Detection of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in oral fluid specimens

by

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ABSTRACT

Understanding pig behavior and reducing stress in the pig in commercial productions settings is important for economical, social and ethical reasons. Stress occurs when pigs are eating, mating or simply when they are in a novel environment. Changes in production and housing environments have occurred very rapidly both in the context of the evolution of the species and in the history of the domestication of the pig. As a result, welfare problems can arise as a result of a mismatch between the pig's behavioral needs and its environment.

As reviewed in Chapter 1, understanding the behavior of pigs can provide useful information regarding the type and function of pig behaviors, their range of choices, and their response to the environment. This information should be used to provide a foundation upon which to build a better environment for pigs therefore improving production. A significant part of the work described in this thesis involved studying the exploratory behavior of the pig for the purpose of developing oral fluid-based diagnostic methods for use in monitoring and surveillance of infectious diseases. Because it is based on natural pig behavior, oral fluid sampling offers an "animal friendly" method to collect diagnostic specimens.

The objective of the first study (Chapter 2) was to determine whether individually-housed mature boars could be trained for oral fluid collection. The objective measures used to evaluate the success of this project were the rate of successful collections and the quantity of sample collected. Three trials of 24 boars each (6 months to 3.6 years in age) were conducted to determine if oral fluids could be collected from individually-penned adult animals. Among 70 trained boars, 524 samples were collected in 560 attempts (93.6%) over an 8-day observation period. Statistically significant differences in sample volume were associated with observation day, boar age, and for the interaction of trial x day, but not with trial or the interactions of boar age x trial or boar age x day.

The objective of the second study (Chapter 3) was to determine whether oral fluid samples could be used to monitor individually-housed adult boars for porcine reproductive and respiratory syndrome virus (PRRSV) infection. In 3 trials, 24 boars were intramuscularly (IM) inoculated with a modified-live PRRSV (MLV) vaccine (Trial 1), a Type 1 PRRSV isolate (Trial 2), or a Type 2 isolate (Trial 3). Oral fluid samples were collected daily and

serum samples were collected twice weekly. Following the completion of the study, samples were randomized and blind-tested for PRRSV by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). PRRSV was detected in oral fluids at DPI 1 and all oral fluid specimens were PRRSV qRT-PCR positive at DPI 4. Although PRRSV was detected in both serum and oral fluid specimens through DPI 21, a comparison of matched samples from individual boars showed that oral fluid was equal to serum for the detection of PRRSV at DPI 7 and more likely to be positive than serum on DPI 14 and 21. Overall, oral fluid was superior to serum for the detection of PRRSV using PCR over the 21 day observation period in this study. The results of this experiment suggest that individually-penned oral fluid sampling could be an efficient, cost-effective approach to PRRSV surveillance in boar studs and other swine populations. This manuscript has been accepted for publication in Virus Research.

CHAPTER 1. INTRODUCTION: THESIS ORGANIZATION

This thesis consists of four chapters. Chapter 1 is the general introduction and review of the literature, "Understanding our friend, the pig". This manuscript will be submitted to Animal Health Research Review. Chapter 2, "Oral fluid diagnostic samples can be collected from individually-penned boars" is in preparation for submission to Journal of Applied Animal Welfare Science. Chapter 3, "Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: Will oral fluid replace serum for PRRSV surveillance?" has been accepted for publication in Virus Research. References, tables, and figures for each research manuscript follow the discussion section of each. The last chapter contains the general conclusions of the thesis research.

CHAPTER 2. UNDERSTANDING OUR FRIEND, THE PIG

For submission to Animal Health Research Reviews

Apisit Kittawornrat, Jeffrey J. Zimmerman

1. Introduction

Domestication of *Sus scrofa* may have occurred as early as 10,000 B.C. in Southeast Asia (Lekagul and McNeely, 1977). Pig production was common in early agricultural societies because pigs were highly resilient, matured quickly, had large litters, and ate low-quality feedstuffs. Pigs were introduced into North America by many of the early explorers and settlers, but pork production began to flourish in the U.S. after the practice of finishing pigs on corn became established in the late 1600s (McGlone and Pond, 2003). The practice was so successful that, by 1847, there were nearly twice as many pigs as people in the U.S. (35 million pigs vs. 20 million people) and Cincinnati was the largest pork market in the world (USDA, 1981). U.S. swine inventory numbers are available as far back as 1850. For most of this period, but particularly between 1890 and the present, the total number of pigs in the U.S. has remained at 50 to 60 million animals (USDA, 2008). However, since about 1900, and more recently elsewhere, the total number of swine farms has been declining (USDA, 2008) resulting in fewer farm with more pigs per farm.

Initial provisions for housing swine were rudimentary, as reflected in Spencer's (1919) assessment that: "*The ordinary sty with a yard attached is unhealthy for a growing or matured pig, but in the colder weather it is simply cruel for newly born pigs*". An evolution in swine housing occurred concurrently with the decline in the number of producers in the 20th century. Experiments in the early 1900's led Danish agriculturalists to conclude that housing swine indoors ("intensive" production) provided for more efficient use of available land, protected animals from weather, eliminated fighting, and improved feed efficiency (Shaw, 1938). Spencer (1919) marveled at the 2-story barn he observed near Aarhuss,

Denmark in which the pigs were fed downstairs and slept upstairs, which they reached by walking up a ramp. Shaw (1938) made the noteworthy comment that removal of manure "at least daily" was required to maintain sanitation under confinement conditions. A reflection of the abundant farm labor available at the time! The trend toward production of swine in intensive systems greatly accelerated after 1980 and has resulted in sophisticated, highly-engineered, capital-intense facilities designed to reduce manual labor and increase efficiency.

Concomitant with the 20th century's remarkable changes in swine husbandry were equally dramatic changes in human demographics. World population more than tripled during this period as the 1.65 billion people present in 1900 swelled to 6 billion in the year 2000 (Gelbard et al., 1999). Simultaneously, a vast migration occurred as people left the farms, moved into the cities, and found new means of livelihood. The 41% of the U.S. labor force employed in agriculture in 1900 fell to 16% in 1945 and just 1.9% in 2000 (Dikitri et al., 2005). As a result of the increase in world population, and as per capita incomes rise in developing countries, there is increased demand for animal protein. It is estimated that the demand for pork, and thus the need for raising pigs, will increase between 6 and 14% over current levels, especially driven by increased demand in South East Asia (EC, 2007).

In the midst of the dramatic and dynamic changes we are witnessing, it is important for those of us responsible for animal welfare and health to pause and reflect. Above all, it is essential that we continue to assess, scrutinize, and improve the welfare of pigs in modern production systems, even as the industry evolves and new health challenges are arise. Therefore, this paper reviews basic sensory and behavioral aspects of swine with the objective of reaching a better understanding of our Friend, the Pig.

2. Sense of sight

The pig’s eyes are deeply placed and appear small, especially in over-conditioned adults. The eye has a diameter of approximately 24 mm. The overall ocular power of the pig’s eye has been calculated as 78 diopters (Coile and O’Keefe, 1988). Pigs have wide angle of vision (310°, Prince, 1977), thus, a panoramic view of their surroundings. Tanida et al. (1996) found that piglets have a tendency to move towards a more brightly illuminated area

and respond negatively to the sharp contrast of black and white on the floor. Moreover, they suggested that a beam of light in the darkness could affect the movement of the piglets, even when it is directly in their eyes. This is not in agreement with Grandin (1982) who recommended that lamps should not shine directly into the eyes of the approaching livestock. However, Tanida et al. (1996) also recommended the light should be even and diffuse in order to encourage movement into poorly illuminated areas.

The radiation wavelengths to which pigs are maximally sensitive are slightly lower than those for humans. Within the range of 465 to 680 nm, pigs can distinguish wavelength differences as small as 20 nm (Signoret et al., 1975). Pigs have good color vision and may respond to the novelty of a change in the color of the handlers' uniform (Hemsworth, 2007). The retina contains a substantial population of two types of cones with sensitivity peaks at about 439mm (indigo) and 556mm (green-yellow) in the spectrum of visible light (Neitz and Jacobs, 1989). The pig is thus believed to have dichromatic color vision. However, the presence of a particular photo pigment is a weak indicator of the information actually supplied by activation of this pigment. Tanida et al. (1991) reported pigs discriminated only blue from gray among three primary colors of red, green, and blue. Similarly, the wild boars succeeded in recognizing bluish color and failed to discriminate green or yellow (Tanida et al., 1991). In a study evaluating responses to water dispenser color, Stelios et al. (2006) reported that newborn pigs were not attracted to the green water dispenser and their behavioral reaction to red and blue was gender driven. Females preferred the blue colored dispenser visiting it more times than the males, while males preferred the red colored dispenser. However, there were no significant effects of trough color (yellow, red, and black) on feed intake, average daily gain, and FCR (Edge et al., 2004).

3. Sense of taste

The pigs famous appetite and food preferences are in part due to their sense of taste. Pigs have taste papillae on the tongue, epiglottis, and soft palate epitheliums (Montavon and Lindstrand, 1991). Each papillus contain high numbers of garlic clove-shaped taste buds, with each taste bud composed of groups of 50 to 120 sensory cells, each of which projects a number of microvilli to the mucus layer of the tongue (Roura and Tedó, 2009). Taste buds

are composed of at least four different types of cells; three taste-type cells (I, II, III) and one basal-type cell believed to be a progenitor of the other three. Type I cells are sour-sensing, type II are sweet, umami, and bitter sensing, and type III cells play an intermediate signaling role between the true taste cells and (type I and II) and the sensory neurons.

As described by Kumar and Bate (2004) pigs have five types of taste papillae: filiform, fungiform, foliate, vallate, and conical. Filiform papillae have either sharp or blunt apical tips. Large numbers of densely-packed sharp-tipped papillae are distributed throughout the tongue, except in the mid raised area of tongue, which is mainly covered with blunt tip papillae. Large, circular fungiform papillae located on the lateral side of the tongue contain surface taste pores, but fungiform papillae on the dorsal surface of the tongue lack taste pores. Foliate papillae are located principally on the caudal third and lateral part of the tongue are composed of four to five leaves and additional pseudopapillae. Two vallate papillae, found in the caudal third and dorsal of the tongue, have well-defined vallum and pseudopapillae on their surface. Finally, two types of conical papillae are present at the root of the tongue directed caudally in a shingle-like arrangement. The first type of conical papilla has a broad base and a tapering pedicle-like apex whereas the second type of conical papillae was cone shaped and displayed a blunt apex. All papillae except the filiform have taste buds on their surface. In particular, the fungiform and vallate papillae are involved in taste (Ojima et al., 2001), whereas the filiform papillae and conical papillae play a role in the mechanical transport of food and liquid towards the pharynx (Ojima et al., 1997).

Food preference, among other factors, is a result of flavor-taste papilla interaction. In humans, the sense of taste differentiates the taste qualities of sour, salty, sweet and bitter. It is not possible to know whether the gustatory sensations of animals are comparable to those of humans, rather it can be assumed that each animal species has its own sensory spectrum. Taste perception in pigs may not be fully developed at birth (Oakley, 1998), but develops quickly develop after birth since taste perception is intimately related to the quality of food and to diet selection.

McLaughlin et al. (1983) investigated flavor additives consisted of 248 trials and conducted to determine the preference for 129 flavors, including flavor combinations from eight major

flavor groups: buttery, cheesy, fatty, fruity, green, meaty, musty, and sweet. Pigs showed highest rate of preferred over non-preferred flavors were recorded for buttery, green, and meaty, respectively. Pigs reject foods that taste bitter to humans (Nelson and Sanregret, 1997). Nelson and Sanregret (1997) studied pigs detect and avoid taste compounds that humans perceive as bitter-tasting. Plant extracts and pharmaceutical compounds resulted in aversion behaviors in pigs some of which were found to be dose dependent.

Pigs have a strong preference for sucrose and a more moderate preference for glucose, lactose, and salt. Therefore, flavoring agents, e.g. sugar, have been used for weanling pigs to create interest in them for solid food (Forbes, 1995). The first work reported on sugar preferences was published in the mid 50s (Salmon-Legagneur and Fevrier, 1956). Kennedy and Baldwin (1972) have examined the taste preferences of pigs for nutritive and non-nutritive sweet solutions over a wide range of concentrations and found that young pigs prefered sucrose solution at concentration above 10.3 g/L. Pigs also preferred water containing greater than 1.8 g/L for glucose solutions, although the consumption volume was lower than sucrose. This indicated that sucrose was the more strongly preferred sugar. Saccharin was also preferred at concentrations from 1 to 2 g/L compared to non-supplemented water, but higher concentrations (above 18 g/L) decreased the preference substantially. The rejection of high concentrations of saccharin may be due to the bitter taste associated with high concentrations of the chemical. Not all non-nutritive sweeteners stimulate attraction in pigs. Nofre et al. (2002) investigated the responses of pigs to 60 artificial sweetener compounds and found that 35 compounds were attractive to pigs, but less so in pigs than in humans. Lugduname and carrelame, the two most potent sweeteners in humans, are also the most effective compounds in pigs.

4. Sense of smell

Certainly the most celebrated anatomical feature of the pig is its snout, the disc-like movable tip of the muzzle that incorporates the central part of the upper lip. The snout is supported by the rostral bone - a structure unique to the pig (Dyce et al., 2010). Olfactory cells, present in the nasal epithelium, convert incoming odor molecules into electrical signals, which are then transmitted via the olfactory nerve to the brain. In addition, the vomeronasal organ, located

in the upper air passages, contains receptors that have access to the central nervous system through the accessory olfactory bulb.

The sense of smell develops early in pigs and is important to survival because piglets are very mobile at birth. They can and need to follow chemical cues learned earlier in life, e.g., the odor of their mother. Morrow-Tesch and McGlone (1990) demonstrated that piglets preferred sow fecal odors and did not prefer to be near novel odors (orange and banana odors). Campbell (1976) fed sows a flavored diet during lactation and fed piglets the same flavor diet at weaning. When given no alternative diet, weaned pigs from dams that had eaten the flavored diet ate more feed in the 2 weeks post weaning compared to those that had not been exposed to the flavor.

Pigs have a highly sensitive sense of smell relative to other animals. Feral pigs use their sense of smell to find food, detect potential predators or prey, and mark territory. In one experiment, sows learned to distinguish between otherwise identical cards that they had previously touched or not touched. The odor they deposited in this way was still perceptible to them after several hours and even after the cards were washed (Signoret et al., 1975). Pigs use a wide range of olfactory cues in their natural behavior e.g., piglets use olfactory cues to recognize their dam and teat position, older pigs use olfactory information as the predominant basis for individual recognition (Curtis et al., 2001). In young or adult pigs, odor preference or aversion varied depending on the individual. Even cloned pigs had various responses to taste (Archer et al., 2003).

Odors related to feed are important; indeed, smell is usually experienced prior to consumption and can be used to increase the palatability of feeds. The smells of cod-liver oil, petrolatum, rancid fishmeal and fish oil, vegetable oils, or sour chicken (Fletcher et al., 1990) were attractive when presented in baits for feral hogs. Inversely, most repellents are based on predator smells.

Krebs (2007) tried to reduce aggressive behavior in group of weaned piglets through aroma conditioning ("aroma therapy"). Amyl acetate which has a scent similar to bananas and apples was applied to room. The results showed that amyl acetate did not reduce stress

within 2 hours of onset of weaning. Odors increased lying behavior, but had no apparent effect on cortisol concentration.

5. Sense of hearing

The ears of pigs are oval with fairly wide base attached to the sides of the high caudal part of the head. They have a pointed tip and hang down over the face in some domestic breeds, but stand erect in others and in the wild *Sus scrofa* (Dyce et al., 2010). Pigs' hearing range is similar to that of humans, but with a shift toward ultrasound. The frequency range for reasonable detection varies between 42 Hz and 40.5 kHz, with a region of best sensitivity from 250 Hz to 16 kHz (Heffner and Heffner, 1990). Spatial discrimination of the source of a sound depends on the difference in time it takes for the sound to reach each ear. Pigs have a sound localization threshold of approximately 4.6 degrees. This is well above the average sound localization threshold of 12 degrees for mammals, although it is still less acute than that in human, at approximately 1.3 degrees (Heffner and Heffner, 1989).

Pigs show an aversion to sudden loud noise. Pigs tested in an open-field situation showed increased heart rate and retreated in response to loud noise. These responses were stronger for a frequency of 8 kHz than for 500 Hz and for an intensity of 97 dB than for 85 dB, although habituation occurred relatively quickly (Talling et al., 1996).

Effects of music on behavior and/or welfare are largely under-investigated in pigs and the experiments conducted to date have produced ambiguous results. One study on the effects of music in weaned piglets reported no positive effect of music on piglet vocalization during stressful events like castration and weaning (Cloutier et al., 2000). In contrast, de Jonge et al. (2008) reported that music can facilitate play behavior in piglets after weaning when music had been presented pre-weaning as a contextual cue associated with access to a playroom. They also suggested that music elicited play behavior - a positive welfare indicator because play behavior is sensitive to adverse environmental and physical conditions.

6. Pig behavior, welfare, and health issues

For pigs, as for most species, stress is a normal part of life and occurs in the course of social dynamics, e.g., feeding, mating, aggressive interactions, and as part of coping with the environment, e.g., heat, cold, novel environmental situations. Pigs show similar reactions to

challenging events over time and across related situations, but demonstrate large variations in behavior among individuals (Forkman et al., 1995; Lawrence et al., 1991; Spoolder et al., 1996). For example, piglets showing a higher number of escape attempts (proactive behavior) in response to manual restraint (back test) are more aggressive in later social interactions (Hessing et al., 1993; Ruis et al., 2000). When faced with non-social challenges, e.g., exposure to novel situations or objects, reactive individuals show higher initial levels of passive avoidance, but spend a longer time exploring after they gain familiarity. In contrast, proactive individuals more quickly approach novel stimuli, but show more superficial exploratory behavior. As a general concept, however, welfare issues arise when there is a mismatch between the pig's instincts and its environment, i.e., when instinctual behavior is thwarted or behavioral impulses are inappropriately directed.

Pigs are normally active during the day, and spend 75% of their active time in foraging-related activities, including rooting, grazing, and exploring with their snout (Stolba and Wood-Gush, 1989). Although domestic pigs in commercial production are routinely supplied with the basic necessities of life (food, water, and shelter), they have an internal need to perform explorative activities (Van Putten and Dammers 1976). A study by Wood-Gush and Vestergaard (1991) demonstrated that pigs preferentially select environments (pens) with novel objects to explore. The typical response to a novel object is chewing, but it is unclear whether chewing reflects feeding motivation, exploratory motivation, or a combination of both (Day et al., 1995). This uncertainty is reflected in the literature, i.e., some authors conclude that chewing behavior is mediated by feeding motivation (Fraser, 1987) and others that chewing behavior represents exploratory motivation (Van Putten and Dammers, 1976). Day et al. (1996) proposed that the initial exploratory chewing of a novel substrate may lead to nutritional feedback being acquired which could modify subsequent foraging behavior.

The effects of environmental enrichment on the behavior, performance and welfare of pigs have been extensively researched. Scientific studies of enrichment for pigs housed in indoor systems have focused on several types of 'toys', including objects such as tires, chains, rubber hoses or dog toys (Apple and Craig, 1992; Pearce and Paterson, 1993; Blackshaw et

al., 1997; Hill et al., 1998). In general, these studies investigated effects of the enrichment, e.g. the preferences of the pigs for the objects offered, aggression, performance and productivity. Feddes and Fraser (1994) concluded from their study into the stimulus features of non-nutritive chewing, that pigs used objects (cotton cords or rubber strips) more if they could alter the object by chewing. Blackshaw et al. (1997) tested whether the characteristics 'loose on floor' or 'attached' had effects. They found that the presence of fixed and free toys did not influence relative growth rate but did affect pig behavior by resulted in a lower level of aggressive behavior. Zonderland et al. (2001) tested several characteristics of four materials (rope, wood, chain and metal pipe) in a more systematic way. They concluded that a combination of 'flexibility' and 'destructibility' might be relevant. Orientation of the object ('horizontal' or 'vertical') did not affect the level of interactions.

Many studies have found that a number of enrichment properties, such as being ingestible, destructible, chewable, and odorous (Feddes and Fraser, 1994; Van de Weerd et al., 2003), and presentation methods, e.g. suspended as opposed to on the floor (Blackshaw et al., 1997), are attractive for pigs. On the other hand, some studies report that the properties of individual enrichment objects could be responsible for generating an unfulfilled behavioral expression, leading to agonistic behaviors (Van de Weerd et al., 2006; Day et al., 2008) which might be further stimulated by increased competition due to spatially limited access to a particular object (Docking et al., 2008). Furthermore, habituation to point source objects can occur rapidly in pigs (Apple and Craig, 1992; Van de Weerd et al., 2003). Thus, novelty of an object is an important property involved in initiating exploration (Gifford et al., 2007), and has been reported to be intrinsically rewarding to pigs (Wood-Gush and Vestergaard, 1991). One way that novelty can be attained is by replacing familiar objects with new objects, although little is known about how long a pig can remember a particular object (Gifford et al., 2007).

Pigs' natural tendency to chew objects in their environment can be mis-directed at other pigs. Van Putten (1969) described that ear and tails are the easiest to chew, but ear-chewing is more likely to provoke an attack than tail biting. According to Van Putten (1969), the bitten animal reacts by waving its tail vigorously, but this attracts bites by other pen-mates. Tail

biting is a behavior and welfare problem that has been attributed to a complex of factors including crowding, lack of bedding, poor ventilation, uncomfortable temperatures, disease (David, 1987), diet, e.g., low fiber, inadequate or poor-quality protein, excessive dietary energy, and deficiencies or imbalances of minerals (Gadd, 1967; Ewbank, 1973). Van Putten (1969) argues that the tail biting is actually mis-directed behavior derived from the quiet, low-intensity chewing and rooting on pen-mates.

Fraser (1987) first developed a cotton rope tail model, with some ropes soaked in whole pig's blood, to measure the pigs' attraction for blood and their tendencies to chew. Pigs showed a preference for the blood-flavored models compared to the untreated rope. Jankevicius and Widowski (2003) studied tail models with balanced colored to determine whether the color of the tail models affected pigs attraction to those flavored with blood. Regardless of color, pigs preferred to chew on tail models soaked in blood, which suggested that pigs uses olfactory or taste cues to discriminate among the different models.

Various strategies have been used to counter outbreaks of tail biting, including providing materials to allow natural biting, rooting and play behavior. Enrichment strategies, such as provision of an object suitable for chewing and rooting, may present a stimulus or route for eliciting and reinforcing exploratory activities involving the snout and mouth (Van de Weerd et al., 2003) and result in a reduction in adverse behaviors, such as tail-biting and belly nosing (Fraser et al., 1991; Peterson et al., 1995). For example, providing growing pigs with straw is known to reduce harmful social behavior, such as ear and tail biting, and to increase behavior directed towards the substrate (de Jong et al., 1998). An experiment by Beattie et al. (1993) showed that growing pigs housed indoors and given a substrate to root in increased the amount of time spent exploring and decreased the time spent inactive and in behaviors directed towards their pen-mates, such as chewing ear and tails. Peat, mushroom compost, and sawdust were the preferred substrates, followed by sand, with wood bark and straw being preferred only to concrete flooring. Pigs' preferences are apparently determined more by particle size or texture than moisture content and growing pigs seem to be attracted to substrates with in texture similar to earth (Beattie et al. 1995).

Pigs' tendency to chew objects in the environment can also be exploited for other purposes, e.g., monitoring and surveillance of infections. Prickett et al., (2008a,b) used cotton rope to collect oral fluid samples under experimental and field conditions. Recently, Kittawornrat et al. (2010) studied oral fluid collection from individual penned boar by hanging cotton rope flavored with 50% sucrose in apple juice. In this study, 70 of 72 boars were successfully trained for oral fluid collection and the method was successfully applied to the detection of porcine reproductive and respiratory syndrome virus detection in oral fluids.

The use of novel objects is an application that exploits both the pigs' tendency to chew and their flavor preferences for the oral delivery of pharmaceuticals and biologics, e.g., vaccines, fertility control agents, and toxicants, to feral swine. Fletcher et al. (1990) demonstrated the feasibility of oral vaccine delivery to feral swine and Kavanaugh and Linhart (2000) showed that bait could be used to deliver oral pharmaceuticals to feral swine, as well. Ballesteros et al. (2009) proved the use of bait for the oral delivery of vaccine formulations to 2- to 4-month-old wild boar by demonstrating antibody titers and recombinant *E. coli* in the feces after consumption of bait.

Flavor formulation of baits is important. Fletcher et al. (1990) reported 95% bait consumption by feral swine using fish meal–based bait; in contrast to 63% bait consumption of bovine-flavored bait (Mitchell, 1998) and 31 to 72% consumption of kangaroo-based bait (Fleming et al., 2000). Ballesteros et al. (2009) prepared bait using a matrix of wild boar feed, wheat flour, paraffin, saccarose, and cinnamon-truffle powder attractant with polyethylene capsules of vaccine within the matrix. However, in addition to feral pigs, baits can attract other species. Campbell et al. (2006) showed that 51% of baits were taken by raccoons (*Procyon lotor*), 22% were taken by feral swine, and 20% were taken by collared peccaries (*Tayassu tajacu*). Thus, bait flavorings should be highly specific to feral swine to avoid adverse effects in non-target species. For example, wild pseudorabies virus (PRV) strains (Platt et al., 1983) and gene-deleted vaccines (Weigel et al., 2003) can replicate in susceptible species, resulting in death and/or the potential for transmission to other wild or domestic animals. Likewise, in fertility-control products, gonadotropin-releasing hormone (GnRH) is a common hormone found in wild and domestic animals. Consequently, any

animal that ingests baits containing the GnRH vaccine and subsequently produce and maintain anti-GnRH antibodies at sufficient titers would likely experience impaired reproductive capacity (Campbell et al., 2006).

7. Conclusion

The changes in husbandry that occurred over the course of the 20th century metamorphosed pig production from small, extensive (outdoor), labor-dependent enterprises into large, intensive (confined), capital-dependent, production systems. Concurrently over the last several years, both production and consumption of pork has risen. World pork production doubled between 1977 and 1998 (Cameron, 2000) and the demand for pork is expected to increase between 6 and 14% over current levels, primarily driven by increasing demand in South East Asia (EC, 2007). The development of large-scale swine production has stimulated debate concerning its impact on animal / human health, environmental effects, and concerns for the ethical care of animals. Safeguarding animal welfare and health is good for pigs, pork producers, and the animal-conscious public. In a very tangible way, the future of pork production depends on effectively addressing the consuming public's animal welfare and health concerns. A good place to start is by understanding the biology behind the welfare of our Friend, the Pig.

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CHAPTER 3. ORAL FLUID SAMPLES FOR HEALTH MONITORING CAN BE COLLECTED FROM INDIVIDUALLY-PENNED BOARS

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Abstract

Oral fluid specimens offer the means to efficiently monitor a variety of parameters relevant to swine welfare and health, e.g., acute phase proteins, infectious agents, hormones, and drugs. The objective of the present study was to determine whether oral fluid samples could be routinely collected from individually-housed adult boars. Three replicates of 24 boars each (6 months to 3.6 years in age) were conducted. Among 70 trained boars, 524 samples were collected in 560 attempts (93.6%) over an 8-day observation period. Statistically significant differences in sample volume were associated with observation day, boar age, and for the interaction of replicate x day, but not with replicate or the interactions of boar age x replicate or boar age x day. Venipuncture for collecting serum from adult sows and boars on a routine basis is difficult, potentially dangerous, and welfare-unfriendly. In contrast, this study showed that oral fluids could quickly and easily be collected by one person. Thus, oral fluid specimens offer the potential to monitor specific health and welfare indicators in commercial swine herds on a routine basis.

1. Introduction

"Oral fluid," the liquid collected by placing an absorptive device in the mouth (Atkinson et al., 1993), is a combination of serum transudate and saliva. Serum transudate enters the mouth via the oral mucosa (oral mucosal transudate) and gingiva (gingival crevicular fluid) from capillaries located in the oral mucosa, crevicular gap, and the gingival tissues (Cameron and Carman, 2005; Delima and Van Dyke, 2003). In pigs, the major salivary glands are the

parotid, submandibular, and sublingual glands (Sisson, 1975). The composition of saliva differs among salivary glands, but saliva is mostly water in which are suspended a variety of molecules with a range of biological functions, e.g., mucin, amylase, lysozyme, and lipase (Llena-Puy, 2006).

Oral fluid has been widely used in human medicine and forensics for the diagnosis or detection of a variety of infectious agents (Malamud, 1992), hormones (Lippi et al., 2009), and drugs (Danhof and Breimer, 1978). In particular, the development of oral fluid-based diagnostics in humans, stimulated by the detection HIV and anti-HIV antibodies in oral fluids (Archibald et al., 1986; Groopman et al., 1984), has had a tremendous impact, i.e., surveillance and monitoring for HIV is primarily done by testing oral fluid specimens using rapid (20 minute) point-of-care assays. The approach is technically simple, e.g., in England, oral fluid samples were collected from 11,698 children at home by their parents and mailed to the laboratory for antibody testing (Bartington et al., 2009).

Although the oral fluid testing has not been widely applied to livestock health and wellness management, the veterinary literature on the presence of antibodies, pathogens, and acute phase proteins in oral fluids from animals reflects the findings in humans (Prickett and Zimmerman, 2010). Under experimental and field conditions infectious agents, cortisol, acute phase proteins, and progesterone have all been monitored in swine using oral fluids (Gutieérrez et al., 2009; Moriyoshi et al., 1996; Parrott and Misson, 1989; Prickett et al., 2008a,b).

Oral fluid has been collected from swine using several methods. Wills et al. (1997) collected oral fluid by placing a sterile swab underneath the tongue of anesthetized pigs. Loftager et al. (1993) collected oral fluid by inducing salivation with azaperone or by inserting a cotton wool swab soaked in ascorbic acid into the mouth of the pig for a few minutes. The investigators to first describe oral fluid collection without restraint allowed pigs to chew on large cotton buds, from which the sample was then extracted (Parrott and Misson 1989). As described elsewhere, oral fluid samples are easily collected in the field by allowing pigs to chew on ropes and then extracting the fluid from the rope (Prickett et al., 2008a,b). However, the collection of oral fluid samples from unrestrained, individually-housed adult

swine has not been reported. If possible, this would allow for the expansion and improvement of swine welfare and preventive medicine to this population in commercial swine herds. Thus, the objective of this research was to determine whether oral fluid samples could be reliably and routinely collected from mature boars housed in crates. The objective measures used to evaluate the responses of the animals were the percent of animals from which oral fluid samples were collected and the quantity of sample collected.

2. Materials and Methods

2.1 Experimental design

An experiment with three replicates of 24 individually-penned boars was conducted to determine if oral fluids could be routinely collected from adult animals. The study was conducted using boars ranging from 6 months to 3.6 years of age under the ownership of PIC North America (Hendersonville, TN USA). Housing, procedures, and protocols were approved and supervised by the PIC USA Health Assurance and Welfare department. Data collected on a daily basis included ambient temperature (maximum and minimum) in the facility, observations on boar behavior and health, oral fluid sampling exposure time, and volume of oral fluid recovered daily from each boar.

2.2 Animals and animal care

The commercial production facility where the boars were housed was equipped with concrete slatted flooring, side curtains, and tunnel ventilation. Feeder space, water delivery, square footage per animal, sanitation, ambient temperature, and ventilation all met or exceeded PIC animal care requirements. Once on site, animals were housed individually in crates (Hog Slat, Inc., Newton Grove, N.C.) and fed a commercial corn/soy swine diet (Land O' Lakes® Farmland Feed, Roland, Iowa USA) at a rate of 4 pounds per animal per day for acclimation/training and then 7 pounds per animal per day after training.

2.3 Collection materials

Oral fluid samples were collected using 5/8", 3-strand, 100% cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL 60010). Prior to sample collection, the rope was measured and cut into 22” (55.9 cm) lengths using a PVC pipe cutter (Orbit water master®, Irrigation

Products, Inc. North Salt Lake, UT 84054). Approximately 4" (10.2 cm) of one end was untwisted and soaked overnight in a flavoring solution (unsweetened apple juice with 50% (v/v) sucrose) and then air-dried. To prevent environmental contamination, ropes (n = 25) were packaged in vacuum-sealed bags in the laboratory prior to use (ITW Space Bag, San Diego, CA 92154).

2.4 Collection process

Boars were trained for two days before the start of each replicate. To familiarize the animals with the process, treated ropes were placed on the floor in front of the boars for ≤ 20 minutes in the morning and afternoon for two days. Thereafter, ropes were placed in brackets fixed at the front of each pen for ≤ 20 minutes and oral fluids were deposited as the boars interacted with the rope.

After two days of training, oral fluid samples were collected daily for 8 days following a specific routine. That is, all samples were collected before 8:00 am and animals were fed immediately after oral fluid collections. Samples were collected on the farm by placing the ropes in brackets constructed by welding a pipe (1.0" (2.5 cm) ID) to a 1.0" (2.5 cm) angle iron at a 45 degree angle. The device was secured to the one end of each crate using U-bolts such that the location of the bracket and angle of the pipe made the hanging rope readily accessible to the animals. Ropes were presented to individually-housed boars by threading the rope through the pipe and tying an overhand knot in the top to prevent the rope from slipping through the pipe. The ropes were left in place for 20 minutes, during which time the boars deposited oral fluids as they chewed the rope. To extract oral fluid samples from the rope, the "boar end" of the rope was inserted into a one-gallon, re-sealable plastic bag (#921279, Johnson & Son, Inc., Racine, WI 53403), the rope was cut 6 inches from the bottom, and the bag sealed with the rope inside. Ropes remained sealed within the plastic bags for the remainder of the process. The plastic bags were immediately transported in a cooler with ice packs. At the laboratory, oral fluid was extracted by passing the plastic bag with the rope inside through a hand wringer (BL-38, Dyna-Jet Products, Overland Park, Kansas USA 66204). After the oral fluid had accumulated in a bottom corner of the bag, the bag was cut, and the contents decanted into a 50 ml centrifuge tube (C-3394-3, ISC

BioEexpress®, Kaysville, Utah USA 84037) and the volume recorded. Oral fluid samples were then centrifuged in a refrigerated centrifuge (4°C) for 10 min at 1,000 x *g*.

2.5 Statistical analysis

The objective of the analysis was to determine the effect of replicate (1, 2, 3), day (1 to 8), boar age (6 months to 3.6 years), and boar type (select vs cull) on the success (Y/N) of oral fluid collection from individual boars and the volume (ml) of oral fluid collected. Logistic regression models with repeated measures were applied to analyze the effects of factors on the success of collecting oral fluid data using the Glimmix procedure in SAS® Version 9.2 (SAS® Institute Inc., Cary, North Carolina USA). Replicate, day, and boar age were included as fixed effects, with "boar" nested within replicate and treated as a random effect in the model. Oral fluid volume data were analyzed with repeated measures analysis of variance (ANOVA) model using the Mixed procedure in SAS. Replicate, day, age, and their interactions (replicate x age; day x age; replicate x day) were included as fixed effects in the model, and "boar" was nested within replicate and treated as a random effect in the model. Zeros (no sample collected) were excluded from the analysis of oral fluid volume data.

3. Results

Ambient temperatures during the observation periods (June to August 2009) were unusually moderate for Iowa USA, ranging from 57° to 78° F (14° to 26°C). All boars were clinically healthy throughout the observation period, i.e., all boars ate well and no signs of respiratory or gastrointestinal disease were observed. Among the 72 boars, 70 were quickly and easily trained to provide oral fluid samples. Two cull boars in Replicate 2 were not trained, i.e., no samples were collected from boar #45 and only two samples were collected from boar #43. Therefore, the data from these animals was excluded from the statistical analysis.

The percent of oral fluid samples collected by observation day is summarized in Figure 1. Analysis of these data showed that replicate ($p = 0.0339$) and observation day ($p < 0.001$) were statistically significant sources of variation, but boar age ($p = 0.9525$) was not. Among a potential total of 560 oral fluid samples, 524 (93.6%) samples were collected. In Replicate 1, 188 (98%) samples were collected, 155 (81%) in Replicate 2, and 181 (94%) in Replicate

3. The rate of successful collection initially increased during the observation period and then stabilized. That is, samples were collected from 56 of 70 (80%) boars on day one, 61 of 70 (87%) boars on day 2, 66 of 70 (94%) boars on day 3, and 68 or 69 (97% or 98%) of 70 boars thereafter.

A summary of oral fluid volumes collected by observation day is shown in Figure 2. The mean volume of oral fluid collected per boar across all replicates was 17.9 ml (range: 1 to 39 ml). Analysis of the sample volume data showed that day ($p < 0.0001$), age ($p < 0.05$), and in the interaction of replicate x sampling day ($p < 0.001$) were statistically significant sources of variation.

4. Discussion

The objective of this research was to determine whether individually-housed mature boars could be trained for oral fluid collection. Most boars investigated the rope placed on the floor of their pen on the first day of training. This agrees with comments by Signoret et al. (1975) regarding the inherent curiosity of pigs. Thereafter, 70 of 72 boars quickly learned to interact with a rope suspended at the front of the crate. Boars continued to learn over the observation period and the rate of success stabilized at about 98% at day 4 and later. No statistically significant differences in success rate by boar age were detected, although oral fluids from older boars had significantly greater volume, probably as a result of their larger body size. Given the "learning curve" observed over the course of the observation period, training animals to ropes at a young age could facilitate compliance when they enter a breeding herd.

The procedures used in this study were compatible with previous reports on swine behavior. Scott et al. (2009) found that swine preferred hanging objects (toys), thus the fact that ropes swung freely in brackets at the pig's eye level may have promoted prolonged play behavior and may, in part, explain the large sample volumes collected (mean volume = 17.9 ml). Suspending ropes avoided a problem discussed by Blackshaw et al. (1997). That is, pigs lose interest in objects that become soiled with excreta. Compliance was reinforced by intermittent reinforcement, i.e., exposure to ropes for only 20 minutes daily helped retain

their novelty. In addition, cotton rope is chewable, destructible, and in this experiment, flavored (50% sucrose in apple juice) - properties previously shown to stimulate sustained attention (Van de Weerd et al., 2003). This research did not test the effect of flavorings on the training response, but most boars appeared to respond to apple flavoring (data not shown). The positive effect of sweeteners (glucose, sucrose, sodium saccharin) on feed or water intake is well-documented (Kennedy and Baldwin, 1972; Salmon-Legagneur and Fevrier, 1956). Cumulatively, these reports suggest that flavorings may be useful for training individual adult animals to ropes and/or for maintaining continued interest in interacting with ropes.

Collecting serum from sows and boars on a routine basis is stressful and potentially dangerous to both human and animal participants. In contrast, this study found that oral fluids could quickly and easily be collected from adult swine by one person. Thus, oral fluid testing offers the potential to collect swine welfare and health surveillance data on a routine basis. From the consumers' perspective, oral fluid sampling offers a "welfare-friendly" method to proactively address animal welfare and health issues.

5. Conclusions

Oral fluid samples can be collected routinely and frequently from individually-housed adult animals. Compared serum samples, this approach provides significant improvement in the ease, timeless, and cost of collecting samples for welfare or health monitoring of individual responses under experimental and/or field conditions. Importantly, this method offers an “animal-friendly” approach for addressing animal welfare and health issues of concern to producers and consumers.

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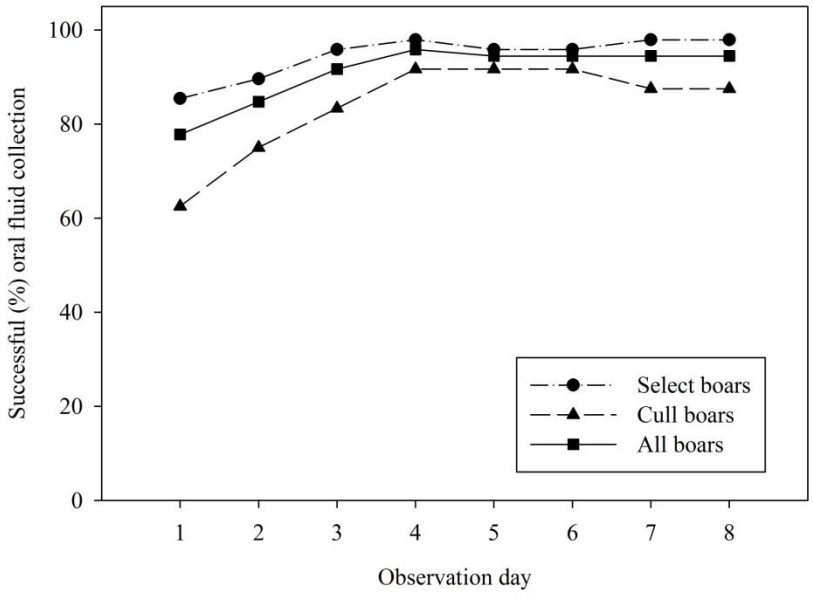

Figure 1 Successful (%) oral fluid collection by observation day

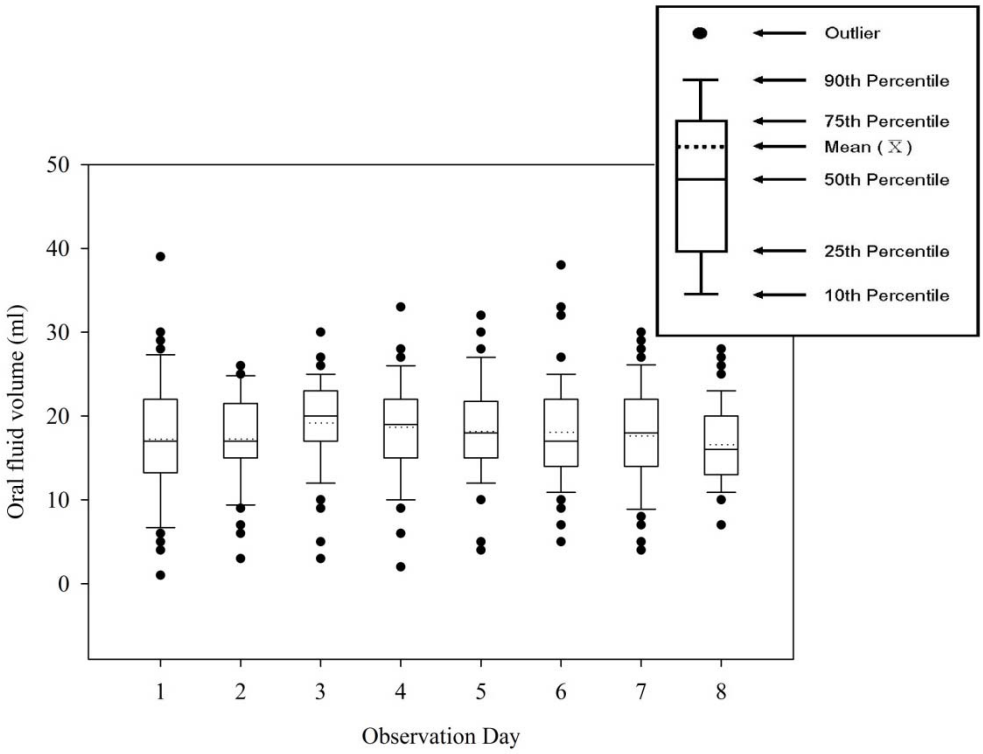

Figure 2 Summary of oral fluid collection by observation day

CHAPTER 4. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) IN SERUM AND ORAL FLUID SAMPLES FROM INDIVIDUAL BOARS: WILL ORAL FLUID REPLACE SERUM FOR PRRSV SURVEILLANCE?

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Abstract

The purpose of this study was to determine whether oral fluid samples could be used to monitor individually-housed adult boars for porcine reproductive and respiratory syndrome virus (PRRSV) infection. In 3 trials, 24 boars were intramuscularly (IM) inoculated with a modified-live PRRSV (MLV) vaccine (Trial 1), a Type 1 PRRSV isolate (Trial 2), or a Type 2 isolate (Trial 3). Oral fluid samples were collected daily and serum samples were collected twice weekly. Following the completion of the study, samples were randomized and blind-tested for PRRSV by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). PRRSV was detected in oral fluids at DPI 1 and all oral fluid specimens were PRRSV qRT-PCR positive at DPI 4. Although PRRSV was detected in both serum and oral fluid specimens through DPI 21, a comparison of matched samples from individual boars showed that oral fluid was equal to serum for the detection of PRRSV at DPI 7 and more likely to be positive than serum on DPI 14 and 21. Overall, oral fluid was superior to serum for the detection of PRRSV using PCR over the 21 day observation period in this study. The results of this experiment suggest that individually-penned oral fluid sampling could be an efficient, cost-effective approach to PRRSV surveillance in boar studs and other swine populations.

Keywords: PRRSV, surveillance, monitor, diagnosis, detection, oral fluid, serum

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) monitoring of boar studs is mandated by the need to provide PRRSV-free semen to breeding herds for artificial insemination. Historically, boar stud monitoring has relied on the detection of PRRSV in serum or semen using convenience samples. Both specimens present challenges in terms of labor, time, and worker safety and neither approach is amenable to achieving sampling requirements for a targeted level of disease detection in terms of sample numbers and random selection of animals.

In human diagnostic medicine, oral fluid specimens have been increasing used in place of serum for disease surveillance and monitoring; particularly since Archibald et al. (1986) reported the detection of anti-HIV antibodies in the oral fluid samples from AIDS patients. Indeed, subsequent studies showed that the diagnosis of infection with HIV based on specific antibody in oral fluid was equivalent to serum in accuracy (Malamud, 1992).

Following this line of thought, Prickett et al. (2008a, b) demonstrated that PRRSV could be detected in swine oral fluid samples collected under field or experimental conditions. These results corroborated an earlier report of PRRSV isolation from buccal swabs collected up to 42 days post inoculation (DPI) from experimentally-inoculated pigs (Wills et al., 1997a, b). Therefore, the objective of this research was to evaluate oral fluid sampling as method of monitoring individually-housed adult boars for PRRSV infection.

2. Materials and Methods

2.1 Experimental design

The study was conducted in 72 boars ranging from 6 months to 3.6 years of age under the ownership of PIC North America (Hendersonville, TN USA). Housing, feed rations, animal care guidelines, and sampling protocols were approved and supervised by the PIC USA Health Assurance and Welfare department. In 3 trials, 24 boars were intramuscularly (IM) inoculated either with a modified-live PRRSV (MLV) vaccine (Trial 1), a Type 1 (European-like) PRRSV isolate (Trial 2), or a Type 2 isolate (MN-184). Oral fluid samples were collected daily from all boars beginning 7 days prior to inoculation and continuing for 21

days post inoculation (DPI). Serum samples were collected from all boars on DPI -7, 0, 7, 14, 21 and from 4 boars randomly selected at the beginning of each trial for additional blood sampling on DPI 3, 5, 10, 17. Data collected on a daily basis included ambient temperature within the facility (maximum and minimum), clinical and behavioral observations, length of time boars were allowed access to rope, and volume of oral fluid recovered from each boar. After the completion of Trial 3, oral fluid samples (DPI -7, 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17, 21) and serum samples (DPI -7, 0, 3, 5, 7, 10, 14, 17, 21) were randomized and blind-tested for the presence of PRRSV by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.2 Animals and animal care

The boars used in this study originated from two Midwest breeding stock sources historically monitored as free of PRRSV infection. The boars in the study included culled boars ranging from one year to 3.6 years of age (n = 24) and select boars ranging from 5 to 6 months of age (n = 48). The boars were housed in a commercial production facility equipped with nipple drinkers, concrete slatted flooring, curtains, and tunnel ventilation. Feeder space, water delivery, square footage per animal, sanitation, and ventilation all met or exceeded PIC animal care requirements. Once on site, animals were housed individually in crates (Hog Slat, Inc., Newton Grove, North Carolina USA) and fed a commercial corn/soy swine diet (Land O' Lakes® Farmland Feed, Roland, Iowa USA) at a rate of 4 pounds per animal per day for acclimation/training and 7 pounds per animal per day thereafter.

2.3 Porcine reproductive and respiratory syndrome viruses

Boars in Trial 1 were inoculated IM with 2 ml of a commercial MLV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO 64506) rehydrated and administered according to the instructions provided by the manufacturer.

Trial 2 boars were inoculated IM with 2 ml of PRRSV isolate D09-012131 at an estimated concentration of $1 \times 10^{5.5}$ median tissue culture infectious dose ($TCID_{50}$) per ml. Isolate D09-012131 is a Type 1 virus that was isolated from serum samples submitted to the University of Minnesota Veterinary Diagnostic Laboratory (St. Paul, Minnesota USA) in March 2009 as part of a routine monitoring program in a 2,000 sow breed-to-wean herd

located in Illinois USA. The herd showed no signs of clinical PRRS, i.e., no evidence of reproductive disease, increased mortality, or decreased piglet quality. Sequencing of open reading frame (ORF) 5 identified the isolate as European-like, sharing 88.6% ORF5 identity with Lelystad Virus, the prototypic Type 1 (European) PRRSV.

Primary isolation of D09-012131 was performed in pulmonary alveolar macrophage (PAM) cells in 48-well plates (Nalge Nunc International, Rochester New York USA). The cells were plated 4 hours prior to inoculation by resuspending them in RPMI-1640 medium (BioWhittaker, Inc., Walkersville, Maryland USA) supplemented with 455 µg international units (IU) per ml penicillin, 455 µg per ml streptomycin, 150 µg per ml neomycin, 1.5 µg per ml Amphotericin B (Sigma Chemical Co., St. Louis, Missouri, USA), 50 µg per ml gentamicin (Mediatech, Inc., Manassas, Virginia USA), and 8% fetal bovine serum (Sigma). Prior to inoculation, cells were washed with Hanks' balanced salt solution once and a spurt of media was added to all wells. An undiluted and 1:10 dilution of each serum sample was inoculated into one well each. Two wells were maintained as negative control wells. These wells received RPMI-1640 media (BioWhittaker, Inc.) instead of serum sample. Plates were incubated at 37°C for one hour to allow for virus adsorption, after which the inoculum was removed and fresh RPMI-1640 was added to all wells. The cells were allowed to incubate for 5 days and were observed microscopically for development of cytopathic effects (CPE). Cells showing CPE were stained by direct immunofluorescent assay for confirmation using anti-PRRSV antibody conjugate. Virus propagation was carried out following the same procedure except that multiple wells on 48-well plates were inoculated with the virus.

Trial 3 boars were IM inoculated with 2 ml of a Type 2 (North American) PRRSV isolate (MN-184, GenBank accession no. AY656992) at a concentration of $10^{4.5}$ $TCID_{50}$ per ml. MN-184 (kindly provided by Dr. Scott Dee, University of Minnesota) was propagated on MARC-145 cells, a clone of the African monkey kidney cell line MA-104 (Kim et. al., 1993). Cells were grown in 162 cm^2 flasks (Corning, New York, USA) using Dulbecco's modified Eagles Medium (DMEM) (Mediatech, Inc.) supplemented with 300 IU per ml penicillin (Sigma), 300 µg per ml streptomycin (Sigma), 0.25 µg per ml Amphotericin B (Sigma), 50 µg per ml gentomycin (Sigma), 0.5 molar L-glutamine (Fisher Scientific,

Hampton, New Hampshire, USA), 1.0% nonessential amino acids (HyClone, Logan, Utah), HEPES buffer (Sigma), and 10.0% heat-inactivated fetal bovine serum (FBS) (Sigma). When cells were confluent (72 to 84 hrs), the media was discarded and the flasks inoculated with 5 ml DMEM (without L-glutamine) containing PRRSV isolate MN-184 at a virus titer of 1 x $10^{3.5}$ $TCID_{50}$ per ml. Flasks were placed on a rocking platform in a 37°C humidified 5% CO_2 incubator for 90 min, then 40 ml of DMEM growth medium, with 4% heat-inactivated fetal bovine serum (FBS) in place of 10% FBS, was added and the flasks returned to the incubator for 36 hrs. Cell culture supernatant was harvested by flask freeze-thaw (-80°C / 25°C) and centrifugation (3000 x *g* for 20 minutes at 4°C).

For virus titration, samples were serially 10-fold diluted (10^{-0} to 10^{-5}) in DMEM growth medium without FBS and assayed in triplicate on confluent (72 hr) MARC-145 cells propagated in 96-well tissue culture plates (Corning). Growth medium was discarded from plates, 5 wells were inoculated with 100 µl of sample at each dilution, and the plates placed in a 37°C humidified 5% CO_2 incubator for 90 min. The inoculum was then discarded, 100 µl per well of growth medium containing 4% FBS was added to each well, and the plates incubated at 37°C in a humidified 5% CO_2 incubator for 24 hrs. Following incubation, cells were fixed with cold, 80% acetone/water solution and stained with a fluorescein isothiocyanate-conjugated (FITC) monoclonal antibody specific for PRRSV (SDOW17, Rural Technologies Inc., Brookings, South Dakota, USA). Results were determined by visualization of PRRSV-specific fluorescence reaction.

2.4 Oral fluid sample collection

Oral fluid samples were collected daily from boars housed individually in crates using a protocol described in detail by Engle et al. (2010). In brief, oral fluid samples were collected using 5/8", 3-strand, 100% cotton rope (Web Rigging Supply, Inc., Lake Barrington, Illinois USA). Prior to sample collection, the rope was measured and cut into 22” (55.9 cm) lengths using a PVC pipe cutter (Orbit water master®, Irrigation Products, Inc. North Salt Lake, Utah USA). Approximately 4” (10.2 cm) of one end was untwisted, soaked overnight in a flavoring solution (unsweetened apple juice with 50% (v/v) sucrose), and then air-dried. To collect the sample, ropes were positioned at shoulder height in "rope holders" fixed at the

front of each pen for approximately 20 minutes, with oral fluid deposited as the boars chewed on the rope. After the exposure period, the wet portion of the rope was inserted into a 1-gallon re-sealable plastic bag, bottom portion of the rope was cut, and the bag sealed with the wet rope inside. At the laboratory, the each bag with the rope inside was compressed through a clothes wringer, causing the oral fluid to pool in the bottom of the bag. Thereafter, the bottom corner of the bag was cut and the accumulated oral fluid was decanted into a 50 ml centrifuge tube. Samples were centrifuged at 1,000 x *g* for 10 minutes at 4°C, aliquoted into 5 ml plastic tubes (Becton, Dickinson and Company, Bedford, Massachusetts USA), and stored at -80°C until assayed.

2.5 Serum collection

Blood was collected by jugular venipuncture using serum separation tubes (Corvac®, Tyco Healthcare Group LP, Mansfield, MA, 02048). Samples were centrifuged at 1,000 x *g* for 10 minutes and the serum was aliquoted into 5 ml plastic tubes (Becton, Dickinson and Company) and stored at -80ºC until assayed.

2.6 PRRSV Antibody ELISA

To determine whether animals had prior exposure to PRRSV, serum samples collected at DPI -7 and 0 were tested for anti-PRRSV antibodies using a commercial ELISA (HerdChek* PRRS X3, IDEXX Laboratories, Inc, Westbrook, Maine USA). Samples were assayed according to the manufacturer's instruction. As per the manufacturer, a positive serum sample was defined as having a sample-to-positive (S:P) ratio ≥ 0.4.

2.7 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

PRRSV RNA extraction from serum Nucleic acid extraction from serum was performed using a commercial RNA extraction kit (Ambion® MagMax™-96 Viral RNA isolation kit, Applied Biosystems™, Foster City, California USA) as per the manufacturer's instruction.

PRRSV RNA extraction from oral fluid A protocol based on a commercial RNA extraction kit (Ambion® MagMax™-96 Viral RNA isolation kit, Applied Biosystems™)

was developed to optimize the extraction of PRRSV RNA from oral fluid samples (Chittick et al., 2010). Lysis/binding solution was prepared by combining 40ml of lysis/binding solution with 623µl of carrier RNA without the addition of isopropanol. The solution was mixed thoroughly and stored at room temperature until used. All other reagents were prepared according to the kit manual. An initial volume of 300µl of sample was added to 450µl of lysis solution. Plates were covered with a plate seal and placed on an orbital plate shaker at 1000 RPM for 5 minutes. Plates were then centrifuged at 2500 x *g* for 5 minutes. After centrifugation, 600µl of the lysate was added to 350µl of isopropanol and 20µl of bead mix in a new deep-well plate. Plates were washed two times with 300µl per well with wash solution 1. Plates were then washed two times with 450µl per well of wash solution 2. Next, 90µl per well of elution buffer was added to the final plate. An automated extraction system was used for the extraction process (Kingfisher-96 Magnetic Particle Processor (Thermo Fisher Scientific, Inc., Waltham, Massachusetts USA) using program "AM_1836_DW300_v2" obtained from the extraction kit manufacturer (Applied Biosystems™).

PRRSV RNA Amplification and Detection via Real-Time PCR Real-time PCR was performed with commercially-available reagent sets (TaqMAN® NA and EU PRRSV Reagents and TaqMAN® NA and EU PRRSV Controls, Applied Biosystems™). In this assay, North-American and/or European PRRSV RNA are reverse-transcribed into cDNA and amplified by Taq DNA polymerase in a single tube, one-step differential (PRRSV Type 1 vs Type 2) PCR reaction. Detection of amplified target is accomplished by Taqman hydrolysis probe chemistry. This master mix also contains primers and probes targeting an internal positive control RNA sequence (Xeno™ RNA-01). The internal positive control was spiked into the RT-PCR master mix at a concentration of 100 copies per µl to monitor PCR amplification and allow for detection of failed PCR reactions. Master mix component volumes differed slightly for oral fluid and serum, but the reactions were performed identically. For serum, master mix component volumes per well consisted of 12.5µl of 2X RT-PCR buffer, 2.5µl of 10X PRRSV primer probe mix, 1.25µl of 20X multiplex RT-PCR enzyme mix, 0.35µl of 100 copies per µl of Xeno™ RNA-01internal control, and 0.4µl of nuclease-free water. For oral fluid, master mix component volumes per well were identical

except that the 20X multiplex RT-PCR enzyme mix was increased to 2.5µl and no water was used. Ultimately, 17µl of master-mix was combined with 8µl of RNA extract onto a 96-well PCR plate. Real-time RT-PCR was performed (ABI 7500, Applied Biosystems™) using the following cycling conditions: 1 cycle at 45°C for 10min, 1 cycle at 95°C for 10min, 40 cycles of: 97°C for 2 sec, 60°C for 40 sec.

Quality control of the extraction process included negative (nuclease-free water) and positive (PRRSV isolate ISU-P) controls, i.e., nuclease-free water and PRRSV extraction-positive controls. Each 96-well PCR plate included a positive amplification control (TaqMAN® NA and EU PRRSV controls provided by Applied Biosystems™) and a negative amplification control (nuclease-free water).

For PCR quantitation, plasmid-derived standards purchased from the manufacturer (TaqMan® NA and EU PRRSV RNA controls, Applied Biosystems™) were used. Eight serially-diluted standards ranging from 10^0 to 10^7 copies per µl were run on each PCR plate and the Ct values from these were used as the basis for the standard curve. Sample were quantified (geq per µl) by fitting the sample Cts to the standard curve using the using the AB7500 Fast System SDS Software (Applied Biosystems™).

2.8 Statistical analysis

Statistical analyses were performed using SAS® Version 9.2 (SAS® Institute Inc., Cary, North Carolina, USA) and MedCalc® 9.2.1.0 (MedCalc Software, Mariakerke Belgium). To meet the distribution requirements for valid statistical analyses, the raw real-time qRT-PCR results (geq per µl) were transformed into natural logarithms ($\log_e$) prior to analyzing the data.

The possible adverse effect of PRRSV infection on animal behaviors impacting oral fluid sample collection was evaluated in terms of successful collection and sample volume. For these analyses, data collected over the 4 days before inoculation were used to establish a baseline and compared to the period 7 days post inoculation. Logistic regression models with repeated measures were applied to analyze the effects of trial (1, 2, 3), DPI, and boar age on the success of oral fluid collection using SAS® PROC GLIMMIX. Trial, DPI, and

boar age were included as fixed effects, with “boar” as the subject of repeated measures. The effect of PRRSV infection on oral fluid sample volume was analyzed using mixed models with repeated measures. Oral fluid volume data were analyzed with a linear mixed model with repeated measures using SAS® PROC MIXED. Trial (1, 2, 3), DPI, boar age, and their pairwise interactions were included as fixed effects in the model and “boar” was the subject of repeated measures

Virus concentration (geq per µl) over time in serum and oral fluid samples was analyzed using a linear mixed model with repeated measures in SAS® PROC MIXED. Trial (1, 2, 3), DPI, boar age, oral fluid volume, and their pairwise interactions were included as fixed effects and "boar" was the subject of repeated measures. To evaluate possible differences in virus concentration between serum and oral fluid samples, results were fit into an overall model with the sample type (serum or oral fluid), trial (1, 2, 3), DPI, and their pairwise interactions as fixed effects. Tukey’s Honestly Significantly Different (HSD) test was used to determine statistically significant differences between virus concentration and sample type by DPI.

McNemar's test (SAS® PROC FREQ) for paired samples was used determine whether the true proportions of qRT-PCR-positive oral fluid and serum samples were significantly different by trial (1, 2, 3) and DPI. This analysis only included cases in which results matched oral fluid and serum results were available, i.e., excluded animals from which either sample was missing.

To evaluate the overall relationship between virus concentration in oral fluid vs serum samples, the onset, duration, and magnitude of virus shedding was summarized for each sample type by boar using area under the curve (AUC) analysis (MedCalc®). Thereafter, the relationship between AUC (oral fluid) vs AUC (serum) was assessed using Pearson’s correlation coefficient (SAS® PROC CORR).

3. Results

3.1 PRRSV-negative status

All serum samples collected on DPI -7 and 0 tested negative for anti-PRRSV antibodies using a commercial ELISA (IDEXX Laboratories, Inc.). All serum samples (n = 140) and 139 of 140 oral fluid samples collected prior to PRRSV inoculation were PRRSV qRT-PCR negative. One oral fluid sample collected on DPI 0 in Trial 1 tested PCR positive, but this result was interpreted as a false positive on the basis of the cumulative ELISA and PCR results.

3.2 Oral fluid collection

Overall, 70 of 72 boars readily learned to chew the cotton rope suspended at the front of the crate - a necessary prerequisite for collecting oral fluid specimens. Two boars in Trial 2 showed no interest in the rope and no oral fluid specimens were collected from these animals. Therefore, these animals were eliminated from all statistical analyses. Among the remaining boars (n = 70), oral fluid samples were collected with increasing success as the animals became accustomed to the process over the 7 days prior to inoculation. Samples were collected from 56 of 70 boars (80%) on DPI -7, 61 (87%) on DPI -6, 66 (94%) on DPI -5, 69 (99%) on DPI -3, and 66 to 70 (94 to 100%) on DPIs -2 to 7. For the period DPI 1 to 21, 1,429 of 1,470 (97%) attempts at oral fluid collection were successful. Oral fluid volume (mean and standard error) by trial and DPI are displayed in Figure 1.

The possible adverse effect of PRRSV infection on animal behaviors involved in oral fluid sample collection was evaluated in terms of successful collections and sample volume. A comparison of the 4 days before PRRSV inoculation versus the 7 days post inoculation found no statistically significant difference in the success rate ($p = 0.7875$), suggesting that PRRSV infection did not impact animal behaviors related to oral fluid collection. Comparison of sample volume for the 4 days prior to inoculation (17.6 ml; range 4 to 38 ml) to the 7 days after inoculation (15.7 ml, range 1 to 37 ml) showed a statistically significant difference ($p = 0.0447$), suggesting that PRRSV infection affected sample volume. Regardless, all successful oral fluid collections produced sufficient sample volume for PRRSV qRT-PCR testing.

3.3 PRRSV qRT-PCR results in serum samples

Serum samples were collected from all boars on DPI -7, 0, 7, 14, 21 and from 4 boars randomly selected at the beginning of each trial for additional blood sampling on DPI 3, 5, 10, 17. The PRRSV qRT-PCR results are displayed in Figure 1 and Tables 1 and 2. All serum samples from DPI -7 and 0 and (n = 140) were qRT-PCR negative. All serum samples from boars tested on DPI 3 (n = 12) and DPI 7 (n = 70) were positive (Table 1). Statistical analysis of qRT-PCR data showed that differences in the level of viremia (geq per µl) were associated with trial ($p < 0.0001$), DPI ($p < 0.0001$), and the interaction between trial and DPI ($p < 0.0001$). As detailed in Table 2, the mean serum concentrations of MN-184 ($1 \times e^{11.8}$), D09-012332 ($1 \times e^{7.9}$), and Ingelvac® PRRS MLV ($1 \times e^{6.9}$) were significantly different at DPI 7, but these differences had largely resolved by DPI 14 and later. Boar age at the time of inoculation had no significant effect on viremia.

3.4 PRRSV qRT-PCR results in oral fluid samples

Oral fluid samples collected on DPI -7, 0, 1, 2, 3, 4, 5, 6, 7, 10, 14, 17, 21 were tested by PRRSV qRT-PCR (Figure 1, Tables 1 and 2). Virus was detected in 7 of 69 (10%) boars on DPI 1, 52 of 68 (76%) on DPI 2, 66 of 70 (94%) on DPI 3, and 67 of 67 (100%) boars on DPI 4. Statistical analysis found no significant difference among trials in the increasing number of positive samples by DPI.

Factors significantly associated with the concentration of PRRSV in oral fluid included trial ($p < 0.0001$), DPI ($p < 0.0001$), the interaction of trial and DPI ($p = 0.0001$), oral fluid volume ($p = 0.0146$), and the interaction oral fluid volume and DPI ($p = 0.0313$). Boar age at the time of inoculation had no statistically significant effect on the concentration of virus in oral fluid samples. As shown in Table 2 and Figure 1, the mean concentration of MN-184 in oral fluid was significantly higher than the other two viruses at DPI 7 and 14, but no difference in virus concentration was present at DPI 21.

3.5 Comparison of PRRSV detection in serum and oral fluid

Serum and oral fluid PRRSV qRT-PCR categorical results by DPI are presented by trial in Table 1. McNemar's test was used to detect statistically significant discordance between the numbers of qRT-PCR positive and negative results for each pair-wise sample combination

(serum vs oral fluid) by trial, DPI, and trial by DPI. It is important to note that McNemar's test requires both serum and oral fluid results from the same boar. Thus, denominators presented in Table 1 may differ slightly from those used in the analysis. As detailed in Table 1, statistically significant differences in the number of positive results from serum vs oral fluid samples were detected in Trial 1 at DPI 14, Trial 2 at DPI 21, the cumulative results at DPI 14 and 21. In every case, a higher number of oral fluid samples were qRT-PCR positive than serum samples.

The concentration of virus (geq per µl) in serum and oral fluid samples demonstrated similar patterns over time (Figure 1), but statistically significant differences in the virus concentration between sample type (serum or oral fluid) and by trial were identified at DPI 7, 14, and 21 (Table 2). In general, serum contained a higher concentration of virus than oral fluid at DPI 7, concentrations were approximately equal at DPI 14, and oral fluid contained a higher concentration of virus than serum at DPI 21. To evaluate the overall relationship between virus concentration in oral fluid samples vs serum samples, the onset, duration, and magnitude of virus shedding was summarized for each boar and sample type using area under the curve (AUC) analysis. A correlation analysis based on serum AUC vs oral fluid AUC estimated Pearson's correlation coefficient as $r = 0.68$ (Figure 2).

4. Discussion

Current methods for monitoring boars in commercial production for PRRSV infection are based on testing semen specimens using PCR-based assays (Swenson et al., 1994; Christopher-Hennings et al., 1995a, b) or blood specimens (Reicks et al., 2006) using antibody- or PCR-based assays. Both approaches present challenges in terms of labor, time, and worker safety. In addition, neither approach is amenable to achieving sampling requirements for a targeted level of disease detection in terms of sample numbers and random selection of animals. Therefore, the purpose of this study was to measure qRT-PCR-detectable PRRSV in oral fluid and serum specimens collected from individually-housed boars with the intent of determining whether oral fluid could reliably replace serum as a diagnostic specimen for monitoring boar studs. If successful, this approach could resolve many of the shortcomings of the current methods.

Oral fluid was collected from individually-housed adult boars on a daily basis by one person and with no risk to either party. The majority of animals were quickly acclimated to oral fluid sampling, as evidenced by the fact that samples were collected from 56 of 70 boars even on DPI -7. On the basis of their overt behavior, the majority of boars seemingly anticipated the daily 20 minute interaction with the rope; to the extent that samples were successfully collected even during acute PRRSV infection. Although 70 boars readily adapted to oral fluid sampling, all attempts at collecting oral fluid specimens from two boars in Trial 2 were unsuccessful. As reviewed by Roura and Tedó (2009), sweet flavorings generally have a positive effect on feed or water intake. Likewise, apple and strawberry flavors are attractants for feral swine (Campbell and Long, 2008). In this study, a combination of sucrose and apple juice were used to flavor ropes, but to no effect in these two boars. Collection of oral fluid from non-cooperative animals is an issue, but venipuncture can present similar issues either due to non-cooperative animals or the level of technical skill of the persons collecting the blood sample. In both cases, the solution is to select more than the minimum number of animals required to fulfill the sampling objective. Future research on swine behavior, the use of attractants, and the presentation of the oral fluid sampling device could lead to improvements in sample collection and greater compliance.

In this experiment, the serum concentration of MN-184 was significantly greater that the two other viruses at DPI 7, but not at DPI 14 or 21. Likewise, the oral fluid concentration of MN-184 was significantly greater than the other two viruses at DPI 7 and 14, but not DPI 21. Isolate-specific differences in the levels of PRRSV replication have been reported previously (Johnson et al., 2004). Despite differences in the level of virus replication, all viruses were detected in oral fluids early in the course of infection, i.e., 7 of 69 (10%) boars on DPI 1, 52 of 68 (76%) on DPI 2, 66 of 70 (94%) on DPI 3, and 67 of 67 (100%) boars on DPI 4. The early detection of PRRSV observed in this study is compatible with previous reports of PRRSV detection within 24 hours post-exposure in buffy coat samples (Rossow et al., 1994), lung (Halbur et al., 1995), serum (Christopher-Hennings et al., 1995a; Halbur et al., 1995; Wasilk et al., 2004), nasal turbinates (Rossow et al., 1996), and tonsil (Halbur et al,. 1995; Rossow et al., 1996) following inoculation with Type 1 or Type 2 isolates. Although PRRSV

was detected by qRT-PCR in both serum and oral fluid through DPI 21, a comparison of matched samples from individual boars showed that oral fluid was equal to serum for the detection of PRRSV at DPI 7 and more likely to be positive than serum on DPI 14 and 21. Thus, oral fluid was superior to serum for the detection of PRRSV using PCR over the 21 day observation period in this study.

Cumulatively, the data show that oral fluid offers distinct advantages over serum for the purpose of monitoring boar studs for PRRSV infection using qRT-PCR. Advantages include easier sample collection, the ability to collect samples frequently (even daily) without incurring animal or employee resentment, the ability to select animals at random for sampling, and the cumulatively greater likelihood of detection in oral fluids vs serum. By extension, oral fluid could also replace blood sampling for monitoring PRRSV in other swine populations (commercial sow herds, growing pigs) and in other applications, such as PRRSV elimination/eradication programs.

As reviewed elsewhere (Prickett and Zimmerman, 2010), a variety of pathogens and pathogen-specific antibodies are present in swine oral fluid. In addition to PRRSV, pathogens present in oral fluid include bovine virus diarrhea virus (Terpstra and Wensvoort, 1997), classical swine fever virus (C-C Chang, personal communication), foot-and-mouth disease virus (Alexandersen et al., 2003), porcine circovirus type 2 (Prickett et al., 2008a, b), pseudorabies (Aujeszky's disease) virus (Bouma et al., 1996), and vesicular stomatitis virus (Stallnecht et al., 1999). Surveillance and monitoring is rarely done in swine populations because of the technical, logistical, and economic challenges of bleeding and testing a statistically sufficient numbers of pigs. Oral fluid-based testing offers the opportunity to collect infectious disease data cheaply, routinely, and in a "welfare-friendly" method in order to proactively address animal health issues.

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Table 1. Serum and oral fluid PRRSV qRT-PCR positive results by day post inoculation (DPI)

	Sample	DPI 0 positive / tested	DPI 7 positive / tested	DPI 14 positive / tested	DPI 21 positive / tested
Trial 1: Ingelvac® PRRS MLV	Oral fluid	1 / 24 (4.2%)[1]	24 / 24 (100%)	20 / 24 (83%)[2]	21 / 24 (88%)
	Serum	0 / 24	24 / 24 (100%)	16 / 24 (67%)	21 / 24 (88%)
Trial 2: Type 1 isolate (D09-012332)	Oral fluid	0 / 22	22 / 22 (100%)	20 / 20 (100%)	20 / 21 (95%)[2]
	Serum	0 / 22	22 / 22 (100%)	18 / 22 (90%)	15 / 22 (68%)
Trial 3: Type 2 isolate (MN-184)	Oral fluid	0 / 24	23 / 23 (100%)	24 / 24 (100%)	19 / 22 (86%)
	Serum	0 / 24	24 / 24 (100%)	22 / 24 (92%)	19 / 24 (79%)
TOTAL	Oral fluid	1 / 70 (1.4%)	69 / 69 (100%)	64 / 68 (94%)[2]	60 / 67 (90%)[2]
	Serum	0 / 70	70 / 70 (100%)	56 / 70 (80%)	55 / 70 (79%)

[1] Singleton false-positive PRRSV qRT-PCR reaction

[2] Statistically significant difference using a comparison based on matched oral fluids and serum samples (McNemar's test, $p < 0.05$)

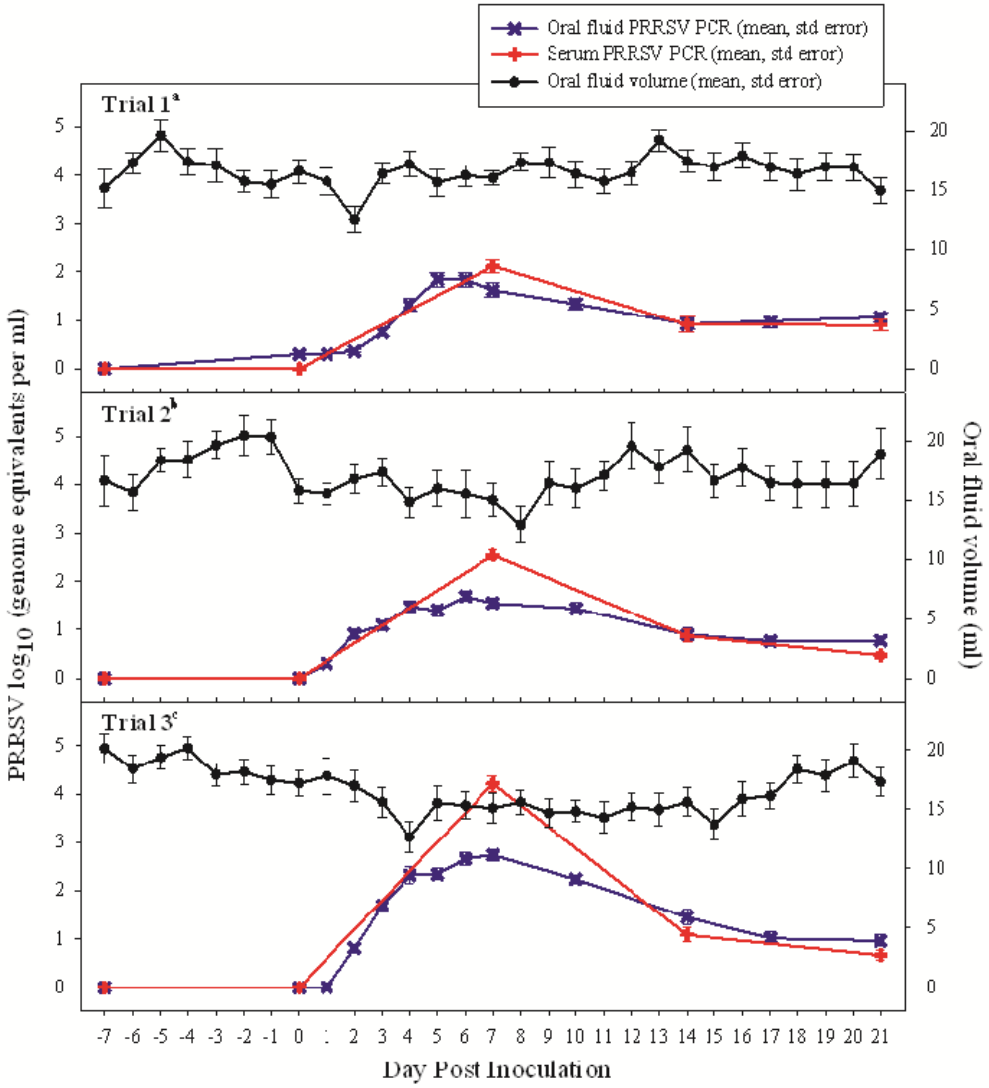
Table 2. Serum and oral fluid PRRSV qRT-PCR quantitative results by day post inoculation (DPI)[1,2]

	Sample	DPI 0	DPI 7	DPI 14	DPI 21
Trial 1: Ingelvac® PRRS MLV	Oral fluid	0.8[3]	5.8 (5.1, 6.4) **d**	3.9 (3.1, 4.6) **e,f**	4.3 (3.7, 5.0) **e**
	Serum	0	6.9 (6.3, 7.6) **c**	3.7 (2.7, 4.7) **e,f,g**	3.3 (2.5, 4.2) **f,g**
Trial 2: Type 1 isolate (D09-012332)	Oral fluid	0	5.6 (5.1, 6.1) **d**	3.8 (3.0, 4.6) **e,f**	3.6 (3.0, 4.1) **e,f,g**
	Serum	0	7.9 (7.4, 8.4) **b**	3.9 (3.1, 4.6) **e,f**	2.3 (1.7, 3.0) **h**
Trial 3: Type 2 isolate (MN-184)	Oral fluid	0	8.4 (7.8, 8.9) **b**	5.1 (4.3, 6.0) **d**	3.4 (2.5, 4.2) **f,g**
	Serum	0	11.8 (11.0, 12.5) **a**	4.3 (3.4, 5.1) **e**	2.9 (2.1, 3.6) **h,g**

[1] PRRSV qRT-PCR least square means (1 x e^x genome equivalents per µl) and 95% confidence intervals

[2] All data within the table are included in the comparison. Means with the same letter are not significantly different (Tukey's Honestly Significantly Different (HSD) test, $p > 0.05$).

[3] Singleton false-positive qRT-PCR reaction



a. Ingelvac® PRRS MLV
b. Type 1 field isolate (D09-012332)
c. Type 2 field isolate (MN-184)

Figure 1 PRRSV qRT-PCR results (serum and oral fluid) and oral fluid collection volume by trial and day post inoculation

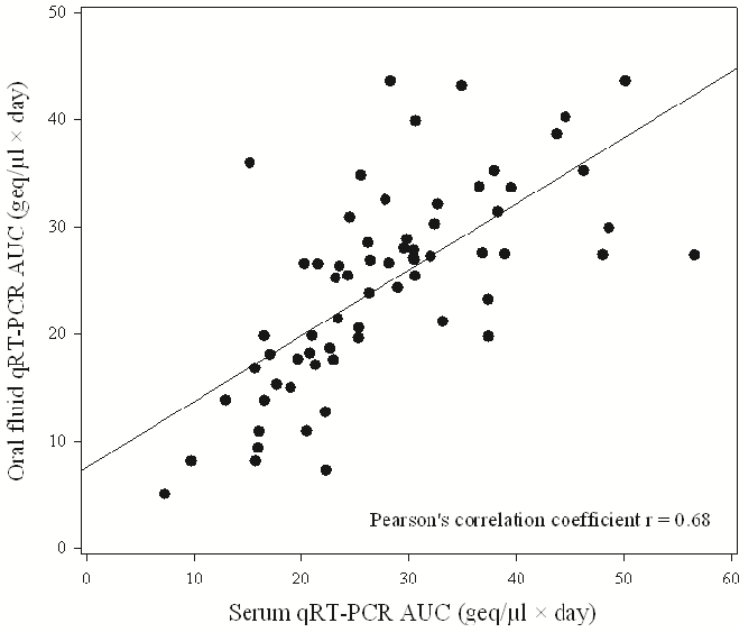


Figure 2 Correlation between virus concentration (qRT-PCR) in serum and oral fluid from individual boars using area under the curve (AUC) as a summary statistic (Pearson's correlation coefficient r = 0.68)

GENERAL CONCLUSIONS

Over the last few years, the world-wide production and consumption of pork has risen. It is estimated that the demand for pork and thus for raising pigs will increase between 6 and 14% over current levels, especially driven by increasing demand in South East Asia (EC,2007). As a result, increasing pig production to supply higher demand is evitable. An important effect of this increasing demand is the impact on animal welfare and the surrounding media attention. Mismatches between behavioral characteristics and pig husbandry conditions a are potential source of welfare problems in domesticated pigs. Therefore, studying the unrestricted behavior of pigs in a natural setting can provide useful information about the range and role of behavior that pigs perform, and their choices and use of habitat could provide clues to their housing needs

Cumulatively, the use of oral fluid as a diagnostic based offers advantages over serum for the purpose of monitoring boar studs for PRRSV infection using qRT-PCR. Advantages include the simple and non-invasive sampling methodology required. Moreover, the oral fluid collection is increasing awareness of animal welfare. Not only the impact on the animals' welfare but also the saved labor of workers. Furthermore, oral fluid collection can repeated sampling over short time intervals which facilitates ongoing animal/disease monitoring and samples can be collected by individuals with modest levels of training.

The research presented in this thesis is the first description of monitoring individually-housed adult boars for porcine reproductive and respiratory syndrome virus (PRRSV) infection. There are many issues yet to be addressed. For example, it would be highly beneficial to detect anti PRRSV antibody among individual animals. As reviewed elsewhere (Prickett and Zimmerman, 2010) a variety of pathogens and pathogen-specific antibodies are present in swine oral fluid, therefore additional research is necessary to detect antigen and antibody of swine infectious. Although much work remains to be done, the review of the literature presented in chapter one and the supporting data in chapter two and three suggest that oral fluid sampling in swine populations holds the potential to be powerful method of obtaining the data crucial to improving the health management of herds.

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