feedlot cattle (Smith et al., 2004, 2005; Renter et al., 2004; Standford et al., 2005), the detection of foot-and-mouth disease virus (FMDV) has been the major focus of research in bovines. The earliest work on oral fluid reported the presence of anti-FMDV neutralizing antibody, presumably locally produced, in calves following nasal inoculation with live type 0 FMDV (Figueroa et al., 1973). Archetti et al., (1995) measured total anti-FMDV immunoglobulin and IgA in oral fluid and probang (oropharyngeal fluid) samples of
vaccinated or FMDV-infected animals. Vaccinated cattle did not generate an antibody response detectable in either sample type, but cattle exposed to, or inoculated with, live vaccine produced detectable IgA in oral fluid. Later work led to the concept that secretory IgA in oral fluid may be used to differentiate vaccinated from FMDV-infected animals (Parida et al., 2006). Parida et al. (2006) developed an ELISA to detect IgA antibody against non-structural proteins (NSP) in oral fluid under the premise that vaccines used in response to an FMDV outbreak would not include NSPs. Considering their work preliminary, they reported a diagnostic specificity of 99.4% and suggested a potential application for detecting persistently infected, sub-clinically infected, or vaccinated animals.

Porcine Detection of antibodies in oral fluid The first report of antibodies in oral fluid from pigs was published in 1976 when Corthier (1976) reported that intranasal vaccination of pigs with the Thiverval strain of classical swine fever virus (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). Strong responses were measured in both sample types, indicating both systemic and local immune responses. The strongest local response (in oral fluid) was observed after intranasal inoculation at the highest dose used.

DeBuysscher and Dubois (1978) inoculated pigs with E. coli strain 1261 via oral or Thiry-Vella loop routes and examined the submandibular and sublingual salivary glands post mortem by staining for anti-E.coli plasma cells of the IgA, IgM, and IgG isotypes. They reported IgA-secreting plasma cells to be most numerous followed by approximately equal numbers of IgM- and IgG-secreting cells, but found no significant differences in the number and isotype of plasma cells in submandibular and sublingual salivary glands between pigs orally or Thiry-Vella loop inoculated pigs.

A few years later DeBuysscher and Berman (1980) performed essentially the same experiment, but with transmissible gastroenteritis virus (TGEV). They observed substantial increases in the number of IgA-secreting cells in salivary glands, followed by IgM-secreting
cells, and a small increase in the number of IgG-secreting cells in both orally and Thiry-Vella loop inoculated pigs. These findings supported the conclusion that corollary increases in antigen-specific plasma cells in intestinal mucosa and distant secretory tissues, i.e., salivary glands, result from the secretory component receptor-conveyor mechanism.

Loftager et al. (1993) collected samples from pigs intranasally inoculated with *Actinobacillus pleuropneumoniae* (APP) and pigs naturally infected with APP. IgA concentrations in oral fluid and serum collected over time were measured using a whole-cell ELISA. IgA was detectable in oral fluid before it appeared in serum and declined more rapidly than serum IgA or IgG. These investigators concluded that oral fluid IgA detection could serve as a practical method to screen for early infection with APP.

**Detection of pathogens in oral fluid** Several swine pathogens are known to be present in detectable levels in oral fluid samples. Vesicular stomatitis virus (VSV) has been isolated from oral fluid, but not serum, from pigs infected via mechanical vectors or by direct contact with infected pigs (Stallnecht et al., 1999). In pigs inoculated with FMDV (O TAW 3/97), virus was recovered by virus isolation for up to 10 DPI and by PCR for up to 11 DPI (Eblé et al., 2004). Recently, the detection of porcine respiratory and reproductive syndrome virus (PPRSV) and porcine circovirus type 2 (PCV2) was reported in swine oral fluid samples collected under both experimental and field conditions (Prickett et al., 2008a,b).

**Future applications of oral fluid diagnostics in veterinary medicine**

Cumulatively, the literature strongly supports implementation of oral fluid-based diagnostics. Pathogen-specific IgA, IgM, and IgG antibodies have all been demonstrated in oral fluid collected from diverse domestic animal species in response to infection. A variety of infectious agents, both local and systemic, are shed in oral fluid, including some of the most economically significant pathogens of production animals, e.g., FMDV, CSFV, and PRRSV. From the perspective of animal welfare, oral fluid sampling also offers a more "animal friendly", non-invasive method to collect diagnostic specimens. Taken together, the diagnostic properties of oral fluid present an opportunity to formulate diagnostically sensitive and specific oral fluid-based assays.
Application of oral fluid-based testing facilitates monitoring, surveillance, and detection of disease in animal populations. For example, despite our best efforts, PRRSV, M. hyopneumoniae, influenza, PCV2, and other endemic pathogens continue to cause significant economic losses in large U.S. swine production systems (Holtkamp D, Rotto H, Garcia R. 2007. The economic cost of major health challenges in large U.S. swine production systems. Proc 2007 American Association of Swine Veterinarians Annual Meeting. Orlando, Florida, pp. 85-89). In part, the disease status quo is maintained by the lack of timely information on the circulation of pathogens. Oral fluid testing offers an opportunity to easily collect herd-level disease data on a periodic basis. Integration of longitudinal disease data with herd records (interventions, morbidity, mortality, and production parameters) would provide for (1) appropriately timed and targeted interventions; (2) "real time" evaluations of interventions; and (3) improved, herd-specific estimates of the impact of specific pathogens on pig health and productivity. This proactive approach to disease monitoring translates diagnostic costs into improved growth performance rather than a historical vignette of past disease events.

Once established for the routine management of endemic diseases, an oral fluid surveillance infrastructure would facilitate rapid data collection in situations such as regional or national disease control and/or eradication programs or foreign animal disease (FAD) contingency efforts. Collection of diagnostic specimens by on-site personnel would profoundly impact the time to detection and effectiveness of control of FAD outbreaks.

The purpose of this review was to summarize the history of the development and implementation of oral fluid diagnostics for infectious diseases of human and domestic animals. A tangential objective is to promote research in diagnostic applications of oral fluid in veterinary medicine, with the hope that animal health can eventually realize the benefits offered by oral fluid diagnostics. At this point, research is necessary to develop and standardize collection methods, optimize diagnostic assays for the oral fluid specimens, and establish sampling protocols for pathogens of interest. Ultimately, point-of-care rapid assays, i.e., cow-side, sow-side, or pen-side tests, will revolutionize our delivery of health management services.
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glands and mucosal immunity. Immunology 57:171-180.
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92:636-638.
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vaccination surveillance. Vaccine 24:1107-1116.


Table 1. Selected examples of diagnostic applications for the detection of infectious diseases of humans based on oral fluid specimens

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Target analyte</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>IgG</td>
<td>Litt et al., 2006</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>virus (PCR)</td>
<td>Wang et al., 2004</td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td>IgG</td>
<td>Moss et al., 2004</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>IgG, IgA</td>
<td>Hochman et al., 1998</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>IgG, IgA</td>
<td>Cuzzubbo et al., 1998</td>
</tr>
<tr>
<td>Diphtheria toxoid</td>
<td>IgG, IgA</td>
<td>Newcomb et al., 1969</td>
</tr>
<tr>
<td>Ebola virus</td>
<td>IgG, PCR</td>
<td>Formenty et al., 2006</td>
</tr>
<tr>
<td>Epstein-bar virus</td>
<td>IgG, IgA</td>
<td>Hochman et al., 1998</td>
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<tr>
<td>Hantaan virus</td>
<td>IgG, IgM</td>
<td>Petraityte et al., 2007</td>
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<tr>
<td><em>Helicobacter pylori</em></td>
<td>IgG</td>
<td>Luzza et al., 1995</td>
</tr>
<tr>
<td>Hepatitis A, B, C</td>
<td>IgM</td>
<td>Amado et al., 2006</td>
</tr>
<tr>
<td>Human herpes virus type 1</td>
<td>virus (PCR)</td>
<td>Scott et al., 1997</td>
</tr>
<tr>
<td>Human herpes virus type 6</td>
<td>virus (PCR)</td>
<td>Collot et al., 2002</td>
</tr>
<tr>
<td>Human leptospirosis</td>
<td>IgM</td>
<td>da Silva et al., 1992</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>IgA</td>
<td>Waldman, 1968</td>
</tr>
<tr>
<td>Japanese encephalitis</td>
<td>IgG, IgM, IgA</td>
<td>Prasad et al., 1994</td>
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<tr>
<td>Measles</td>
<td>IgM</td>
<td>Brown et al., 1994</td>
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<td>Mumps</td>
<td>Neutralizing antibody</td>
<td>Chiba and Nakao, 1972</td>
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<tr>
<td>Mumps</td>
<td>IgM</td>
<td>Frankova and Sixtova, 1987</td>
</tr>
<tr>
<td>Norwalk virus</td>
<td>IgG, IgA</td>
<td>Moe et al., 2004</td>
</tr>
<tr>
<td>Polio virus</td>
<td>IgA</td>
<td>Berger, et a., 1967</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>IgA</td>
<td>Ward et al., 1992</td>
</tr>
<tr>
<td>Rubella</td>
<td>IgG</td>
<td>Ben Salah et al., 2003</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>IgA</td>
<td>Herath, 2003</td>
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<tr>
<td><em>Treponema pallidum</em></td>
<td>IgG</td>
<td>Baguley et al., 2005</td>
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<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>IgG</td>
<td>Pinho et al., 1999</td>
</tr>
<tr>
<td>Varicella-zoster virus</td>
<td>virus (PCR)</td>
<td>Furuta et al., 2004</td>
</tr>
<tr>
<td><em>Yersinaia entercolitica</em></td>
<td>IgG</td>
<td>Grönblad and Mäkelä,1986</td>
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</tbody>
</table>
CHAPTER 2. DETECTION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) INFECTION IN PORCINE ORAL FLUID SAMPLES: A LONGITUDINAL STUDY UNDER EXPERIMENTAL CONDITIONS


J. Prickett, R. Simer, J. Christopher-Hennings, K.-J. Yoon, R.B. Evans, J.J. Zimmerman

Abstract

Isolation of porcine reproductive and respiratory syndrome virus (PRRSV) from oral fluids was first reported in 1997. The objective of the present study was to determine whether PRRSV and/or anti-PRRSV antibodies were present in oral fluids at diagnostic levels. The level and duration of PRRSV and anti-PRRSV antibodies in serum and oral fluids was evaluated in three age groups of pigs (4, 8, or 12 weeks of age) inoculated with a type 2 (North American) PRRSV isolate. Serum, buccal swabs, and pen-based oral fluid samples were collected for 63 days following inoculation. Specimens were assayed for PRRSV by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and for anti-PRRSV antibodies by enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA). PRRSV was detected by real-time qRT-PCR in serum for approximately 5 weeks and in oral fluids for approximately 4 weeks postinoculation. Pig age at the time of inoculation had no effect on the quantity or duration of virus in oral fluid samples. Low levels of anti-PRRSV antibody were detected in oral fluid samples by ELISA and IFA. Although the approach remains to be validated in the field, the results of this experiment suggest that pen-based oral fluid sampling could be an efficient, cost-effective approach to PRRSV surveillance in swine populations.
Introduction

‘Oral fluid’ is a mixture of saliva and mucosal transudate. Saliva is produced by the parotid, submandibular, and sublingual salivary glands, as well as the minor salivary glands located on the lips, tongue, palate, cheeks, and pharynx. Mucosal transudates originate from the gingival and buccal mucosa and contain serum-derived antibodies.

The presence of porcine reproductive and respiratory syndrome virus (PRRSV) in oral fluids was first reported in 1997. Virus was isolated from buccal swabs collected from experimentally-inoculated young pigs on 7, 14, 21, 28, 35, and 42 days postinoculation (DPI). The isolation of PRRSV was also reported from oropharyngeal (“tonsil scraping”) samples. Virus was recovered from 3 of 4 pigs sampled on 56, 70, and 84 DPI, and one pig sampled on 157 DPI. These reports suggested the possibility that porcine oral fluid samples could be used to monitor PRRSV infection. The purpose of the present study was to determine if PRRSV and/or anti-PRRSV antibodies were present in oral fluids at consistently detectable levels and, if so, how long PRRSV and/or anti-PRRSV antibodies were present in oral fluids and whether this was affected by pig age.

Materials and Methods

Experimental design

The level and duration of PRRSV and anti-PRRSV antibodies in serum and oral fluids was evaluated in three age groups of pigs (4, 8, or 12 weeks of age at time of inoculation). Each age group consisted of 16 pigs (12 PRRSV-inoculated, 4 negative controls) housed in pens of four pigs each, with the exception of 4-week-old PRRSV-inoculated pigs, which were housed in two pens of six pigs each. Pigs were randomly assigned to treatment groups. Serum samples collected 8 days before the start of the experiment and day 0 were assayed by enzyme-linked immunosorbent assay (ELISA) to confirm the absence of PRRSV infection. The pigs were intramuscularly inoculated on day 0 with 2 ml of a preparation containing 1 x 10^1.7 50% tissue culture infective dose (TCID50) of PRRSV per ml. After PRRSV inoculation, serum, buccal swabs, and oral fluids were collected at regular intervals for 63 days. At the end of the experiment, all samples were randomized, relabeled, and submitted.
for testing. Samples were assayed for the presence of PRRSV by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and for the presence of anti-PRRSV antibodies by ELISA and indirect fluorescent antibody (IFA) assay.

**Animals and animal care**

Pigs were obtained from a commercial swine herd known to be free of PRRSV. Pigs were received at 3, 7, or 11 weeks of age and housed in the Livestock Infectious Disease Isolation Facility, College of Veterinary Medicine, Iowa State University (Ames, IA). Animals were housed on the floor in pens that were cleaned daily. Feeder space, square footage per animal, ambient temperature, and room air exchanges all met or exceeded guidelines and requirements set forth in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. Animals were fed ad libitum an age-appropriate commercial feed that met or exceeded the nutritional requirements for swine as determined by the National Research Council.

**PRRSV**

A type 2 (North American) PRRSV isolate (ISU-P) was used in this study. The isolate was initially recovered from a homogenate prepared from a pool of lung tissues collected in October 1990 from young pigs in a herd experiencing an acute outbreak of PRRSV in Illinois. As described elsewhere, the virus was initially isolated on porcine alveolar macrophage (PAM) cultures. Subsequently, the isolate was cloned by three rounds of limiting dilutions in PAMs and twice by plaquing in MA-104 cells. The working stock of virus used in the present study represented the fourth passage in MA-104 cells.

**Preparation of PRRSV inoculum**

The PRRSV inoculum was prepared as previously described. Each dilution was run in duplicate and the means were used to calculate the virus titer using the Spearman-Karber method. The titer of the inoculum was estimated at $1 \times 10^{1.7}$ TCID$_{50}$ per ml.

**Collection of biological samples**

Blood samples were collected from all pigs twice weekly through 14 DPI, then weekly through 63 DPI. Samples were collected using a single-use blood collection system. Blood
samples were centrifuged at 1000 x g for 10 min, after which the serum was harvested and stored at –20°C.

Buccal samples were collected from all pigs twice weekly through 21 DPI using polyester swabs. Following collection, swabs were placed in 5-ml snap-cap tubes containing 1 ml of PBS and stored at –20°C.

Pen-based oral fluid samples were collected twice weekly through 61 DPI by suspending a length of 3-strand twisted cotton rope in each pen of four or six pigs. To facilitate oral fluid collection, each pen was equipped with a 90° bracket with a one inch hole in the center of the horizontal surface. Brackets were fixed in place by bolting the bracket to a back plate through the vertical bars of the pen. One bracket was located in each pen such that the rope could be placed and recovered without the need to enter the pen. For sample collection, one end of the rope (1.59 cm or 1.27 cm [5/8 inch or 1/2 inch]) was knotted and the opposite (unknotted) end threaded through the hole in the horizontal surface of the bracket. The rope was cut to length so that the end was at shoulder height to the animals. Ropes were left in place for 20 to 30 min. Pigs actively sought out and chewed the rope, leaving the strands moistened with oral fluids. To recover the oral fluid sample, the bottom 30.48 cm (12 inch) of the rope was inserted into a 17.78 cm by 30.48 cm (7 inch by 12 inch) stomacher filter bag while still suspended from the bracket and cut from the upper portion of the rope. Oral fluids were extracted from the rope by mechanical compression (wringer), decanted into 5-ml snap-cap tubes, and stored at –20°C until assayed.

**PRRSV real-time qRT-PCR**

For purposes of comparison, oral fluid samples were submitted to two laboratories (A and B) for PRRSV real-time qRT-PCR analysis; serum samples only to laboratory A.

**Laboratory A**. qRT-PCR to detect and quantify PRRSV RNA (ORF7) was performed as previously described. In brief, PRRSV RNA was extracted from 0.14 ml of sample with a QIAamp viral RNA mini kit. Real-time RT-PCR was performed using an ABI Prism 7900 HT sequence detection system using oligonucleotide primers and minor groove binder (MGB) probes specific for ORF7. The thermal profile for amplification of PRRSV viral
RNA was a reverse transcription at 50° C for 30 minutes, followed by enzyme activation at 95°C for 15 minutes, then 40 cycles of denaturation at 94°C for 15 seconds and a combined annealing/extension step at 60°C for 60 seconds. For each assay, a standard curve was generated using virus standards (10^1 to 10^6 TCID₅₀ equivalents per ml) and positive and negative control samples were tested with the unknowns.

**Laboratory B.** Oral fluids were shipped overnight on ice to laboratory B. Samples were not frozen when received, but were kept cold and processed the same day as arrival. qRT-PCR was performed as previously described.³⁵ This assay has minor differences in comparison to Laboratory A. Specifically, an MGB 5’ nuclease probe and primers were designed from the 3’UTR PRRSV genomic region by alignment of GenBank isolates and based on conserved areas of the 3’UTR primer and probe region. A PCR reaction was considered positive if the cycle threshold (Ct) level was obtained at ≤39 cycles. A standard curve used for qPCR, consisted of known amounts of serially diluted in vitro transcript RNA product (1 × 10⁻¹ through 1 × 10⁶ copies per μl). Copy per ml concentrations of the unknown samples were determined by linear extrapolation of the Ct values plotted against the known concentration of the 3’UTR transcript product.

**PRRSV indirect fluorescent antibody**

Oral fluid samples were assayed for the presence of specific anti-PRRSV IgG and IgA antibodies by IFA using fixed MARC-145 cells infected with PRRSV as previously described.³⁹ with a few modifications. Known positive and negative controls were diluted 1:20 in 0.01M PBS (pH 7.2) with 0.05% Tween® 20 (PBST) while oral fluid samples were tested after being diluted 1:2 in PBST. For detection of anti-PRRSV antibodies, FITC-conjugated anti-porcine IgG and FITC-conjugated anti-porcine IgA were used as the secondary antibody for detection of anti-PRRSV IgG and IgA respectively.

**PRRSV ELISA**

Both oral fluid and serum samples were tested for the presence of antibodies against PRRSV using the HerdChek® PRRS Antibody 2XR Test Kit. Serum samples were diluted 1:50 and assayed according to the manufacturer’s instruction. Oral fluids were assayed according to
the manufacturer’s instructions, with the exception that samples were centrifuged at 1000 x g for 10 min and diluted 1:2 in dilution buffer provided by the manufacturer prior to being assayed. All ELISA results were expressed as sample/positive (S/P) ratios.

Statistical methods
All statistical analyses were performed using JMP 6.0.0.1 Serum and oral fluids results for ELISA and real-time qRT-PCR were analyzed using multivariate analysis of variance (MANOVA) with time as the repeated measure and age as a model effect. When statistically significant (p < 0.05) age-by-time interactions were observed, Tukey’s Honestly Significantly Different (HSD) test was used to determine when in time (DPI) the response by age differed.

Linear regression was used to evaluate the relationship between oral fluid samples and serum samples with respect to the quantity of viral RNA and, in a separate analysis, ELISA S/P values. Consolidation of data was necessary to conduct linear regression, despite its recognized effect on type 2 error rate. That is, oral fluid samples were collected from pens (not individual pigs) at 2- to 4-day intervals, whereas serum samples were collected from individual pigs twice a week through day 14, then weekly. Thus, the linear regression analyses (real-time qRT-PCR, ELISA) were based on a weekly average of oral fluid results for each pen and weekly average of serum results for individual pigs within the same pen.

Results

PRRSV real-time qRT-PCR
Oral fluid samples were assayed by real-time qRT-PCR at two laboratories (A and B). All oral fluid samples collected from negative control pens were negative in both laboratories (n = 55). Overall, 88% of the qualitative results from laboratories A and B were in agreement (i.e., 37 positive samples and 147 negative samples of the 209 samples assayed). Categorical results from the two laboratories are given by week postinoculation in Table 1. Real-time qRT-PCR estimates of virus concentration were based on extrapolation to TCID₅₀ standards (laboratory A) or transcript RNA product standards (laboratory B). Linear regression analysis showed a positive correlation (r² = 0.60) between the quantitative estimates from the
two laboratories. Age at the time of inoculation had no effect on the quantity of virus present in oral fluid samples (MANOVA: laboratory A, \( p = 0.21 \); laboratory B, \( p = 0.42 \); Figs. 1, 2; Tables 2, 3).

Given that real-time qRT-PCR results from the two laboratories were comparable, buccal swabs and serum samples (reported below) were only assayed at laboratory A. Buccal swab samples were collected from individual pigs twice weekly through 21 DPI. All samples collected prior to inoculation (\( n = 48 \)) were real-time qRT-PCR negative. Of 216 buccal swabs collected from PRRSV-inoculated pigs on 3, 7, 10, 14, 17, and 21 DPI, 29 were positive by real-time qRT-PCR. One of 72 buccal swab samples collected from negative control pigs during the observation period tested positive. All other specimens (serum, oral fluid, buccal swab) collected from negative control pigs tested negative by qRT-PCR and ELISA. This supports the interpretation of this one result as a false positive. Pig-matched serum and buccal swab samples were collected from PRRSV-inoculated pigs on 7, 14, and 21 DPI. Of 108 paired samples, 12 were real-time qRT-PCR–positive for both specimens; 92 were serum-positive and buccal swab–negative; and 4 were negative for both samples.

Serum samples were collected from all pigs twice weekly through 14 DPI, then weekly through 63 DPI. Analysis (MANOVA) of the real-time qRT-PCR data detected a difference \( (p = 0.0001) \) in the level of viremia by the age of the pig at the time of inoculation. Overall, the youngest pigs had the highest level of viremia followed by the next youngest group (Fig. 3, Table 4). Statistically significant differences in the level of PRRSV viremia by age group were identified (Tukey HSD) at 7, 10, and 42 DPI (Table 4).

Comparison showed that the levels of virus in serum and oral fluid samples followed a similar pattern, with oral fluid consistently containing a lower concentration of virus (Fig. 4). Both serum and oral fluid samples were real-time qRT-PCR positive from 3 DPI to 4 to 5 weeks postinoculation, with sporadic positives thereafter. Linear regression analysis estimated the correlation \( (r^2) \) between virus concentration in serum and oral fluids at 0.56 (Fig. 5).

The diagnostic sensitivity of real-time qRT-PCR for serum and oral fluid samples
corresponded to the percentage of inoculated animals (or pens) that tested positive (Table 1). For oral fluid samples, the mean diagnostic sensitivity for the first 4 weeks postinoculation was 88% for laboratory A and 81% for laboratory B. By comparison, the mean diagnostic sensitivity of laboratory A on individual pig serum samples for the same 4-week period was 89%. Neither laboratory reported positive real-time qRT-PCR results for serum or oral fluid samples from negative controls (i.e., diagnostic specificity was 100%).

**PRRSV ELISA**

Statistical analysis of the serum antibody response found a significant difference in ELISA S/P values by pig age at the time of inoculation (MANOVA: $p = 0.0001$). The youngest pigs had the highest S/P response, followed by the next youngest group (Fig. 6, Table 5). Statistically significant differences in S/P values by age group were identified (Tukey HSD) from 14 to 63 DPI, with the 4-week-old group consistently showing the highest S/P response.

In contrast, a comparison of oral fluid ELISA results found no significant difference in least square means between PRRSV-inoculated and negative-control pens (MANOVA: $p = 0.72$). Likewise, no significant difference was detected in the ELISA response within the inoculated groups by pig age at time of inoculation (MANOVA: $p = 0.15$).

**PRRSV indirect fluorescent antibody**

Anti-PRRSV IgG was detected by IFA in 20 of 154 oral fluid samples from inoculated pens, with positives sporadically distributed across the postinoculation observation period. No anti-PRRSV IgA was detected in oral fluid samples with the protocol described. Samples from negative control pens were negative for both IgG and IgA.

**Discussion**

In humans, viral infections in which the agent is present in oral fluids include hepatitis A, hepatitis B, hepatitis C, hepatitis G, herpes simplex virus type 1, human herpesvirus 6, human immunodeficiency virus, measles virus, mumps virus, Mycobacterium tuberculosis, rubella virus, severe acute respiratory syndrome-associated coronavirus, transfusion transmitted virus, and varicella-zoster virus. Some viral infections were also
demonstrated to produce detectable levels of specific antibodies in oral fluids: Dengue virus, hepatitis A, hepatitis B, hepatitis C, human immunodeficiency virus, measles virus, Norwalk virus, rubella virus, and others. In human diagnostic medicine, the presence of pathogens and/or antibodies have stimulated important developments in diagnostic medicine, e.g., in 2004, the FDA approved a rapid assay (20 min) for detection of antibodies against HIV-1 in oral fluid, blood, or plasma samples (Anonymous: 2004, FDA approves oral fluid rapid HIV test. Dentistry Today 23:42). Likewise, IgG-based assays for oral fluid have been developed for Epstein-Barr virus, hepatitis A and B viruses, parvovirus B19, and rubella virus and assays based on the IgM antibodies have been developed to detect recent infections by hepatitis A and B viruses, measles virus, mumps virus, parvovirus B19, and rubella virus.

In animals, the presence of pathogens in oral fluid has generally been described in the context of transmission (e.g., rabies virus). In swine, examples of viral pathogens present in oral fluids include bovine virus diarrhea virus, foot-and-mouth disease virus, porcine circovirus type 2, pseudorabies (Aujeszky’s disease) virus, and vesicular stomatitis virus. Pathogen-specific antibodies in oral fluids have also been described in animals, although the research is extremely limited. In swine, the appearance of specific antibodies in oral fluids was shown following inoculation with Actinobacillus pleuropneumoniae, cholera toxin B subunit, and group E Streptococcus spp.

The use of oral fluids in veterinary diagnostic medicine has been minimal; for the most part limited to the diagnosis of feline immunodeficiency virus. Recently, oral fluid samples were reported as a method for the detection of E. coli O157:H7 in feedlot cattle (Renter DG, Visser A, McFall M et al: 2004, Rapid pen-level surveillance of E. coli O157:H7 in finished feedlot cattle. Animal Health Forum 9(1):3).

The research reported here represents a further investigation of the application of oral fluids to veterinary diagnostic medicine. In this experiment, the collection of oral fluid samples from pens of pigs was determined to be easy and efficient. Normal pig behavior was conducive to sample collection, that is, pigs naturally investigate and chew on new objects.
within the pen, i.e., rope. PRRSV was detected by real-time qRT-PCR in oral fluid samples for approximately four weeks. The fact that two laboratories independently arrived at similar results (88% agreement) suggests that oral fluid samples could be used to monitor PRRSV infection in commercial swine herds using currently available PCR-based assays by testing at ≤4-week intervals. Specific anti-PRRSV antibody was detected in oral fluids, but additional research will be required to develop diagnostically sensitive assays. Oral fluid sampling must still be validated in the field, but preliminary data suggest that this approach could offer a significant improvement in the ease, timeliness, and cost of disease surveillance in commercial swine populations.

Acknowledgements

The study was supported in part by Pork Checkoff funds distributed through the National Pork Board, P.O. Box 9114, Des Moines, IA 50306. The authors thank the faculty and staff of the Iowa State University Veterinary Diagnostic Laboratory for advice and technical support.

Sources and Manufacturers

a. Becton Dickinson, Franklin Lakes, NJ.

b. Fisher Scientific, Logan, UT.

c. Ace Hardware Corporation, Colorado Springs, CO.

d. QIAGEN, Inc., Valencia, CA.

e. Applied Biosystems, Foster City, CA.

f. Integrated DNA Technologies, Coralville, IA.

g. GenBank®, Bethesda, MD.

h. Bethyl Laboratories, Montgomery, TX.
i. IDEXX Laboratories, Inc., Westport, ME.

j. SAS Institute, Cary, NC.
References


### Tables and Figures

Table 1. Oral fluid, serum, and buccal swab PRRSV real-time qRT-PCR categorical results by time post-inoculation

<table>
<thead>
<tr>
<th>Week</th>
<th>Pen-based oral fluid samples</th>
<th>Individual pig results†</th>
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<tbody>
<tr>
<td></td>
<td>Pen-based results*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Laboratory A</td>
<td>Laboratory B</td>
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<tr>
<td>1</td>
<td>8 (100%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>2</td>
<td>7 (88%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>3</td>
<td>6 (75%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>4</td>
<td>7 (88%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td>5</td>
<td>2 (25%)</td>
<td>3 (38%)</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>3 (38%)</td>
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<tr>
<td>7</td>
<td>1 (13%)</td>
<td>1 (13%)</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number and percent (%) of real-time qRT-PCR positive pens among 8 pens sampled.

† Number and percent (%) of real-time qRT-PCR positive pigs among 36 pigs sampled.

‡ Pen was classified positive if one or more individual pig serum samples within pen was positive.
Table 2. Laboratory A: Oral fluid PRRSV real-time qRT-PCR least square means by pig age and week post-inoculation

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<th>Pig age (weeks)†</th>
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* PRRSV real-time qRT-PCR results reported as log$_{10}$ TCID$_{50}$ equivalents per ml.

† Pig age at the time of inoculation had no effect on the quantity of virus in oral fluid (MANOVA: $p = 0.21$).
Table 3. Laboratory B: Oral fluid PRRSV real-time qRT-PCR least square means by pig age and week post-inoculation

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* PRRSV real-time qRT-PCR results reported as log10 RNA copies per ml.

† Pig age at the time of inoculation had no effect on the quantity of virus in oral fluid (MANOVA: p = 0.42).
Table 4. Serum PRRSV real-time qRT-PCR least square means by age and day post-inoculation

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* PRRSV real-time qRT-PCR results reported as log_{10} TCID_{50} equivalents per ml.

†,‡ Within columns, means with different superscripts were significantly different by Tukey HSD.
Table 5. Serum PRRSV ELISA sample/positive least square means by pig age and day post-inoculation

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*,† Within columns, means with different superscripts were significantly different by Tukey HSD.
Figure 1. Laboratory A: Oral fluid PRRSV qRT-PCR least square means by pig age and week post-inoculation.
Figure 2. Laboratory B: Oral fluid PRRSV real-time qRT-PCR least square means by pig age and week post-inoculation
Figure 3. Serum PRRSV real-time qRT-PCR least square means by pig age and day post-inoculation.
Figure 4. Serum and oral fluid PRRSV qRT-PCR results by week post-inoculation.
Figure 5. Correlation between serum and oral fluid PRRSV qRT-PCR results: \( r^2 = 0.56 \)
Figure 6. Serum PRRSV ELISA least square means by pig age and day post-inoculation
CHAPTER 3. SURVEILLANCE OF COMMERCIAL GROWING PIGS FOR PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) AND PORCINE CIRCOVIRUS TYPE 2 (PCV2) INFECTIONS USING ORAL FLUID SAMPLES


J Prickett, W Kim, R Simer, K Yoon, J Zimmerman

Summary

Objectives: To validate the use of oral fluids to detect infections with porcine respiratory and reproductive syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) in three commercial swine herds.

Materials and methods: Oral-fluid and serum samples were collected from one barn on each of three PRRSV-infected finishing sites. Six pens per barn (20 to 30 pigs per pen) were sampled repeatedly, beginning when the pigs entered the facilities (3 weeks of age), and then at 5, 8, 12, and 16 weeks of age. Serum samples were tested using a commercial PRRSV ELISA. Both serum and oral-fluid samples were tested for PRRSV by quantitative reverse-transcriptase polymerase chain reaction (PCR), and oral fluids were tested for PCV2 by qRT-PCR.

Results: Site One pigs seroconverted to PRRSV at 12 weeks of age, and Site Two and Three pigs at 5 to 8 weeks of age. All individual serum samples tested PCR-negative for PRRSV in pigs 3 to 5 weeks old, while ≥1 sample tested positive in pigs 8, 12, and 16 weeks old, with 77% agreement between oral-fluid and serum pen-level results. At all sites, ≥1 oral-fluid sample tested PCR-positive for PCV2 beginning when pigs were 8 weeks old.

Implications: Oral-fluid samples may be used to monitor PRRSV and PCV2 infections in commercial production systems. PRRSV virus is detectable in oral fluids for 3 to 8 weeks,
and PCV2 may be detectable for > 8 weeks. Sampling at 2- to 4-week intervals is recommended for surveillance of PRRSV and PCV2.

Introduction

In both humans and animals, antibodies and pathogens may be detected in oral fluids collected from infected individuals. The presence of antibody in oral fluid was demonstrated as early as 1909. Antibody (IgM, IgA, and IgG) is produced locally in salivary glands and lymphoid tissue, but the primary source of antibody in oral fluid is oral mucosal transudate. Pathogens in oral fluids may originate in tissues associated with the buccal cavity (eg, classical swine fever virus replicates in the tonsil of the soft palate) or reach the buccal cavity from the circulatory system via oral mucosal transudate (eg, hepatitis B). Examples in which both the agent and antibody are present in oral fluids include foot-and-mouth disease virus in cattle, Brucella melitensis in humans, and feline immunodeficiency virus in cats.

The body of literature on the use of oral fluids in human diagnostics is extensive, but Archibald et al may have been the first to suggest their use as a primary diagnostic specimen. Thereafter, diagnostic assays using oral fluid became available for a variety of infections and infectious agents, eg, human immunodeficiency viruses, measles, mumps, rubella, hepatitis A, B, and C, and others.

In veterinary medicine, oral fluids have been used for detection of Escherichia coli O157:H7 and Salmonella in feedlot cattle, and feline leukemia virus in cats. In swine, specific antibodies were detected in oral fluid from pigs infected with group E streptococcus, Actinobacillus pleuropneumoniae, and cholera toxin B subunit.

Both porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) have been detected in buccal samples. Recently, under experimental conditions, oral-fluid samples from pigs inoculated with PRRSV were shown to contain diagnostic levels of virus. Here, we report a pilot project validating the use of oral fluids for detection of PRRSV and PCV2 infections in three commercial swine herds.
Materials and Methods

Experimental design
Oral-fluid and serum samples were collected on three PRRSV-infected finishing sites stocked with pigs from endemically-infected sow farms. Pigs on Site One were sourced from one sow farm, and pigs on Sites Two and Three from a second sow farm. On each site, six pens in one barn (20 to 30 pigs per pen) were sampled repeatedly over time. Samples were collected when the pigs entered the facilities at 3 weeks of age, and then at 5, 8, 12, and 16 weeks of age. At each time point, one oral-fluid sample was collected from each pen, and blood samples were collected from a convenience sample of five pigs per pen. At the end of the collection period, all oral-fluid and serum samples were randomized, relabeled, and tested for PRRSV by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In addition, serum samples were tested for anti-PRRSV antibody using a commercial ELISA, and oral fluids were tested for PCV2 by qRT-PCR.

Collection of biological samples
Oral fluids were collected by hanging a length of cotton rope within the pen for 20 to 30 minutes. At each sampling, the rope was positioned at shoulder height for the pigs in the pen, ie, the length of the rope was adjusted as the pigs grew. Pigs are naturally attracted to the rope and deposit oral fluids during the process of interacting with it. After the exposure period, oral fluids were extracted from the rope by wringing the wet end or portion of the rope into a 1-gallon resealable plastic bag and clipping a bottom corner of the bag to drain the fluid into a 50-mL centrifuge tube. Samples were stored frozen until assayed.

Blood samples were collected using a single-use blood collection system (Vacutainer; Becton Dickinson, Franklin Lakes, New Jersey). Blood was centrifuged at 1000g for 10 minutes, and serum was harvested and stored at -20°C.

PRRSV qRT-PCR
Oral-fluid and serum samples were assayed for PRRSV by qRT-PCR as previously described with minor exceptions. Briefly, viral RNA for qRT-PCR amplification was extracted from 0.14 mL of sample using an Ambion viral RNA kit (Ambion, Valencia,
California) according to the protocols recommended by the manufacturer. Real-time RT-PCR quantification was performed using an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, California). Primers specific for PRRSV open reading frame (ORF) 7 were synthesized by Integrated DNA Technologies, Inc (Coralville, Iowa), and minor groove binder probes were synthesized by Applied Biosystems. The thermal profile for amplification of PRRSV viral RNA was a reverse transcription at 50°C for 30 minutes, followed by enzyme activation at 95°C for 15 minutes, then 40 cycles of denaturation at 94°C for 15 seconds and a combined annealing-extension step at 60°C for 60 seconds, with fluorescence data capture at the combined annealing-extension stage. For each assay, a standard curve was generated using standards (10^1 to 10^6 median tissue culture infections dose [TCID50] equivalents per mL), and positive and negative control samples were tested with the unknowns. The unit of expression for PRRSV qRT-PCR results was TCID_{50} equivalents per mL, which represented the quantity of total viral RNA in samples relative to standards in which the amount of infectious PRRSV was quantified using microtitration infectivity assays. A positive sample was defined as a sample that produced a TCID_{50} estimate in the qRT-PCR assay.

**PCV2 qRT-PCR**

The presence of PCV2 in oral fluids was assessed by qRT-PCR using a previously described protocol; serum samples were not available for testing. Briefly, viral DNA was extracted from 50 µL of each oral fluid sample using MagMax total viral nucleic acid isolation kit (Ambion, Valencia, California) according to the manufacturer’s instruction. Real-time PCR was performed with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in 25-µL reaction volumes using 5 µL of extracted template. The PCR primers (Integrated DNA Technologies, Inc) and probe (Applied Biosystems) with 5’ reporter 6-carboxyfluorescein (FAM) and a 3’ TAMRA quencher were designed to detect complementary sequences in ORF1 of PCV2. Primers were added at a final concentration of 20 µM each; the probe was at a final concentration of 25 µM. The PCR amplification was performed on the ABI 7500HT Sequence Detection System (Applied Biosystems). Cycling conditions were as follows: an activation step at 95°C for 20 seconds and then 35 cycles of 3 seconds at 94°C...
and 30 seconds at 60°C. A set of PCV2 preparations with known virus titer (fluorescent focus forming unit, FFU) was used to generate a standard curve. Samples with a threshold cycle of ≤ 35 cycles were considered positive.

**PRRSV ELISA**

Serum samples were tested for antibodies against PRRSV using the HerdChek PRRS Antibody 2XR Test Kit (IDEXX Laboratories, Inc, Westbrook, Maine). Serum samples were assayed according to the manufacturer’s instruction. As recommended by the manufacturer, a positive serum sample was defined as having a sample-to-positive ratio (S:P) value ≥ 0.4.

**Results**

**PRRSV ELISA**

Serum ELISA S:P results are presented as means over time (Figure 1). At all three sites, S:P ratios declined between placement (3 weeks of age) and 2 weeks post placement. In pigs at Site One, S:P ratios increased when pigs were 8 to 12 weeks of age, while at Sites Two and Three, S:P ratios increased when pigs were 5 to 8 weeks of age. In contrast, pigs at Site One seroconverted between 8 and 12 weeks of age. That is, all Site One pigs (n = 30) were ELISA-negative at 8 weeks of age and ELISA-positive at 12 weeks of age.

**PRRSV qRT-PCR**

All individual pig serum samples collected when the pigs were 3 and 5 weeks of age tested PCR-negative. At all three sites, one or more serum samples (n = 30 per site at each sampling) tested PCR-positive in pigs 8, 12, and 16 weeks of age. At the pen level, 77% of PRRSV qRT-PCR oral-fluid and serum results were in agreement (Figure 1). PRRS virus was first detected at Sites Two and Three in oral-fluid samples collected at 8 weeks of age, consistent with the serum qRT-PCR results at each site. However, in all 30 pigs 8 weeks of age at Site One, PCR for PRRSV in oral-fluid samples and serum ELISA for PRRSV antibodies were negative, while serum PCR was positive in 25 of 30 pigs sampled at Site 1 (Figure 1).
**PCV2 qRT-PCR**

Results of testing oral-fluid samples for PCV2 by qRT-PCR are summarized in Table 1. Two or more oral-fluid samples from Site One tested positive at all sampling points, including all six pens at the last sampling point (16 weeks of age). At Site Two, all pens tested negative at the first sampling, and at Site Three, all pens tested negative at 3, 5, and 8 weeks of age.

**Discussion**

Surveillance, ie, on-going efforts to detect a pathogenic agent or disease, is fundamental to the control, elimination, or eradication of an infectious agent. Current surveillance methods for monitoring PRRSV in the production setting require collection of serum samples from individual animals. The number of samples required, labor, and time associated with serum-based testing are often cost-prohibitive. The most frequent consequence is that surveillance is ineffectively executed or abandoned altogether.

Previous data collected under experimental conditions suggested that PRRSV is detectable in oral fluid samples for approximately 4 weeks after exposure. The objective of this study was to conduct a preliminary assessment of the feasibility of detecting PRRSV in oral fluid samples collected in an endemically-infected commercial population. At pen level, 77% of the PRRSV qRT-PCR oral fluid and serum results were in agreement. Pen-based oral fluid sampling offers a simple, nontechnical technique for monitoring PRRSV circulation in a population. Further research under experimental conditions and field settings with matched sera and oral fluid samples is needed to establish sample size and refine sampling protocols. However, the data reported here and the work previously published suggest that a sampling interval of 2 to 4 weeks would be sufficient for timely and effective PRRSV and PCV2 surveillance.

The original experimental design of this study did not include testing for PCV2. Due to the current interest in PCV2, oral fluid samples were tested for PCV2 by qRT-PCR (serum samples were no longer available). Reflecting the ubiquitous distribution of the virus, PCV2 was detected in oral fluids from each of the three sites and, at Site One, two or more PCR-
positive oral-fluid samples were recovered at every sampling point. These data suggested that oral-fluid sampling could be used to collect PCV2 for genetic characterization and to monitor circulation of PCV2 in commercial populations.

**Implications**

- Under the conditions of this study, testing of oral fluids by PCR may be used to detect PRRSV and PCV2 infections in commercial production systems.
- PRRS virus is detectable in oral fluids for 3 to 8 weeks, and PCV2 may be detectable for longer than 8 weeks.
- Sampling at 2- to 4-week intervals is recommended for surveillance of PRRSV and PCV2.

**Acknowledgements**

The study was supported in part by Pork Checkoff funds distributed through the National Pork Board, Des Moines, Iowa.

**References**


Tables and Figures

Table 1. Categorical individual pig serum results: PRRSV qRT-PCR and ELISA positives by site and pen

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Table 2. Categorical pen-level (n = 6 pens) results for PRRSV\textsuperscript{a} and PCV2 by site

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<th>Pen results based on oral fluid samples\textsuperscript{c}</th>
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\textsuperscript{a} PRRSV qRT-PCR oral fluid and serum results showed 77\% agreement at the pen level

\textsuperscript{b} Number of positive pens among 6 pens sampled per site. Pen defined as positive if \( \geq 1 \) pig serum sample tested positive

\textsuperscript{c} One oral fluid sample collected from each pen (6) per sampling point
Figure 1. Cumulative results of testing oral fluids and serum by PRRSV qRT-PCR, testing oral fluids by PCV2 PCR, and serum antibodies by PRRSV ELISA in three commercial finisher sites.
CHAPTER 4. STABILITY OF PCR-DETECTABLE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) AND ANTI-PRRSV ANTIBODY IN SWINE ORAL FLUID

A paper submitted to the Journal of Swine Health and Production

J. Prickett, S Cutler, J Kinyon, N Naberhaus, W Stensland, K Yoon, J Zimmerman

Summary

Objective: The objective of this research was to evaluate the stability of porcine reproductive and respiratory syndrome virus (PRRSV) and anti-PRRSV antibodies in oral fluid as a function of time and temperature.

Materials and methods: A 4-liter pool of swine oral fluid was collected from 16-week old finisher pigs. To ensure uniform, quantifiable levels of virus and antibody over time, the pool was “spiked” with 4 ml of PRRSV isolate ISU-P containing $1 \times 10^{12}$ RNA copies per ml and 10 ml of concentrated hyper-immune anti-PRRSV antibodies. The pool was divided into 3 equal portions: (1) no treatment; (2) chlorhexidine digluconate at 0.01% by volume; (3) isothiazolinone at 3 parts per million. Each treatment was run in triplicate at each of five temperatures (-20°C, 4°C, 10°C, 20°C, 30°C). Samples were removed at specific intervals (0 hr, 12 hr, 24 hr, 48 hr, 72 hr, 144 hr, 216 hr, and 288 hr), stored at -80°C, and then assayed for: (1) PRRSV RNA; (2) IgM, IgA, and IgG; (3) ELISA-detectable PRRSV-specific antibody; (4) culturable bacteria per ml.

Results: The results showed that the stability of anti-PRRSV antibody and PCR-detectable PRRSV was highly temperature-dependent, with antimicrobial treatment providing no improvements in stability at lower temperatures. In particular, both virus and antibody were stable at $\leq 10^\circ C$ over 12 days of storage.
**Implications:** Conventional serum storage protocols (freezing or refrigeration at 4°C) will preserve PRRSV and anti-PRRSV antibody in oral fluid diagnostic samples.

**Introduction**

Oral fluid specimens have been widely used in human medicine and forensics for the diagnosis or detection of a variety infectious agents, hormones, toxins, and drugs. When used in the surveillance of infectious disease, oral fluid testing facilitates the efficient collection of large numbers of diagnostic samples at low cost. For example, the New York City Department of Health and Mental Hygiene performed 166,058 oral fluid rapid HIV antibody tests between March 2005 to May 2008 in 10 walk-in clinics. In the United Kingdom, oral fluid samples were collected from 11,698 children at home and mailed to the laboratory for antibody testing.

Oral fluids have not been widely exploited in veterinary diagnostic medicine, but the detection of pathogens and/or antibodies in swine oral fluids, i.e., porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), influenza virus (Prickett et al., March 2009. Cost effective PRRSV surveillance. Proc AASV. Dallas, Texas, pp. 467-469.), transmissible gastroenteritis virus (TGEV), and *Actinobacillus pleuropneumoniae* suggests this approach could be used in the surveillance of these pathogens in commercial swine herds.

Oral fluids inherently contain endogenous antiviral and antibacterial factors that could theoretically affect the stability of PCR targets. Furthermore, normal oral bacterial flora and environmental bacterial contaminants present in livestock facilities could produce bacterial proteases capable of enzymatic cleavage of immunoglobulins. Therefore, the objective of this research was to evaluate the stability of PCR-detectable PRRSV and anti-PRRSV antibody in oral fluids and develop handling and storage guidelines for porcine oral fluid specimens.
Materials and Methods

Experimental design
The effects of antimicrobial treatment, storage temperature, and storage time were evaluated in respect to: (1) total detectable PRRSV RNA; (2) total porcine IgM, IgA, and IgG; (3) ELISA-detectable PRRSV-specific antibody; (4) total bacteria; and (5) pH. To conduct the experiment, 4 liters of swine oral fluid was collected from 12-week-old commercial finisher pigs and “spiked” with a type 2 PRRSV isolate and anti-PRRSV antibodies. To test the effect of antimicrobials on diagnostic target stability, the pool was divided into 3 treatments: (1) untreated control (no trt); (2) chlorhexidine digluconate (chlx; 0.01% by volume); and (3) isothiazolinone (kat; 3 ppm). To ensure the uniform distribution of virus, antibody, and antimicrobials, the solution was stirred constantly using a magnetic mixer throughout the process of preparation and transfer. To test the effect of treatment by storage temperature and time, 2.5 ml volumes of each antimicrobial treatment were aliquoted into 5ml tubes and held at each of five temperatures (-20°C, 4°C, 10°C, 20°C, 30°C). Three tubes (replicates) from each antimicrobial treatment and temperature combination were removed at specific times (0 hr, 12 hr, 24 hr, 48 hr, 72 hr, 144 hr, 216 hr, 288 hr) and held at -80°C until tested. Thereafter, samples were randomized, tested, and the results evaluated for treatment, time, and temperature effects on concentrations of antibody, bacteria, and PCR-detectable PRRSV.

Porcine oral fluid
A pool of swine oral fluid (4 liters) was obtained from a population of 16-week-old commercial finisher pigs housed in a 2,400 head facility located in Iowa. Pigs were allowed to chew on cotton ropes, after which oral fluids were mechanically extracted, as previously described. The herd had been vaccinated for PVC2 and Mycoplasma hyopneumoniae at approximately 6 weeks of age and had seroconverted to PRRSV between 10 and 14 weeks of age.

PRRSV
Four ml of PRRSV isolate ISU-P (1 x 10^{12} RNA copies per ml) were added to the pool of oral fluid. The isolate was initially recovered from lung tissues collected from young pigs in
a herd experiencing an acute outbreak of PRRSV in Illinois. As described elsewhere, the virus was initially isolated on porcine alveolar macrophage (PAM) cultures and then cloned by three rounds of limiting dilutions on PAMs, and twice by plaquing on MA-104 cells. The working stock of virus used in the present study represented the fourth passage in MA-104 cells.

**Anti-PRRSV antibodies**

Anti-PRRSV antibody (kindly provided by Dr. Fernando Osorio) was produced by hyper-immunizing 13 pigs against several PRRSV isolates, as previously described. In brief, hyper-immunization produced neutralizing antibody titers of 1:32-1:128 in all 13 pigs. Thereafter, animals were euthanized and exsanguinated. Serum antibodies from each animal were precipitated and concentrated by NH$_2$SO$_4$ treatment. The concentrated antibodies used in this study represented a pool from all 13 animals. Prior to adding to the pool of oral fluids, the concentrated antibodies were reconstituted in 10 ml of phosphate buffered saline.

**Anti-microbial agents**

The effect of antimicrobial preservatives on the stability of PRRSV and anti-PRRSV antibody was tested by adding chlorhexidine digluconate or isothiazolinone to oral fluid at concentrations of 0.01% and 0.003% (3 ppm), respectively. Biguanides, i.e., chlorhexidine digluconate, act against non-sporulating bacteria and yeasts by disrupting cell membranes. Isothiazolones inhibit metabolic pathways by reacting with thiol-containing enzymes, thereby leading to cell death. Pilot standard plate count (SPC) assays using a “checkerboard” of antimicrobial concentrations were conducted to select bacteriostatic, not virucidal, concentrations (data not shown).

**Standard plate count**

The total number of bacteria per ml in each sample was quantified using a SPC method. In brief, samples were thawed at 23°C for 20 min and then serially diluted in PBS from 1x10$^{-1}$ through 1 x 10$^{-7}$. To perform the assay, all dilutions, including the undiluted sample, were plated in 10 μl volumes on culture plates containing trypticase soy agar with 5% sheep blood. Plates were incubated in 5% CO$_2$ at 35°C for 72 hr. Colonies were counted manually and the
total bacteria per ml was estimated and reported in log_{10} values, with \( \geq 1 \times 10^8 \) considered the upper limit of detection.

**PRRSV real-time RT-PCR**

PRRSV RNA for qRT-PCR amplification was extracted from oral fluid samples with a commercially available viral RNA isolation kit (Applied Biosystems, Foster City, CA). The manufacturer’s protocol was modified by increasing the initial volume of the sample from 50 \( \mu \)l to 175 \( \mu \)l. This volume of sample was added to 235 \( \mu \)l of lysis/binding solution (provided in the kit) to which carrier RNA had been added (provided in the kit) in a 96-well plate. The plate was sealed and placed onto an orbital plate shaker for 3 min at the highest setting, after which cell debris was removed by centrifugation of the plate at 1500 \( \times g \) for 6 min. Thereafter, 115 \( \mu \)l of the supernatant was transferred to a 96-well plate containing 65 \( \mu \)l of 100% isopropanol in each well. The remainder of the RNA extraction protocol was performed according to the manufacturer’s instruction.

Real-time RT-PCR reactions performed using a commercially available PRRSV PCR kit (Applied Biosystems, Foster City, CA) and analyzed according to manufacturer’s recommendations. Positive and negative plate control samples were run with the oral fluid samples. A standard curve was generated using the genomic copy standards provided in the kit (1 \( \times 10^5 \) to 1 \( \times 10^{10} \) genomic copies per ml) and the results were expressed as PRRSV genomic equivalents per ml (log_{10}).

**PRRSV ELISA**

All oral fluid samples were tested for antibodies against PRRSV using a commercially available PRRSV antibody ELISA (IDEXX Laboratories, Inc., Westport, ME.) and a modified protocol: (1) oral fluid samples were diluted 1:3 using the kit diluent, (2) the diluted oral fluid samples were incubated on the ELISA plates overnight at 4°C, and (3) plates were brought to room temperature in the morning (30 min) and the remainder of the assay was completed according to the manufactures protocol. The results were reported as sample-to-positive (S/P) ratios and the response to temperature, time, and treatment was analyzed as continuous data, i.e., no “cut-off” value was established or used in this study.
Immunoglobulin isotype quantification

All samples were assayed for IgA, IgG, and IgM antibodies using isotype-specific total swine antibody quantification assays following the procedures provided by the manufacturer (Bethyl Laboratories, Inc., Montgomery, TX). Prior to testing, the samples were heat-inactivated at 56°C for 10 min. Preliminary assays were performed to determine dilution ranges required to quantify each immunoglobulin class. Each sample was serially diluted (1:20–1:160 for IgM and 1:50–1:400 for IgG and IgA) in diluent provided by manufacturer in a 96-well round bottom plates. Samples were then transferred to the kit ELISA plates and the assay completed. The optical density (OD) response was used to estimate the concentration of immunoglobulin in each sample using the linear portion of the standard curve, with appropriate adjustment for sample dilution. Samples that were nonresponsive at the initial dilutions were re-assayed at lower dilutions (1:2–1:20 for IgM and 1:2–1:50 for IgG and IgA). By definition, samples that were nonresponsive at the lowest dilution, i.e., 1:2, contained ≤5 ng per ml of respective antibody.

Measurement of pH

The pH of 1 of the 3 replicates collected at each sampling point was measured using a pH meter calibrated using commercially available standards of pH 4, 7, and 10 (Sigma-Aldrich, St. Louis, MO). The pH probe was washed in de-ionized water and blotted dry between each measurement. Calibration of the pH meter was verified at the beginning, middle, and end of testing.

Statistical methods

All analyses were performed using commercial statistical software (JMP® 7.0.2, SAS Institute Inc., Cary, NJ.). Initially, the results of each assay were examined for the effect of temperature and treatment using analysis of variance (ANOVA). Temperature, antimicrobial treatment, and temperature*antimicrobial treatment were denoted as fixed effects in the model. Thereafter, planned orthogonal comparisons by Student’s t-test were used to identify significant differences among treatments and temperatures using the least squares means of the assay results. For clarity in graphical presentation, data were combined if no statistical difference was detected between storage temperatures.
Results

**Standard plate count**
Statistical analysis of main effects (marginal means) showed that both temperature and antimicrobial treatment had significant effects on the level of bacteria detected by the SPC (Table 1, Fig. 1). Two temperature groups were identified on the basis of SPC results (Fig. 3). In order of increasing mean SPC (Fig. 1), the two groups were: \((-20\, ^\circ\text{C} = 4\, ^\circ\text{C} = 10\, ^\circ\text{C}) < (20\, ^\circ\text{C} = 30\, ^\circ\text{C})\). Significant differences were detected among treatments, but chlorhexidine treatment produced the lowest SPC results at all temperatures.

**PRRSV real-time RT-PCR**
Statistical analysis of main effects (marginal means) showed that temperature, but not antimicrobial treatment, had a significant effect on the stability of PCR-detectable PRRSV (Table 2). Three temperature groups were identified on the basis of PRRSV qRT-PCR results (Fig. 2). In order of declining concentrations of virus, the three groups were: \((-20\, ^\circ\text{C} = 4\, ^\circ\text{C} = 10\, ^\circ\text{C}) > (20\, ^\circ\text{C}) > (30\, ^\circ\text{C})\). In particular, the concentration of PRRSV in chlorhexidine-treated samples was significantly lower than other treatments at \(-20\, ^\circ\text{C}, 4\, ^\circ\text{C},\) and \(10\, ^\circ\text{C}\) and significantly higher than other treatments at \(30\, ^\circ\text{C}\).

**PRRSV ELISA**
Statistical analysis of main effects (marginal means) showed that both temperature and antimicrobial treatment had significant effects on the stability of ELISA-detectable anti-PRRSV antibody (Table 3). Three temperature groups were identified on the basis of PRRSV ELISA results (Fig. 3). In order of declining mean ELISA S/P ratios, the groups were: \((-20\, ^\circ\text{C} = 4\, ^\circ\text{C} = 10\, ^\circ\text{C}) > (20\, ^\circ\text{C}) > (30\, ^\circ\text{C})\). In particular, the mean ELISA S/P ratios in chlorhexidine-treated samples were significantly higher than other treatments at \(30\, ^\circ\text{C}\).

**Total swine immunoglobulin isotype quantification ELISAs**
Concentration (log\(_{10}\) ng per ml) of total swine IgG, IgA, and IgM antibody in oral fluid samples by time, temperature, and treatment are shown in Figures 4, 5, 6. For IgG and IgA, two temperature groups were identified on the basis of statistically significant differences: \((-20\, ^\circ\text{C} = 4\, ^\circ\text{C} = 10\, ^\circ\text{C} = 20\, ^\circ\text{C}) > (30\, ^\circ\text{C})\). At \(30\, ^\circ\text{C}\), higher levels of IgG and IgA were measured
in chlorhexidine- and isothiazolinone-treated samples over time relative to no treatment. For IgM, no significant difference was found among results by temperature or treatment.

**Measurement of pH**

Statistical analysis of main effects (marginal means) showed that both temperature and antimicrobial treatment had significant effects on pH levels (Table 4). Three temperature groups were identified on the basis of pH levels: \((-20°C = 4°C = 10°C) > (20°C) > (30°C)\).

**Discussion**

Oral fluids have been shown to be a convenient diagnostic specimen for the detection of infectious agents and antibodies.\(^6\)\(^-\)\(^8\),\(^10\),\(^11\) The present study addressed the question of PRRSV and anti-PRRSV antibody stability in oral fluid as a function of time and temperature, both with and without anti-microbial preservatives. Stability was evaluated over a 12-day observation period to test extreme sample handling and storage on diagnostic results.

Standard plate count data showed that bacterial proliferation was reduced by chlorhexidine at all temperatures tested. The concentration of isothiazolinone tested in this study did not produce a measurable effect on bacterial proliferation. Importantly, untreated samples contained concentrations of PCR-detectable PRRSV RNA and ELISA-detectable anti-PRRSV antibody at levels equal or greater than preserved samples when held at temperatures \(\leq10°C\). Thus, preservatives were not required, if the cold-chain were maintained.

There are few publications with which to compare these data. A search of the literature yielded no prior reports on the stability of PCR-detectable viral or bacterial agents by time and temperature in oral fluids. However, our data are in agreement with published data reporting that the concentration of antibodies in human oral fluids declined as a function of increasing time and higher temperature.\(^22\),\(^24\),\(^25\)
Implications

- PRRSV and anti-PRRSV antibodies are relatively resistant to degradation in oral fluids.
- Use of antimicrobials is not necessary for the preservation of diagnostic targets if appropriate handling, storage, and transport protocols are implemented.
- Appropriate specimen handling protocols, i.e., freezing or refrigeration at 4°C, will maintain the integrity of PRRSV and anti-PRRSV antibodies in oral fluid samples collected for diagnostic testing.

Acknowledgements

We thank Dr. Fernando A. Osorio (University of Nebraska-Lincoln) for generously providing concentrated anti-PRRSV antibody. This project was funded in part by an Advanced PRRS Research Award provided by Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO.

References


### Tables and Figures

Table 1. Standard Plate Count log10 cells per ml (LS means)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Chlorhexidine</th>
<th>Isothiazolinone</th>
<th>No Treatment</th>
<th>Temperature means</th>
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<tr>
<td>30°C</td>
<td>4.2 E</td>
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<td>8.1 AB</td>
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<td>4°C</td>
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<tr>
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<td>0.7 F</td>
<td>6.9 D</td>
<td>7.4 ABCD</td>
<td>5.0 B</td>
</tr>
</tbody>
</table>

Treatment means: 2.0 C, 7.2 B, 7.9 A

Values not connected by the same letter are significantly different.
Table 2. PRRSV qRT-PCR log_{10} genomic copies per ml (LS means)

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<th>No Treatment</th>
<th>Temperature means</th>
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<tr>
<td>4°C</td>
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Values not connected by the same letter are significantly different
Table 3. PRRSV ELISA S/P (LS means)

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<td>1.4 B</td>
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Values not connected by the same letter are significantly different
Table 4. pH (LS means)

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<tr>
<td>Treatment means</td>
<td>8.4 A</td>
<td>8.2 B</td>
<td>8.2 B</td>
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Values not connected by the same letter are significantly different.
Figure 1. Standard plate count (log₁₀ cells per ml) over time by temperature and storage time.
Figure 2. PRRSV qRT-PCR (log10 genomic copies per ml) over time by temperature and storage time
Figure 3. PRRSV ELISA (S/P) over time by temperature and storage time
Figure 4. Total swine IgG (log_{10} nanograms per ml) over time and storage temperature
Figure 5. Total swine IgA (log_{10} nanograms per ml) over time and storage temperature
Figure 6. Total swine IgM (log<sub>10</sub> nanograms per ml) over time and storage temperature
CHAPTER 5. PROLONGED DETECTION OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) AND ANTI-PCV2 ANTIBODY IN ORAL FLUIDS FOLLOWING EXPERIMENTAL INOCULATION

A paper to be submitted to Veterinary Research.

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Jeffrey J. Zimmerman, Tanja Opriessnig

Abstract

The onset, level, and duration of quantitative polymerase chain reaction-detectable PCV2 and anti-PCV2 antibody in oral fluid was evaluated using samples collected from experimentally-inoculated pigs for 98 days post inoculation (DPI). Pigs (n = 24) were obtained at 3 weeks of age and randomly allocated to 4 treatment pens of 6 pigs each: (1) negative control group; (2) inoculated with PCV2a (strain ISU 40895) on DPI 0; (3) inoculated with PCV2a (strain ISU-40895) on DPI 0 and re-challenged at DPs 35 and 70; (4) inoculated with PCV2a (ISU-40895), PCV2b (PVG4072), and PCV2a (ISU-4838) on DPs 0, 35, and 70, respectively. Serum was collected from each animal and one oral fluid sample was collected from each pen (group) every other day from DPI 2 through DPI 14 and weekly through 98 DPI. Oral fluid samples were assayed for the presence of PCV2 by PCR, anti-PCV2 IgG antibody by enzyme linked immunosorbent assay (ELISA), and anti-PCV2 antibody isotypes (IgA, and IgM) by ELISA. Serum was assayed for anti-PCV2 IgG by ELISA. Anti-PCV2 antibodies (IgG, IgM, and IgA) were detected in oral fluid from experimentally inoculated pigs from 14 to 98 DPI. PCV2 was detected by PCR in oral fluid samples from all pens of inoculated pigs at 2 DPI. Thereafter, PCV2 was detected in oral fluid throughout DPI 98. Overall, the data indicated that PCV2 infection in swine populations can be efficiently monitored using oral fluid specimens.
Introduction

Porcine circovirus Type 2 (PCV2) is a small circular DNA virus belonging to the family *Circoviridae* (Tischer et al., 1982). There are currently three known strains of PCV2. PCV2a was demonstrated in tissue samples archived in 1962 (Jacobsen, 2009), PCV2b was first reported in North America in 2005 (Cheng, 2009; Hesse, 2008), and PCV2c was recently reported in Denmark from archived serum samples collected from non-clinical pigs in 1980, 1987 and 1990 (Dupont et al., 2008). PCV2c isolates are more closely related to PCV2b (95%) than PCV2a (91-93.6%) in sequence homology (Dupont et al., 2008).

PCV2 is present in commercial swine herds worldwide and within infected herds, infection approaches 100% of pigs (Opriessnig et al., 2004c). Transmission of PCV2 occurs as a result of direct contact with oronasal secretions, feces, and urine (Bolin et al., 2001; Magar et al., 2000, Shibata et al., 2003). The effect of PCV2 on pig health is highly variable, ranging from subclinical infection to acute mortality. Although essentially all herds are infected and relatively few herds exhibit clinical signs, some affected herds experience massive losses (Opriessnig et al., 2007). To provide clarity to the broad range of clinical consequences of PCV2 infection, the American Association of Swine Veterinarians (AASV) adopted the term "porcine circovirus-associated disease" (PCVAD) and provided case definitions and criteria for the diagnosis of PCVAD (Anon, 2007). According to these guidelines, PCVAD includes subclinical infection, multisystemic disease with weight loss (formerly known as PMWS), high mortality without an alternate etiology, respiratory signs, enteric signs, porcine dermatitis and nephropathy syndrome (PDNS), and reproductive disease.

There is need for reliable and economical methods to monitor PCV2 circulation in swine populations. Oral fluid samples were previously shown to reflect the circulation of pathogens in swine populations (Prickett et al., 2008b) and PCV2 has been detected in oral fluid collected from gnotobiotic (Allan and Ellis, 2000) and finishing pigs (Prickett et al., 2008b). Therefore, the objectives of this research were to determine the onset, level, and duration of PCV2 and anti-PCV2 antibody in oral fluid samples from experimentally inoculated pigs.
Materials and Methods

Experimental design
The onset, level, and duration of PCR-detectable PCV2 and anti-PCV2 antibody in oral fluid was evaluated using samples collected from experimentally-inoculated pigs for 98 days post inoculation (DPI). Pigs (n = 24) were obtained at 3 weeks of age from a commercial swine herd free of PRRSV and influenza virus, but seropositive for PCV2, and housed thereafter in research facilities at Iowa State University in Ames, Iowa. Prior to PCV2 inoculation, animals were monitored for the decay of anti-PCV2 maternal antibody until sample-to-positive (S/P) values were ≤ 0.2. At 11 weeks of age, animals were randomly allocated to four treatment pens of 6 pigs each (Table 1): (1) negative control group; (2) inoculated with PCV2a (strain ISU 40895) on DPI 0; (3) inoculated with PCV2a (strain ISU-40895) on DPI 0 and re-challenged at DPsIs 35 and 70; (4) inoculated with PCV2a (ISU-40895), PCV2b (PVG4072), and PCV2a (ISU-4838) on DPsIs 0, 35, and 70, respectively. All viruses were administered as one ml intramuscular (IM) and 2 ml intranasal (IN) inocula. PCV2a 40895 and PCV2b PVG4072 were used at a dose of $10^{4.5}$ 50% tissue culture infectious dose (TCID$_{50}$) per ml and PCV2b 4838 was used at a dose of $0.5 \times 10^{3.5}$ TCID$_{50}$ per ml. Serum was collected from each animal and one oral fluid sample was collected from each pen (group) every other day from DPI 2 through DPI 14 and weekly through 98 DPI. Oral fluid samples were assayed for the presence of PCV2 by quantitative PCR, anti-PCV2 IgG antibody by enzyme linked immunosorbent assay (ELISA), and anti-PCV2 antibody isotypes (IgA, and IgM) by ELISA. Serum was assayed for anti-PCV2 IgG by ELISA.

Animals and animal care
The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#3-06-6083-S). Throughout the experiment, animals were housed on the floor in pens that were cleaned daily. Feeder space, square footage per animal, ambient temperature, and room air exchanges all met or exceeded guidelines and requirements set forth in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Sciences Societies, 1999). Animals were fed ad libitum an age-appropriate commercial feed that met or exceeded the
nutritional requirements for swine as determined by the National Research Council (National Research Council, 1998).

Porcine circovirus type 2 (PCV2)

PCV2 strain 40895 (PCV2a) (GenBank accession number AF264042) was recovered from an Iowa farm in 1998 and has been well characterized genetically (Fenaux et al., 2000) and in the SPF pig model (Fenaux et al., 2003; Fenaux et al., 2004; Opriessnig et al., 2004a,b,c; Opriessnig et al., 2003). PCV2 strain PVG4072 was isolated from lung tissue homogenate obtained from a pig presenting clinical PCVAD in a farm experiencing approximately 25% mortality in Indiana. PCV2 isolate 4838 (PCV2a) (GenBank accession number DQ397521) was recovered from a subclinically-infected pig on an Iowa farm in 2003 (Fenaux et al., 2002).

Collection of serum and oral fluid samples

Blood samples were collected using a single-use blood collection system (Becton, Dickinson and Company, Franklin Lakes, NJ) and centrifuged at 1000 x g for 10 min. Serum was harvested, aliquoted, and stored at –80°C. Pen-based oral fluid samples were collected by suspending a length of 3-strand twisted cotton rope in each pen for 20 min (Prickett et al., 2008a). Oral fluid was extracted from each rope, aliquoted, and stored at –80°C.

PCV2 quantification

The completed PCR protocol has been described elsewhere (Oprissnig et al., 2004b). In brief, PCV2 nucleic acid extraction from serum and oral fluid samples was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA) according to the manufactures protocol. DNA-extracts were used for quantification of PCV2 genomic DNA by real-time PCR and expressed as genomic copies per ml.

PCV2 serum IgG antibody ELISA

Serum samples were tested in duplicate for anti-PCV2 antibody by ELISA (Nawagitgul et al., 2002). In brief, serum samples were diluted 1:100 in 5% non-fat dry milk in PBS (NFDM) diluents and assayed in two wells coated with partially-purified recombinant open reading frame two (ORF2) protein produced in baculovirus-infected insect cells. To measure
background reactivity, serum samples were concurrently assayed in two wells coated with baculovirus-infected insect cells (SF-9 cells, Invitrogen 11496-015, Carlsbad, CA). Controls consisted of PCV2 antibody-positive serum diluted with PBS to the appropriate range of reactivity and antibody-negative serum from a cesarean-derived, colostrum-deprived pig determined to be free of PCV2. Antibody-antigen reactions were detected using horseradish peroxidase-labeled anti-swine IgG antibody (KPL 78526) diluted 1:2000 in PBS followed by the addition of tetramethylbenzidine (TMB, KPL 52-00-03, Gaithersburg, MD). The response was read at 405 nm and measured as optical density (OD). The results were corrected for non-specific reactivity and reported as sample-to-positive (S/P) values:

\[
S/P = \frac{\text{mean sample ORF2 well OD} - \text{mean sample baculovirus well OD}}{\text{mean positive control ORF2 well OD} - \text{mean positive control baculovirus well OD}}
\]

S/P results \( \geq 0.30 \) were considered positive for anti-PCV2 antibody.

**Oral fluid PCV2 IgM-, IgG-, IgA-specific antibody ELISAs**

*Preparation and purification of PCV2 nucleocapsid antigen*  
An ELISA based on PCV2 nucleocapsid protein was used to characterize the anti-PCV2 antibody response (IgM, IgA, IgG) in oral fluids. To produce PCV2 nucleocapsid protein, a 549bp sequence of the PCV2 capsid gene lacking 39 amino acids in the amino terminal and 10 amino acids in the carboxyl terminal was amplified with PCV2-specific primers containing BamH1 (New England Biolabs, MA) and Zho1 (New England Biolabs, MA) restriction enzyme sites: 5’ CGC GGA TCC ATG AAA AAT GGC ATC TTC AAC ACC CGC CT 3’ and 5’ CCG CTC GAG TTC TCT GAA TTG TAC ATA CAT GGT3’. PCR amplification was carried out in a 50 \( \mu \)l reaction tube containing 1x PCR buffer, 0.5ng per \( \mu \)l template, 0.25 \( \mu \)M of each primer, 0.2mM of dNTPs, and 0.25\( \mu \)l of taq polymerase (AmpliTaq Gold®, Applied Biosystems, 5 units per \( \mu \)l). After an initial denaturation at 95°C for 10 min, PCV2 capsid gene was amplified by 45 cycles of denaturation at 94°C for 30s, annealing at 55°C for 35s, extension at 72°C for 40s, and final extension at 72°C for 7 min. After PCR amplification, the product was tested on 1.2% agarose gel for the correct band size and then purified (PCR purification kit, Qiagen®, Valencia, CA) and digested with restriction enzymes BamH1 and Zho1 for 2hrs at 37°C. The digested PCR product was then re-purified (PCR purification kit,
Qiagen®, Valencia, CA). A modified pET24b vector (Novagen, Madison, WI) with a 5’ myc tag and a terminal 3’ His tag was used to clone the truncated PCV2 capsid gene. Cap PCV2-pET24b was then transformed to XL-10 gold competent cells (Stratagene, La Jolla, CA) and spread onto Luria Bertani (LB) agar plates (Difco, Lawrence, KS) containing 30µg per ml kanamycin (Sigma-Aldrich, St. Louis, MO) and screened by colony PCR using T7 forward and reverse primers of pET24b plasmid. Purified plasmids from positive colonies were further confirmed by DNA sequencing for the integrity of capsid genes. Once the sequence was confirmed, recombinant plasmid was transformed into BL21 (DE3)-RP cells (Stratagene, La Jolla, CA) and spread onto LB plates containing kanamycin at 30µg per ml and chloramphenicol (Sigma-Aldrich, St. Louis, MO) at 35µg per ml. Ten well-grown isolated colonies were transferred and grown overnight at 37°C in LB medium with antibiotics and then 200 µl of each colony was inoculated into 10ml of 2xYT containing kanamycin at 30µg per ml. These cultures were grown at 37°C to an OD (600 nm) of 0.5 to 0.6. Half of the culture was then removed for an un-induced control and the remaining half was induced with isopropyl β-D-thiogalactoside (IPTG; Calbiochem, San Diego, CA) at a concentration of 1.0mM. The induced cultures were incubated at 37°C for 4hrs and then 200 µl from induced and un-induced cultures were analyzed by SDS-PAGE. After confirming the expression in small scale culture, E. coli BL21 RP-containing recombinant plasmid was grown in one litre culture and the expressed protein was purified by Ni-NTA agarose bead affinity chromatography (Qiagen®, Valencia, CA). Briefly the culture was centrifuged at 17,600 x g for 10 min and supernatant was discarded. The pellet was suspended in 50ml suspension buffer containing 50mM tris-HCl (Fishers), 500mM NaCl, 10mM β-ME (beta mercaptobenzothiazole) Gibco 1mM PMSF (phenylethyl sulphonyl fluoride Sigma-Aldrich, St. Louis, MO), lysozyme (0.2mg per ml; Sigma-Aldrich, St. Louis, MO), Triton X-100 (10%,sigma), and benzonase® nuclease(8 units per ml; Novagen, Madison, WI). After brief sonication, the suspension was re-centrifuged, supernatant was harvested, and the pellet was re-suspended in 50ml double deionized water containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 10mM β-ME. SDS-PAGE was performed to determine if the expressed protein was present in the supernatant or inclusion bodies. Results indicated PCV capsid proteins were present in the pellet as inclusion bodies. Recombinant PCV capsid protein was
recovered by re-suspending the pellet in buffer containing 10mM tris-HCl, 100mM NaH2PO4, 6M guanidine hydrochloride, 10mM β-ME and 1mM PMSF. The mixture was rotated at room temperature for 30min after which the solution was centrifuged at 31,000 x g for 15 min at 4°C. Recombinant PCV capsid proteins were purified by Ni-NTA agarose bead affinity chromatography (Qiagen®, Valencia, CA) according to manufacturer’s instructions. Purified recombinant protein was analyzed by SDS-PAGE to determine the purity and concentration was measured by Bradford assay according to manufacturer’s instructions using a spectrophotometer. The purified protein (more than 90% pure based on SDS-PAGE) was stored in -80°C.

**Oral fluid anti-PCV2 IgM-, IgG-, IgA-specific antibody quantification**

Oral fluid samples were assayed in duplicate for anti-PCV2 IgM, IgA, and IgG immunoglobulins using indirect ELISAs specific for each isotype. Plates were prepared by diluting PCV2 nucleocapsid proteins in carbonate buffer (15mM NaCO3, 35 mM NaHCO3, pH 9.6) to a concentration of 2 ug per ml. Antigen concentration of 200ug per well was achieved by transferring 100ul of the protein solution to each well of a 96 well plate (COSTAR 3590, 96 well EIA/RIA plate, Corning, NY) and incubating the plate overnight at 4°C. Plates were washed 3 times with 1X phosphate buffered saline and 0.05% Tween20® (PBST, pH 7.4). The last wash remained in the wells for 5 min at room temperature before the plate was aspirated and blotted dry. To prevent non-specific binding, blocking buffer (5% Non-Fat Dry Milk (NFDM) in PBST, pH 9.6) was added to the plates and incubated at room temperature for 2 hr. The plates were then washed as previously described. Oral fluid samples were diluted 1:3 in 5% NFDM and 1% sodium azide in PBST and 100ul of diluted sample was incubated on the plates for 18 hr at 4°C. Each antibody isotype ELISA used a horseradish-peroxidase conjugated secondary antibody specific for swine IgM, IgA, or IgG (E100-102, E100-104, E100-100, Bethyl Laboratories, Inc., Montgomery, TX). The secondary antibody for each assay was diluted 1:25,000 with 5% NFDM in PBST and 100ul was added to each well and incubated for 2 hr at room temperature. Next, 100ul of enzyme substrate solution at room temperature (TMB Peroxidase Substrate System, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added to each well and incubated at room
temperature for 20 min. At precisely 20 min, 100μl of stop solution (1M phosphoric acid) was added to each well. The plates were read at 450nm and results expressed as raw OD values.

**Statistical methods**

Two-way analysis of variance (ANOVA) was performed (JMP® 8.0, SAS, Cary, NC) on log$_{10}$ transformed data to test for significant differences among treatment groups. Time and treatment were included as fixed effects in the model. Thereafter, differences in treatment effects were analyzed using pair-wise comparisons with Tukey adjustment to account for Type 1 errors associated with multiple comparisons. In all analyses, significance was defined as $p \leq 0.05$. All figures were generated using Sigmaplot® 11.0.0.75 (Systat Software Inc., San Jose, California)

**Results**

**Oral fluid PCR**

Overall, 56 of 57 oral fluid samples from PCV2-inoculated pens were PCR positive through the observation period and 19 of 19 oral fluid samples from the negative control pen tested PCR negative. This corresponded to a diagnostic sensitivity and diagnostic specificity of 98% (95% CI: 94.8, 100.0) and 100%, respectively. Two-way ANOVA analysis of PCR results showed significant differences in PCR log$_{10}$ genomic copies per ml by DPI and treatment, but pair-wise comparisons found no significant differences between inoculated groups. All pens were PCV2 positive by two DPI and all groups continued to shed PCV2 in oral fluids through the end of the collection period (Figure 1).

**ELISA**

**PCV2 Serum IgG antibody ELISA**

Two-way ANOVA analysis of serum IgG results showed that there were significant differences in S/P values by DPI and treatment. Pair-wise comparisons detected no significant differences between inoculated groups. Based on the 0.30 cut-off of the PCV2 serum ELISA, the means of all inoculated groups indicated seroconversion between 14 and 21 DPI and thereafter a high level of serum IgG was maintained throughout the duration of the study (Figure 2).
**Oral fluid antibody ELISA’s**  Two-way ANOVA analysis of oral fluid IgG results showed significant differences in S/P values by DPI and treatment. Pair-wise comparisons detected no significant differences in oral fluid IgG results (means shown in Table 2). The onset of anti-PCV2 IgG in oral fluid was between 14 and 21 DPI and IgG remained detectable through the end of the study. The means of the inoculated pens and the negative control pen over-time are shown in Figure 3.

Two-way ANOVA analysis of oral fluid IgM results showed significant differences in S/P values by DPI and treatment. Pair-wise comparisons showed that the level of IgM was significantly different among inoculation groups (means shown in Table 2), but changes in oral IgM concentrations did not coincide with re-inoculation events. In inoculated groups, the onset of anti-PCV2 IgM in oral fluid was between 10 and 14 DPI and IgM remained detectable through the end of the study. The means of the inoculated pens and the negative control pen over-time are shown in Figure 4.

Two-way ANOVA analysis of oral fluid IgA results showed significant differences in OD values by DPI and treatment. Pair-wise comparisons indicated no significant differences in the amount of anti-PCV2 IgA in oral fluid samples (Table 2). The onset of anti-PCV2 IgA in oral fluid was between 14 and 21 DPI and IgA remained detectable through the end of the study. The means of the inoculated pens and the negative control pen over-time are shown in Figure 5.

**Discussion**

The use of oral fluid specimens in diagnostic medicine is supported by a broad foundation of research and application in human medicine. Both PCR- and antibody-based assays using oral fluids have been employed in the diagnosis of a variety of human pathogens, e.g., HIV, hepatitis A, B, and C viruses, measles, mumps, and others (Madar et al., 2003). A diagnostic approach based on oral fluid specimens has facilitated the collection of large amounts of epidemiological data, e.g., HIV in Africa (Connolly et al., 2004; Fylkesnes and Kasumba, 1998) and Thailand (Frerichs et al., 1994), and measles in Europe (Ramsay et al., 1997), Ethiopia (Nigatu et al., 2008), Brazil (de Azevedo Neto et al., 1995; Oliveira et al., 1998),
In swine, a number of viral pathogens may be detected in oral fluid, including influenza virus (Heinen et al., 2001), PCV2 (Prickett et al., 2008b; Shibata et al., 2003), PRRSV (Prickett et al., 2008a,b; Wills et al., 1997), and vesicular stomatitis virus (Stallnecht et al., 1999). Likewise, antibodies against a variety of swine pathogens have been reported in oral fluid, e.g., *Actinobacillus pleuropneumoniae* (Loftager et al., 1993), *E. coli* (Duysscher and Dubois, 1978), classical swine fever virus (Corthier, 1976; Corthier and Aynaud, 1977), and transmissible gastroenteritis virus (Duysscher and Berman, 1980).

The objective of this research was to provide improved estimates of the onset, level, and duration of PCV2 and anti-PCV2 antibody in oral fluid samples from experimentally inoculated pigs. Anti-PCV2 antibodies (IgG, IgM, and IgA) were demonstrated in oral fluid from experimentally inoculated pigs from 14 to 98 DPI, but the small number of pens in the study precluded a robust statistical analysis of assay performance and cut-off selection. Shibata et al. (2003) previously reported the detection of PCV2 at 70 DPI in serum, feces, nasal, and oropharyngeal secretions from gnotobiotic pigs. The current work expanded the previously reported period of shedding and showed that prolonged shedding of PCV2 in oral fluid is not restricted to gnotobiotics. That is, PCV2 was detected by PCR in oral fluid samples from all pens of inoculated pigs at 2 DPI, i.e., the first sampling point. Thereafter, PCV2 was detected in oral fluid samples from all inoculated pens throughout the remainder of the study. At the final sampling point (DPI 98), the mean virus concentration in oral fluid was $1 \times 10^{5.6}$ genomic copies per ml. Overall, the data indicated that PCV2 infection in swine populations can be efficiently monitored using oral fluid specimens.

**Acknowledgements**

We would like to thank the faculty and staff of the Veterinary Diagnostic Laboratories at Iowa State University and the University of Minnesota for their contributions to this work.
References


### Tables and Figures

Table 1. Experimental design: inoculation schedule and PCV2 strains

<table>
<thead>
<tr>
<th>Group designation</th>
<th>Day Post Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>(1) Neg. Controls (n=6)</td>
<td>---</td>
</tr>
<tr>
<td>(2) PCV2a (n=6)</td>
<td>PCV2a&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>(3) R-PCV2a (n=6)</td>
<td>PCV2a&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>(4) R-PCV2a/b (n=6)</td>
<td>PCV2a&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A</sup> Strain 40895

<sup>B</sup> Strain 4838

<sup>C</sup> Strain PVG4072
Table 2. Oral fluid ELISA: significant differences in ELISA S/P results by inoculation group

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Controls (n=6)</td>
<td>0.09 (B)</td>
<td>0.05 (C)</td>
<td>0.06 (B)</td>
</tr>
<tr>
<td>(2) PCV2a (n=6)</td>
<td>0.88 (A)</td>
<td>0.09 (B)</td>
<td>1.23 (B)</td>
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<tr>
<td>(3) R-PCV2a (n=6)</td>
<td>1.20 (A)</td>
<td>0.15 (A)</td>
<td>1.53 (B)</td>
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<tr>
<td>(4) R-PCV2a/b (n=6)</td>
<td>0.99 (A)</td>
<td>0.12 (AB)</td>
<td>1.29 (B)</td>
</tr>
</tbody>
</table>

* Means not connected by the same letter are significantly different.
Figure 1. Mean log$_{10}$ PCV2 genomic copies per ml in oral fluid over time
Figure 2. Mean PCV2 serum IgG ELISA S/P results over time
Figure 3. Mean PCV2 oral fluid IgG ELISA S/P results over time
Figure 4. Mean PCV2 oral fluid IgM ELISA S/P results over time
Figure 5. Mean PCV2 oral fluid IgA ELISA S/P results over time
GENERAL CONCLUSIONS

The U.S. swine industry has experienced dramatic change over the past several decades marked by intense consolidation of pig production. The U.S. Census of Agriculture numbers showed that the average total inventory among U.S. swine operations in 1959 was 37 animals (USDA, 1997). This increased to a mean of 81 per operation in 1969, 130 in 1978, 215 in 1987, 301 in 1992, and the trend continues today. Historically, medication of individual animals prevailed in health management, with sporadic outbreaks of disease controlled with vaccination or depopulation-repopulation. These measures were generally effective because small herd size was amenable achieving herd immunity, thereby limiting the severity and frequency of outbreaks.

Health challenges have drastically changed as a result of the increased population size of production units, but changes in health management have lagged behind. An important, but generally unrecognized, effect of this change is the impact of increasing herd size on the expression of clinical disease. In today's high density production systems, pathogens that were previously economically and clinically unimportant have exerted a larger impact on health as a consequence of changing disease ecology. While experimental research has provided information regarding the effects of co-infections on swine health, these research models cannot recreate the diversity or magnitude of co-infections that drive the ecological relationships among the pathogens that circulate in modern swine populations. The economic burden of disease on pork producers is already significant, but will be exacerbated in the future by increasing energy and feed costs. This fact drives the necessity to improve the health management of swine populations to protect swine health and enterprise profitability.

To reduce the economic burden of disease and improve health management of herds, it is imperative to quantify the cost of specific pathogens and co-infection to compromised pig performance and the benefit of interventions in terms of return on investment. The data to answer these questions can only be collected from commercial swine herds. Historically, the estimates of the burden of infection have been based on analysis of serum samples collected from individual animals. However, these data are cost-prohibitive because of the labor and
number of samples required to sufficiently represent the population.

The research presented in this dissertation is the first foray into the surveillance of swine pathogens with pen-based oral fluid samples and there are many issues yet to be addressed. For example, it would be highly beneficial to devise a method to collect oral fluid samples from individual animals. These data would make it possible to quantify the variability of antibody/pathogen shedding among individual animals. Oral fluid testing of individual animals would also have applicability in the swine industry, i.e., intensive disease monitoring of boar studs could benefit from an easy to collect and reliable diagnostic sample.

Thus, the results of the research presented in this dissertation begin to lay the foundation for a new approach to field research in the area of swine health. Although much work remains to be done, the review of the literature presented in chapter one and the supporting data in chapters two to five suggest that oral fluid sampling in swine populations holds the potential to be a powerful method of obtaining the data crucial to improving the health management of herds.

References

ACKNOWLEDGEMENTS

I would like to thank the faculty and staff of the Iowa State University Veterinary Diagnostic Laboratory for their contributions to my graduate student education. The environment of the VDL is uniquely conducive to graduate student learning due to the open, frank, and friendly people that work there. My experiences as a graduate student in the VDL will serve me well as a future veterinary diagnostician. I am also grateful for the intellectual and economic support I received from swine producers, biologic companies, and practitioners. In particular, I extend special thanks to the National Pork Board, Boehringer Ingelheim Vetmedica, Inc., IDEXX Laboratories, Inc., Murphy-Brown, LLC., and Pfizer Animal Health.