Oral fluid as a non-invasive alternative diagnostic medium for disease monitoring in pigs

By

Lorna Louise Dawson

MSci. Biomedical Sciences

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School of Agriculture, Food and Rural Development

Newcastle University

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Abstract

Current pig disease serological surveillance often involves blood sampling which is costly to the farmer due to veterinary fees and labour. Oral fluid (OF) has been highlighted as a promising non-invasive alternative to blood serum for diagnostics, however research into the collection and handling of samples, as well as validation against the current Gold Standard serum is required. This thesis aimed to address these key areas to support the application of OF diagnostics in the pig industry.

Collection methodologies were investigated, including the use of flavours on cotton chewing ropes to increase sample yield, and the provision of multiple ropes to increase sample representation in larger groups. Flavour did not affect OF yield, however the provision of multiple ropes did improve representation in weaner, but not finishing pigs.

A method to store porcine OF samples at ambient temperature for diagnosis of Porcine Reproductive and Respiratory Syndrome virus (PRRSv) infection via reverse transcription polymerase chain reaction (RT-PCR) was developed. Flinders Technology Associates (FTA) cards preserved PRRSv RNA within spiked OF samples stored at ambient temperature for up to 4 weeks.

An existing protocol designed for use with serum was modified for the detection of anti-Salmonella antibody in porcine OF. Field testing resulted in 56% sensitivity and 97% specificity compared with serum.

In order to validate the use of OF to assess PRRS status, blood and OF samples were collected from pigs in eight commercial pig production flows, comprising 29 pig units of breeder, nursery and finisher stages. Comparison with current Gold Standard serum testing yielded 90% sensitivity and 76% specificity by pen. This was followed by a longitudinal OF study of pigs from 8 units through nursery and growing stages to track PRRS antibody dynamics over time. Three specific patterns of anti-PRRSv antibody were detected in different populations of pigs between, as well as within the same pig flows studied.

The results of this research will support the successful implementation of OF diagnostics for health management in pigs and has identified areas where further work is necessary.

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Glossary of Abbreviations and Technical Terms

Abbreviations

DNA: Deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

FMD: Footh and mouth disease

FTA card: Flinders Technology Associates card

HIV: Human immunodeficiency virus

IAV: Influenza A virus

Ig: immunoglobulin

MALDI-TOF: matrix associated laser desorption ionisation / time-of-flight

MS: mass spectrometry

OF: oral fluid

PCV-2: Porcine circovirus type-2

PRRS: Porcine reproductive and respiratory syndrome

RNA: Ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

SD: standard deviation

SEM: standard error of the mean

Technical Terms

Ct value: The number of replicative cycles at which the quantity of cDNA produced reaches a threshold value. Lower values indicate a greater starting quantity of material.

Gold Standard: A test with performance of superior quality serving as a point of reference for which other tests' performance can be compared against.

Sensitivity: A term describing the ability of a diagnostic assay to detect a target. Calculated by dividing the number of true positive OF outcomes by the total number of positive outcomes by Gold Standard blood serum testing.

S:N ratio: Sample-to-negative ratio in blocking ELISA testing, which describes the proportion of assay reaction occurring per well relative to the internal kit control wells. A higher ratio indicates a lower antibody titre.

Specificity: A term describing the ability of a diagnostic assay to detect the given target only, without detection of contaminants or other compounds. Calculated by dividing the number of true negative OF outcomes by the total number of negative outcomes by Gold Standard blood serum testing.

S:P ratio: Sample-to-positive ratio in indirect ELISA testing, which describes the proportion of assay reaction occurring per well relative to the internal kit control wells. A higher ratio indicates a higher antibody titre.

Chapter 1: Infectious disease, the need for surveillance and oral fluid as an alternative diagnostic medium

1.1 General Introduction

Herd health is a key and highly sensitive determinant of productivity within any livestock husbandry system with small deviations from the maintenance of optimal herd health being capable of causing significant consequences for farmers and livestock. This is of particular importance for the pig industry due to the highly intensive nature of pig husbandry. The UK herd currently stands at 4,383,000 pigs, of which 398,000 comprise the breeding herd (BPEX, 2014c). The majority of UK breeding pigs are situated in the English regions of Yorkshire and Humberside, and East Anglia, together forming 50% of the total UK breeding pig herd (BPEX, 2014a). This distribution, with particularly high density of pigs within the two major regions highlighted, serves to further demonstrate the necessity for effective health management.

The economic and animal welfare impacts of clinical disease outbreaks in the UK are devastating and can be exemplified by the 2001 Foot and Mouth Disease (FMD) epidemic. This was found to have originated from a finishing pig unit in the North of England (Scudamore, 2002) and resulted in over 6 million animals being culled, of which 0.4 million were pigs. Financial losses to British agriculture due to this outbreak totalled £3.1 billion (The Royal Society, 2002). In addition to clinical disease which presents epidemically, there are a number of disease agents which exist sub-clinically, that is, they circulate within a population without the presentation of clinical symptoms. Such agents are thus deemed endemic and yet can have severe economic impacts due to sub-optimal growth performance as a result of energy investment in the immunological response to infection and reduced appetite. The lack of clinical signs associated with sub-clinical disease is a source of great frustration to farmers who may be aware that animals are performing sub-optimally but cannot determine the cause. In these cases great emphasis is therefore placed upon monitoring and surveillance for pathogens via the regular collection of biological samples from animals for diagnostic testing and veterinary treatment interventions where necessary. This has been recognised by the British Pig Executive (BPEX), an English levy board which launched the Pig Health

Improvement Project (PHIP) in 2011 with the aim to increase surveillance and monitoring for some of the key endemic diseases in UK pigs (BPEX, 2011a).

Current methods by which routine disease monitoring is conducted consist largely of the collection of blood and environmental (i.e. floor swabs for Salmonella) samples from farms for subsequent analysis in a laboratory (DEFRA, 2008). Additionally, there are some endemic diseases e.g. Salmonellosis, enzootic pneumonia which are tested for at slaughter, either by collection of a meat juice sample or examination of organs for specific lesions (Davies *et al.*, 2003; Holt *et al.*, 2011). Although these methods deliver substantial and reliable information, they are not without limitations. Firstly, blood sampling for routine health monitoring is usually performed by a veterinary surgeon (Veterinary Surgery (Blood Sampling) Order 1983) proving costly to the farmer, and requires a second person to catch and restrain each pig for bleeding, incurring additional cost. The most commonly used method of blood sampling pigs for disease diagnostics is via jugular venepuncture as this allows the collection of an adequate volume (typically 3-5ml) of blood for multiple tests. An alternative method is to puncture an ear vein if smaller volumes are sufficient for the purpose, i.e. only one or two tests required. Both bleeding methods require the restraint of the pig using a snare device which is stressful for the animal (Farmer *et al.*, 1991; Roozen *et al.*, 1995) and time consuming for the personnel involved. There is also a small risk of damage or even death to the pig undergoing a blood sample. Surveillance methods which rely upon slaughterhouse samples give accurate information about disease status at the point of slaughter. However, such a retrospective approach provides limited options for investigation of intervention strategies which may be implemented during the animal's lifetime. A sample medium which could be collected non-invasively throughout the animal's lifetime that still offers accurate diagnostic information would therefore be very useful for monitoring endemic disease.

Oral fluid (OF) is a mixture of salivary secretions and oral mucosal transudates which, due to the latter component, can provide an insight into the constituents of the bloodstream (De Almeida *et al.*, 2008). It is through the mucosa that both pathogens, as well as antibodies produced by the host in response to these and

circulating within the blood, can be transferred into the oral cavity and detected in the resulting OF. Pigs possess a natural behavioural tendency to bite and chew on objects presented to them, thus an OF sample can be collected from a pen of pigs via the provision of a length of absorbent cotton rope for the group to chew. The OF can then be easily removed from the rope by hand-wringing for transport to the laboratory and subsequent diagnostic testing.

The use of OF samples for disease diagnostics has not been widely investigated in the UK. The group that performed the original pioneering research into OF diagnostics for pigs in the USA now use this sample type to conduct routine disease surveillance for a number of key pathogens (ISU, 2012). The developmental work performed by the team at Iowa State University will be discussed in detail throughout this review. Only one pilot study has been conducted in the UK, a study which yielded promising results but demonstrated the necessity for more work within the area. In order for successful adoption of OF as a diagnostic medium for UK pig disease surveillance, laboratory diagnostic methods would need to be optimised and fully validated against the current Gold Standard test medium (i.e. blood serum), this is one of the three main aims of this thesis. Secondly, the treatment of samples post-collection should be investigated in order to establish a means by which samples could be collected and shipped relatively easily to the diagnostic facility, to ensure that this could be feasible for farmers. Thirdly, the diversity of pig husbandry systems currently in operation in the UK must also be taken into account, as the original USA work was largely based upon intensive indoor conventional production systems with small group sizes (<30 pigs per pen). Conversely, in the UK there are many types of system ranging from conventional indoor intensive, to free-range outdoor, and often pigs may move from a nursery site to a finishing site as part of the same pig production flow.. Investigation of the robustness of OF sampling for use in all of these diverse systems is necessary for application of this alternative diagnostic method in the UK. This thesis aims to address these three areas, with specific objectives to:

- Establish and optimise laboratory diagnostic tests for use with pig OF
- Review the composition of OF and pig husbandry factors which may affect this

- Develop sample handling and storage protocols for the maintenance of diagnostic viability of OF during shipment
- Investigate the optimal OF sample collection process for pigs kept in diverse housing systems
- Validate the use of pig OF compared to the current Gold Standard blood serum for the diagnosis and monitoring of infectious disease

1.1.1 *Porcine Reproductive and Respiratory Syndrome virus (PRRSv)*

Porcine Reproductive and Respiratory Syndrome virus (PRRSv) poses a significant threat to animal welfare, and is a disease of high economic importance to the pig industry worldwide. This RNA virus belongs to the Arteriviridae family and causes reproductive failure in sows, as well as respiratory symptoms in growing pigs during clinical manifestation. The incubation period of the virus is between 3 to 37 days (AHA, 2004), and the prevalence of the disease in the UK is approximately 40% (Evans, 2008). There are two strains of significance to the UK industry: Type I PRRSv, which is a European strain, and Type II, which is a North American strain. The disease can also exist sub-clinically within pig populations showing few or no typical clinical symptoms, but a marked decrease in productivity. This sub-optimal performance, plus the losses / treatment costs associated with clinical disease lead to significant financial losses over time for the farmer. Iowa State University estimates the loss of productivity to US producers due to PRRSv to cost around $664 million per annum (Holtkamp, 2011). The relative cost of PRRS across Europe has not been quantified as such, however a pamphlet published in 2011 by the British Pig Executive estimated the UK costs to be around £117 per sow per year (BPEX, 2011b), equivalent to £46.5 million based on the current herd size (BPEX, 2014b). These implications have become major contributory factors in the drive to develop novel methods of early disease screening and surveillance. Such methods could also enable the detection of disease non-invasively at the sub-clinical stage. Consequently, PRRSv has become the prime candidate for the application of non-invasive OF diagnostics.

1.1.2 *Salmonella*

Salmonella bacteria are the cause of clinical Salmonellosis infections. The bacteria are zoonotic, posing a significant threat to both animal and public

health. Salmonellosis is characterised by gastrointestinal disease in both humans and pigs, including watery diarrhoea which can lead to dehydration and in severe cases, death. Human infections arise mainly from the consumption of contaminated food products. The most problematic serovars for human infections are *S. enteritidis*, most commonly associated with the consumption of contaminated egg and poultry products, and *S. typhimurium*, which is most commonly associated with the consumption of contaminated pig meat and bovine meat products (European Food Safety Authority, 2014). In the pig, the bacterium has an incubation period of 24 to 48 hours before the onset of clinical signs when Salmonellosis infection presents, although the pigs can also be infected subclinically showing no clinical signs (Straw, 1999). The EU Zoonoses Regulation (EC) No 2160/2003 is the main driver of the detection and control of Salmonella strains significant to public health in EU Member States.The prevalence of Salmonella in pigs in the UK is currently estimated to be 48% and surveillance strategies rely mainly upon meat juice sample antibody testing post-slaughter, faecal bacterial culture and blood serum antibody testing. Although these methods are effective in monitoring this pathogen, they are not without limitations. Firstly, meat juice is taken post-slaughter, therefore options to investigate potential intervention strategies for treatment of Salmonella are limited. Secondly, although faecal culturing provides good information about which animals are actively shedding Salmonella, this only pertains to a particular snapshot in time. Alternatively, antibody monitoring offers more historic information about Salmonella within the pig flow, but at present this can only be tested in blood serum, which is invasive / stressful to the pigs to collect, as well as costly to the farmer since a vet must undertake the blood sampling (Veterinary Surgery (Blood Sampling) Order 1983).A non-invasive means of detecting anti-Salmonella antibody, i.e. using OF could thus provide an invaluable tool for the surveillance of this pathogen for the protection of both animal and public health.

1.2 Introducing oral fluid: basic composition and factors which may affect this

Oral fluid or whole saliva is a complex biological solution, composed of a vast assortment of diverse constituents. The term oral fluid encompasses secretions from multiple salivary glands, principally parotid, submandibular and sublingual,

as well as gingival crevicular fluid and serum and mucosal transudates which originate from the circulation, although the principal salivary glands may differ between species (Schipper *et al.*, 2007). The relative secretory contributions that form oral fluid vary in magnitude and nature, and are mediated by multiple factors, including the autonomic nervous system (Proctor and Carpenter, 2007), diet, age and health status (Farnaud *et al.*, 2010). Investigation of such secretory determinants, and conditions under which they could change, may lead to a deeper understanding of the composition of oral fluid and forms the main aim of the current review section.

1.2.1 *Basic OF Composition*

The composition of oral fluid, as mentioned, can be highly variable, which can often confound studies that investigate oral fluid, and in particular those that aim to eventually utilise OF as a diagnostic medium. There are many constituents that make up the whole saliva, the majority of the solution consisting of water and electrolytes such as sodium, chloride and calcium (Streckfus and Bigler, 2002). Other components such as phosphates and bicarbonate, are involved in pH buffering, an essential characteristic of oral fluid for the maintenance of homeostasis within the mouth (Humphrey and Williamson, 2001).

Additionally, there are a host of proteins which accompany the inorganic content of saliva. This organic portion contributes significantly to the dynamic nature of OF via processes such as post translational protein modification. This involves enzymatic cleavage of proteins after secretion into saliva (Helmerhorst and Oppenheim, 2007). The human salivary proteome has become a focal point for many research groups around the world, with increasing interest in utilising this biological fluid, for which sampling is non-invasive, to investigate oral health in dentistry and for disease diagnostics in human medicine. The human salivary proteome is therefore becoming increasingly well established. However, a lesser proportion of research has investigated the protein content of animal saliva to the level of individual proteins. One group have published such an analysis of swine saliva, with 10 major groups of protein bands identified, as described in Table 1.1(Gutiérrez *et al.*, 2011) . Protein groups were identified via two-dimensional gel electrophoresis and MALDI-TOF/TOF (matrix assisted laser desorption / ionisation–time–of–flight) mass spectrometry, followed by database searches (Gutiérrez *et al.*, 2011). Expected physiological roles of

each of the protein groups based upon what is known from human and animal research are also listed.

Table 1.1: Protein groups identified in swine saliva (Gutiérrez et al., 2011), and their proposed characteristics / physiological significance.

Protein identified in swine saliva	**Relative roles / characteristics**
Serum albumin precursor	Precursor of the major protein in blood plasma, involved in maintenance of colloidal osmotic blood pressure (Miller, 2009)
Adenosine deaminase	Involved in purine metabolism (Castro *et al.*, 2011)
Pancreatic α-amylase precursor	Precursor of protein that is involved in starch hydrolysis in digestion (Maeda *et al.*, 2008)
Carbonic anhydrase VI	Maintenance of acid – base balance (Nishita *et al.*, 2011)
Salivary lipocalin precursor	Precursor of protein that binds sex pheromones (Seo *et al.*, 2011)
Prolactin inducible protein homologue precursor	Precursor of protein homologous to that which, in humans, is involved in cell signalling pathways (Clark, 1999)
Lipocalin 1 precursor	Precursor of protein that binds lipophilic molecules e.g. fatty acids, glycolipids (Wojnar *et al.*, 2002)
Double headed protease inhibitor (submandibular)	Inhibition of proteolytic activity
Cystatin B	Protein which forms part of a complex which may be involved in regulation of proteolytic activity (Lupi *et al.*, 2003)
Calgranulin C	Calcium (Ca^{2+}) binding protein (Wicki *et al.*, 1996)

By considering the various constituents of whole saliva, and the diverse physiological contributions of these, the nature of this secretion as a complex and dynamic biological fluid can be better appreciated.

As mentioned before, there are several salivary glands that contribute to production of OF. The nature of the secretion from each gland varies, depending upon the cells that predominate in the gland, called acini (Pfaffe *et al.*, 2011). The acini can be either serous, producing a watery solution, or mucous, producing a solution that has a high mucin content (Pfaffe *et al.*, 2011). Glands that consist of predominantly mucous acini are therefore likely to produce a more viscous saliva. In contrast, the parotid gland is most densely populated with serous acini cells, and thus plays a significant role in the production of stimulated saliva (Pfaffe *et al.*, 2011).

Taking into account the relative components that form oral fluid, it is possible to extrapolate specific traits, or characteristics of this which may be adaptable to utilise as measurable variables. Examples of such traits include pH, mediated by the relative levels of the inorganic compounds previously discussed, and the proteolytic activity that exists in oral fluid as a result of protease enzymes involved in post translational processing.

1.2.2 *Factors likely to affect OF composition*

1.2.2.1 *Circadian effects on OF*

Evidence within the literature is suggestive of circadian variability in both the flow rate and composition of human oral fluid. Originally investigated by Dawes in 1972, the group reported that total protein concentration and key electrolyte (sodium, chloride, potassium) concentration in stimulated parotid saliva show a degree of circadian variation (Dawes, 1972). A diurnal effect upon the flow rate of unstimulated saliva was also observed, with peak flow rate between around 14:00 and 18:00 hrs (Dawes, 1972). More recently, in 2000 Rantonen and colleagues investigated the composition of stimulated whole saliva specifically. They found that over the 12 hour daytime period that they tested, concentrations of salivary IgA, albumin, amylase and total protein showed significant variation (Rantonen and Meurman, 2000). The effect on IgA seems especially interesting, as initial diagnostic screening typically involves antibody

detection assays, the results of which could potentially be confounded by diurnal variation in the diagnostic target.

Knowledge of the OF variability induced by circadian rhythm may be useful in determining a sample collection time frame for optimal diagnostic results, which could then be incorporated into the OF sample collection protocol.

1.2.2.2 *Diet and salivary flow*

With around 99% of the total content of oral fluid comprising water (Pfaffe *et al.*, 2011), hydration status becomes significant as a factor that may influence OF composition. All pigs reared commercially have access to water *ad libitum*, however the type of diet on which they are fed may differ. Water and dry matter dietary content has been shown to affect OF secretion via altering the requirement for mastication, or chewing of foodstuffs. In general, diets that comprise more dry matter require more chewing and lubrication in the mouth in order to form a bolus for swallowing, and are therefore associated with increased salivary flow rates (Hall *et al.*, 1967). The effect has been demonstrated in rats (Johnson, 1973), and the reverse shown in humans, where a 7 day liquefied diet resulted in decreased salivary volume, amylase and total protein content (Hall *et al.*, 1967). Further evidence to support the hypothesis of different feed types inducing differential chewing times and jaw movements is provided by Brøkner et al. (2008). The group found that different types of roughage provoked variations in actual jaw movements, as well as time spent chewing in horses (Brøkner *et al.*, 2008). Considering this evidence across species, it may be fair to postulate that the same is true of pigs, and that those fed a wet feed may have lower salivary flow rates than those fed dry feed. This may then affect sample collection, and require sample collection times to be specifically tailored to the feed type, in order to collect samples of equal volumes from differentially fed populations.

1.2.2.3 *Stimulation and salivary flow*

Salivary stimulation is induced by mastication. The physiological purpose of this is to lubricate the oral cavity and aid digestion when feeding (van der Bilt *et al.*, 2006), and may induce changes in its composition to occur. An example of one compositional change that may be necessary during feeding behaviour might be

the secretion of more digestive enzymes into stimulated oral fluid. Neyraud and colleagues provide supportive comment on this in their assessment of the variability of human saliva composition. The group reported a reduction in protein concentration, and increased proteolytic activity in saliva stimulated mechanically by mastication, compared to unstimulated (Neyraud *et al.*, 2011).

In humans it is possible to collect unstimulated, as well as gland specific and whole stimulated saliva. This is achieved by asking the human subject to perform specific tasks, for example allowing their natural flow of saliva to drip into a collection vessel without moving the jaw or tongue (Dawes, 1972). Such tasks cannot be asked of the pig, however they do possess a natural tendency to engage in chewing behaviours (Trickett *et al.*, 2009). Consequently, porcine OF sampling relies solely upon salivary secretions that are stimulated, i.e. by chewing on absorbent sponge or cotton rope. A second level of chemical stimulation of salivation may also be introduced, by the application of flavouring to the medium provided for chewing. Various additives have been assessed in their ability to stimulate OF secretion in humans, the most effective to date being citric acid (Streckfus and Bigler, 2002). The use of such a stimulant might be expected to induce compositional changes to occur in OF via increased (i.e., stimulated) salivary flow. However, when Neyraud and colleagues investigated the effects of different taste compounds on the flow and composition of human parotid saliva, they found that taste compound was unlikely to affect pH, but may influence the change in protein concentration associated with the resultant stimulation of salivary flow (Neyraud *et al.*, 2009).

Stimulation of salivary secretion results predominantly in an increased volume of fluid being produced. A measure of this can be obtained by assessing the salivary flow rate of an individual. In humans it is possible to measure either stimulated or basal (unstimulated) flow rates by collecting the fluid produced over a set time period (Streckfus and Bigler, 2002). The resulting flow rate is then expressed as the volume of oral fluid produced per unit time, e.g. ml/min. Human basal secretion rates can be as low as 0.1 – 0.5 ml/min, whereas when stimulated mechanically by chewing, salivary flow can reach rates of up to 5 ml/min (Dawes, 1987). Further, gustatory stimulation by the addition of citric acid can result in flow rates of 7 ml/min (Watanabe and Dawes, 1988). There

may be scope to apply this methodology to the pig, via the addition of citric acid (e.g. fruit juice) to cotton chewing ropes in order to investigate the potential changes in oral fluid composition associated with flow rate, and how this might influence viral RNA / antibody detectability. The increase in proteolytic activity that is associated with salivary stimulation, and hence flow rate could directly affect the recovery of disease markers from OF samples, especially given that porcine OF samples are, by nature, the result of salivary stimulation.

Normal salivary pH in humans lies somewhere between 6 and 7, and has been shown to be positively correlated to salivary flow (Humphrey and Williamson, 2001). Given this finding, plus a report by Jacobs et al. (2010) describing the relative stability of PRRSv RNA at ambient temperatures at neutral pH, it might be anticipated that a change in the pH, i.e. an increase induced by flow stimulation, could be likely to affect RNA stability.

1.2.2.4 *Sample handling and storage*

Methodological implications, and the effects that these may exert on oral fluid composition will now be discussed. Sample collection, handling and storage may each be considered individually as being able to have consequential effects on OF constituents. For most human studies that aim to investigate oral fluid for use in disease diagnostics, the sample is collected into a device such as a Salivette tube, and immediately chilled or frozen until it is processed (Navazesh, 1993). Methodology for OF sampling in the pig is reasonably well established in the literature, involving provision of a cotton rope at shoulder height for chewing, and thus sample deposition. Wringing by hand or centrifugation of the rope then extracts the OF sample into a collection device for subsequent processing (Prickett, 2008b).

Previous work that focussed on Acute Phase Protein (APP) analysis in porcine OF investigated whether the absorptive medium used to collect OF itself may influence the recovery of diagnostic targets. The use of an oral swab device (Salimetrics) resulted in a non significant increase in OF concentration of the APP's Haptoglobin and C reactive protein compared to that collected by allowing pigs to chew on cotton rope (Seddon, 2011).

It is most likely to be the way in which samples are handled and stored after the point of collection that may exert the most profound effects on composition. As mentioned previously, most biological samples including semen, blood serum and oral fluid are chilled as soon as possible after collection and then frozen, often at -80°C, for longer term storage until they are analysed. Samples are treated in this way in order to minimise changes that may occur to the composition of OF. One such example of altered OF composition after the point of collection is reported by Schipper et al. (2007), who documented increased buffering capacity, which will aid the stabilisation of the pH of OF stored at room temperature or -18°C. Storage at -80°C was not found to affect pH, and would therefore be the favourable storage temperature for OF samples for the prevention of such changes (Schipper *et al.*, 2007). Additionally, the same review explains how the pH of OF left to incubate at room temperature will slowly rise due to the release of dissolved carbon dioxide from the OF into air (Schipper *et al.*, 2007). Taking this into account, it may then be possible to estimate how fresh an OF sample is, by assessing relative pH change, from a baseline value for a particular farm. This could be advantageous in routine testing of OF samples as a means of quality control when samples arrive at the diagnostic facility for testing.

Compositional changes in OF may occur due to a variety of reasons, one of which is the proteolytic activity of endogenous enzymes (Helmerhorst and Oppenheim, 2007). These enzymes may be likely to remain active, thereby potentially altering the protein, and therefore antibody content of samples if they were to be stored at ambient temperatures. A study by Prickett et al. (2010) showed that the stability of anti-Porcine Reproductive and Respiratory Syndrome virus (PRRSv) antibodies, as well as viral RNA in oral fluid, is dependent on temperature. Disease markers remained detectable for up to 12 days when samples were stored at 10°C or below, but were less detectable at temperatures above 10°C (Prickett *et al.*, 2010). Taking this evidence into account, it would seem advisable to chill OF samples immediately following collection, and conduct laboratory testing as soon as possible for optimal results. Following investigation of the stability of PRRSv at ambient temperatures, (between 4°C and 30°C), Jacobs and colleagues reported that no significant change in PRRSv RNA detectability was induced by storage of the

virus in stock solution with pH 7.5 at either 4, 10, 20 or 30°C (Jacobs *et al.*, 2010). This finding suggests that something about the OF sample itself gives rise to temperature dependent RNA instability, since in the environment of the stock culture solution PRRSv RNA remained stable as temperature increased considerably. Given what is known about the kinetics and biology of enzymes, it would be plausible therefore to suggest that it is this enzymatic aspect of oral fluid that is most likely affecting the stability, and thus the detectability of viral RNA.

There are a number of different treatments that can be utilised on samples, and have been shown to have a stabilising effect on proteolytic activity. One of these methods is acidification using hydrochloric acid (HCl), though this may also result in protein denaturation, and thus should be carefully considered according to the desired variable. Additionally, the use of “cocktails” of various protease inhibitor compounds is becoming increasingly attractive in the pursuit of achieving biomarker stability in fluids such as OF (Thomadaki *et al.*, 2011).

There can be a great degree of variability in the purity of OF samples. The term “purity” is applied loosely, in order to be able to consider the potential contamination of samples by food particles for instance. In human studies, this issue is circumvented by instructing subjects to perform uniform mouth rinsing activities (Navazesh, 1993). For the pig, the extent to which an OF sample may be contaminated with such debris depends upon how recently an animal’s last feed was prior to sample collection, and the nature of that feed. It may also depend on the availability of bedding substrates in which animals may root and ingest. It is common practice to pellet out all debris in an OF sample, including any food particles, by performing a centrifugation step at the beginning of sample processing (Guarino *et al.*, 1997; Gonçalves *et al.*, 2005; Chittick *et al.*, 2011). This also has what may be described as a “blanket – type” effect across samples, removing all particulate matter present in the sample, thus leaving the supernatant, which may then be regarded as a relatively uniform sampling medium. It has however been highlighted that total particulate removal may not be as advantageous as first anticipated. During work on Hepatitis C virus (HCV) detection in OF, Roy and colleagues proposed the possibility of virus particles remaining associated with sample debris, and thus being lost during the

centrifugation process (Roy *et al.*, 1999). Further to this, Goncalves et al. (2005) described plans to investigate the potentially differential effects of pellet / supernatant RT-PCR analysis for HCV, given that the virus is known to replicate in epithelial cells, which are pelleted out during centrifugation (Gonçalves *et al.*, 2005). Although PRRS virus is not currently known to be associated with any such cells, the possibility of virus being associated with other OF debris exists. Studies looking into PRRSv RNA detection in centrifuged versus non centrifuged samples may therefore be worthwhile. Additionally, the introduction of large particle filtration (e.g. 0.45µm filtering) may be worthwhile, so as to gain substantial, but not total particle removal.

The ways in which OF samples are handled and stored post-collection seem to have a large influence on the maintenance of sample quality for investigative purposes. Methods therefore become critical in the use of OF for diagnostic purposes, as even a subtle change in the sampling conditions could give rise to drastic downstream effects by facilitating processes mediated by the bioactive components in the fluid.

1.3 An introduction to laboratory-based antibody detection methods

At present, there are a multitude of laboratory techniques which enable the detection of antibodies in cell free body fluids (e.g. serum, cerebro-spinal fluid). Serological testing, that is the analysis of serum from blood samples, is probably the most well characterised application of this process. Such tests can be utilised within the clinical setting for diagnostic purposes. Additionally, the technology can, and is increasingly being, applied to investigative questions within the fields of medical and veterinary research. The Enzyme Linked Immunosorbent Assay (ELISA) is the most widely applied antibody detection test, used both on a commercial scale for disease diagnostics, and in research. There are many variations of the ELISA, with differences in diagnostic sensitivity and specificity. The basic principles behind three commonly used ELISA subtypes can be found in Appendix A.

1.3.1 *Human applications*

The exposure of a subject to a pathogen, or in some cases allergen, will elicit an immunological response. Part of this response involves the activation of B cells in the peripheral lymphoid tissues to produce antibodies against the

pathogen. These antibodies have multiple roles within the host to aid the recovery from disease following infection with specific pathogens. Immunological memory is also mediated by antibodies serving to recognise and prevent future infection once a response to a specific pathogen has been elicited. Due to this immune-protective role, antibody presence can be exploited for diagnostic testing, offering the ability to screen subjects for evidence of past infection with a given pathogen.

Antibody detection in human blood samples dates back to 1946, when a strain of St. Louis encephalitis virus was successfully isolated from the peripheral blood of a live human subject (Blattner and Heys, 1946). Although virus isolation was deemed as the main achievement of this study, one of the key markers of infection that was observed was an increase in detectable antibody concentration or titre within the subject's bloodstream. Since then, the use of serum based antibody tests has been widely implemented in medical diagnostics across the globe. Probably the most significant advance within the human medical field was the ability to detect antibodies against Human Immunodeficiency Virus (HIV) in patient serum (Abe *et al.*, 1985; Goedert and Gallo, 1985), allowing rapid testing for HIV positivity. HIV is a pathogen for which antibodies can be successfully detected in saliva samples (Archibald, 1986) and as such this non-invasive collection method allows the same rapid and accurate diagnostic results for widespread screening of adults and children, which is of particular importance in countries where HIV is a significant health issue. Another advantage of antibody testing over other types of diagnostic test, such as molecular methods is cost. For example, the reagents for antibody detection assays are considerably less expensive than the highly sequence-specific primers and probes required for polymerase chain reaction (PCR). This human medical evidence inspired pig veterinary researchers to begin the development of antibody detection assays targeting pig disease, which will now be discussed.

1.3.2 *Pig applications*

The detection of antibodies in swine oral fluid (OF) was first described in 1976 when Corthier vaccinated pigs intranasally with a strain of the Classical Swine Fever virus (CSFV) and monitored the immune response elicited (Corthier,

1976). A detailed review of this work, plus other early reports of antibody detection in porcine OF can be found in Prickett and Zimmerman (2010).
The initial isolation of PRRSv in porcine oral fluid was established by Wills and colleagues in 1997 from oropharyngeal swabs (Wills *et al.*, 1997b). The group demonstrated persistent infection and shedding of PRRSv in pigs for up to 157 days following experimental inoculation with the virus. The study did not investigate anti-PRRSv antibody presence in OF, however the group present integrated serological and OF virus isolation data. These data illustrate the close correlation between virus shedding into the OF and seroconversion during the course of PRRSv infection.

Following the reports of successful pathogen detection in swine OF, Prickett and colleagues initiated a longitudinal experimental infection study to investigate OF use for diagnosis of infection with PRRSv and Porcine Circovirus Type 2 (PCV-2) (Prickett, 2008a). The group targeted the respective pathogens themselves, by virus isolation and PCR methods but, in the case of PRRSv, tests to detect antibody presence were also introduced. Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test (IFAT) were used, as antibody presence would indicate past exposure, and possible immunity to the virus. Prickett (2008a) described a low level of anti-PRRSv antibody detection (i.e., sensitivity) in porcine OF, e.g. only 20 of 154 samples tested positive by the IFAT method. OF ELISA results did not differ significantly between experimentally inoculated and negative control pigs (Prickett, 2008a). The result highlighted the potential of OF use for widespread screening of populations for evidence of pathogen exposure via the detection of antibodies. Improving the low sensitivity of the methods used to detect anti-PRRSv antibodies in OF then became a primary focus for many research groups and industrial contributors such as commercial ELISA kit manufacturers.

It became apparent that successful validation of OF protocols for diagnostic testing would require extensive optimisation, and thus adaptations to serological protocols for use with OF then began to emerge. It was perceived that the highly complex, enzymatically active composition of OF may be detrimental to disease markers such as virus nucleic acids and antibodies. Prickett *et al*. (2010) addressed this by investigating the stability of PRRSv and anti-PRRSv

antibodies in swine OF. A large pool (4 litres) of finisher pig OF was spiked with both a PRRSv isolate, and antibodies produced in pigs following inoculation. The relative stability of the agents was investigated at a variety of storage temperatures and for different time periods. It was suggested that metabolic activity from bacteria present in OF may be detrimental to the stability of viral RNA and / or antibodies. Further to this, it has recently been suggested that the presence of salivary proteins may lead to increased proteolytic activity from specific oral commensal bacteria (Kindblom *et al.*, 2012). In order to test for any bacterial influence, antimicrobials were introduced as an experimental factor, for measurement of any potential effects on disease marker recovery following storage at the different temperatures. Antibodies were detected via the HerdChek PRRS 2XR ELISA (IDEXX Laboratories Inc., Westbrook, ME) with adaptations to the manufacturer's protocol for use with OF. It is important to note that although multiple experiments were conducted in this study, only the findings pertaining to anti-PRRSv antibodies as shown in Figure 1.1 will be discussed in this review.

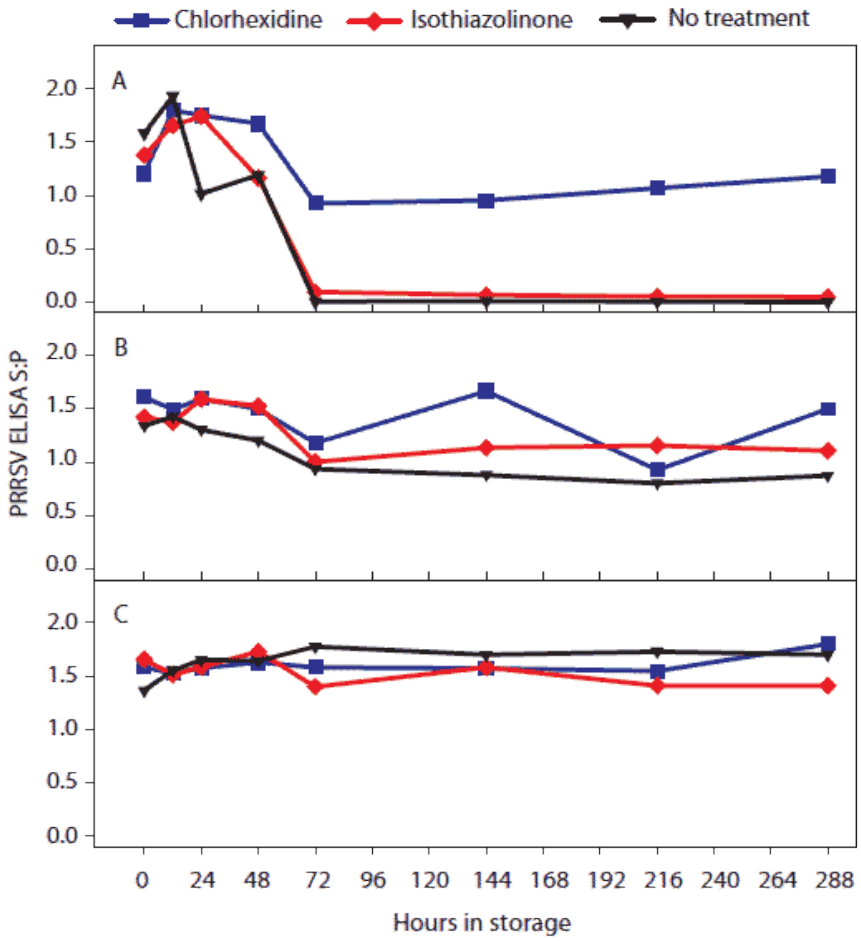

Figure 1.1: ELISA S:P Ratios for PRRSv specific antibody detected in porcine OF stored at differential temperatures over the experimental time period. Relative antimicrobial treatments indicated. Panels represent different temperature conditions: A = 30°C; B = 20°C; C = -20, 4 and 10°C. Figure taken directly from Prickett et al. (2010).

The three temperature groupings pertaining to panels A –C in the figure were formulated following statistical analysis by ANOVA. Lower temperatures (≤10°C) were found to be optimal for maintaining antibody stability over the experimental time course of 12 days, with ELISA sample-to-positive (S:P) ratios decreasing for temperatures of 20 and 30°C. Sample-to-positive (S:P) ratio is an expression which takes into account the optical density of the test sample in indirect ELISA, relative to the optical density yielded by the internal kit positive control, as the optical density in indirect ELISA testing reflects the antibody within the sample which has been successfully detected. The use of the antimicrobial chlorhexidine significantly improved the S:P ratio for the higher temperatures, but no effect of antimicrobial agent was observed at lower temperatures (Prickett *et al.*, 2010). In addition to analysing overall antibody stability, ELISA tests were specifically designed to detect and thus quantify the

different immunoglobulin isotypes; IgA, IgG and IgM. IgG and IgA seemed to be the most sensitive to increasing temperature but, similarly to the result previously mentioned, the two isotypes appeared more stable at 30°C when samples were treated with either chlorhexidine or isothiazolinone (Prickett *et al.*, 2010). Taken together, these findings demonstrate that antibodies can be successfully stored and subsequently recovered from porcine OF kept for at least 12 days by chilling or freezing samples.

The establishment of anti-PRRSv antibody detection in swine OF then sparked interest in applying the technology to other pathogens. Porcine Circovirus Type 2 (PCV-2) has been implicated in Postweaning Multisystemic Wasting Syndrome (PMWS) (Baekbo *et al.*, 2012) and Porcine Dermatitis Nephropathy Syndrome (PDNS) (Phaneuf *et al.*, 2007). Similar to PRRSv, PCV-2 can be sub-clinically prevalent within a herd, showing few or no symptoms. Consequently PCV-2 is another target pathogen for the development of reliable OF diagnostics. In 2010 a longitudinal design field study was published by W.I. Kim, which compared pen-based OF with serum samples from 3 separate farms. 6 pens of 25 pigs each were used for sampling at each of the 3 sites, where pen-OF and individual serum samples were collected at set intervals. Individual sera were taken from 5 pigs chosen at random per pen (i.e. 20% of the population), and pooled in order to compare detection of antibodies across the two diagnostic media.

Extensive antibody testing methods were carried out on the OF. IFAT assessed anti-PCV-2 IgG and IgA, and blocking, indirect and IgG / IgM sandwich ELISA tests were also performed. The blocking ELISA was reported to be able to detect all anti-PCV-2 antibody subtypes, whereas the indirect method was specific to IgG. The sandwich ELISA was able to simultaneously detect both IgM and IgG using the same kit.

This study aimed to compare the abilities of three ELISA methods to detect anti-PCV-2 antibodies in porcine serum and OF samples with that of IFAT. The results of the study demonstrate clear detection of anti-PCV-2 antibody in porcine OF, though at lower concentrations than in serum. IgG antibody was found to be detectable from around 3 weeks of age to between 5 and 8 weeks of age. Both IgG and IgA antibodies were detectable at 16 weeks of age for all pens at only one of the farms. Despite this difference between sites, the three

ELISA methods and IFAT analysis were found to have 88-93% agreement, with kappa (κ) value 0.75-0.87 in OF (Kim, 2010). The group calculated test agreement such that for total test agreement, κ = 1, and for no agreement, κ = 0. The kappa values for serum were calculated to be lower than those for OF: 49-76%, κ = 0.19-0.47 (Kim, 2010). The greater range of agreement for serum testing is suggestive of a greater variability in the sensitivities of the tests when analysing serum compared to OF.

Shortly after this field study, Prickett and colleagues initiated an experimental study in which pigs were inoculated with PCV-2, and in some cases re-inoculated with different strains at set time points throughout the study duration (Prickett *et al.*, 2011). Pooled OF samples from each experimental pen (n=6 pigs/pen), along with individual blood samples from each pig (total n=24) were collected at set intervals for diagnostic testing. The group analysed the response to PCV-2 infection in terms of immunoglobulin isotypes, by designing a specific ELISA test, the method for which is described in detail by Prickett *et al*. (2011). Blood sera were tested only for IgG antibody by ELISA. Anti-PCV-2 antibody isotypes IgG, IgA and IgM were detectable from 14 days post inoculation (dpi) in OF samples, with IgG and IgA remaining until 98 dpi, i.e. 14 weeks post inoculation (Prickett *et al.*, 2011). These results, taken together with those reported by Kim (2010), not only demonstrate the application of antibody testing to a pathogen other than PRRSv, but also illustrate the longevity of antibody presence in OF. This characteristic of the immune response may prove a useful tool in research that aims to investigate such responses and their dynamics in the pig, as well as providing a non-invasive method for studying herd-wide pathogen exposure.

1.3.3 *Current state of the art of serological methods and their application to pig oral fluid*

The potential industrial benefits of using pooled OF as a means of conducting widespread pathogen surveillance in commercial scale pig populations are key motivators in the drive to develop and validate existing serological assays for use with porcine OF. The growth and progression of the field is most evident in North America, with Iowa State University's Veterinary Diagnostic Laboratory performing over 10,000 OF tests in 2010, the first year of offering the service. In 2012 this figure stood at 60,000, and that same number of OF tests have been

performed at the centre between January and July 2014 (Zimmerman, 2014). This demonstration of mounting interest in, and application of, pig OF diagnostics is driving increasingly innovative questions to be asked by research groups within the discipline. One example of this was reported by Panyasing *et al*., (2012), a group that used an ELISA designed to detect anti-Influenza A virus (anti-IAV) nucleoprotein antibody in avian serum to try to detect the anti-IAV antibody in porcine serum initially, and then OF. Modification of the original manufacturer's protocol allowed anti-IAV antibodies to be successfully detected in porcine OF samples from day 7 through to day 42 following experimental inoculation with the virus. Furthermore, the correlation between the sample to negative ratios (S:N) for serum and OF samples was calculated to be 0.796 (Pearson's correlation coefficient, $P < 0.0001$) (Panyasing *et al.*, 2012). Sample-to-negative (S:N) ratio is used to express the results of the blocking ELISA, where the optical density is inversely proportional to antibody detection within the sample, and takes into account the optical density of the test sample relative to the internal kit negative control value. Similarly, an ELISA assay for the detection of anti-PRRSv antibodies in porcine OF has been developed based upon an original assay designed to detect antibodies in porcine serum (Kittawornrat *et al.*, 2012a). Following a number of methodological modifications, one of the most significant reported by the group was the titration of the secondary antibody conjugate against kit controls, an optimal conjugate dilution, and thus working protocol, for OF have been established. Initially this assay required an overnight incubation period to allow potential antibody within the OF sample to bind to the antigen coated on the ELISA plate, however there is now a commercially available PRRS ELISA test kit for use with OF (IDEXX Laboratories, Westbrook, ME), which requires a sample incubation period of only 30 minutes, which is the same as the protocol for serum testing.

A ring test study in the US that investigated the in-house repeatability, and reproducibility between different laboratories, for this assay evaluated performance using commercially configured reagents, i.e. as provided by the manufacturer, as well as allowing labs to use "in-house" protocols with specific reagent modifications (Kittawornrat *et al.*, 2012b). Porcine OF samples of known and unknown PRRSv status were tested by 12 laboratories, and reliability coefficients were calculated for assay performance. These ranged

from 0.942 to 0.997 for within-lab repeatability when the same kit lot was used, and 0.953 to 0.998 when different kit lots were used. In addition, reproducibility reliability coefficients ranged from 0.957-0.932 between the participant labs for all samples tested (Kittawornrat *et al.*, 2012b). These studies have led to the launch of a commercial PRRS ELISA kit for porcine OF, from which the assay can be performed within a number of hours.

The ability to rapidly and reliably provide pen-based diagnostic results to farmers using samples which can be collected non-invasively, and therefore cost effectively, is an achievement of great significance, especially for the economically prominent PRRS virus. Whereas the well characterised molecular methods such as RT-PCR are able to pinpoint viral shedding in OF during the course of infection, the information obtained by ELISA testing, (i.e., antibody presence and/or exposure to virus / prevalence within the herd), could allow veterinarians and farmers to perform detailed epidemiological evaluations of specific pathogens in collaboration .

1.3.4 *Alternative approaches for antibody detection*

The current most widely used indirect ELISA for PRRSv (PRRS X3 Antibody Test, IDEXX Laboratories Inc., Westbrook, ME- formerly IDEXX Herdchek 2XR ELISA) targets antibodies specific to the viral nucleocapsid protein (N) in both serum and OF. Although these tests deliver 98.8% diagnostic sensitivity and 99.9% specificity (IDEXX Laboratories Inc.), ways in which these parameters may be improved further are being continuously researched. Work has been conducted to investigate the potential of other antigenic targets for PRRSv ELISA testing. In 2009, Brown et al. developed a direct ELISA which targeted the PRRSv non-structural protein 7 (nsp7) (Brown *et al.*, 2009). The test successfully detected anti-nsp7 antibodies in swine sera, and in addition was able to differentiate between the two major PRRSv genotypic strains, Type I (European) and Type II (North American). Furthermore, the group progressed to develop a “dual” ELISA, which would simultaneously detect antibodies raised against nsp7 from both Type I and II PRRSv strains. Validation of this test against the IDEXX indirect ELISA using field serum samples yielded a sensitivity and specificity of 97.6% and 97.5% respectively. Consequently, the authors deemed the new test to be comparable to the IDEXX Herdchek 2XR ELISA. Antibody response to nsp7 was detectable by ELISA for 202 days post

inoculation (dpi) in serum from experimentally infected pigs. In contrast, the response detectable by IDEXX ELISA, i.e. that elicited towards the nucleocapsid N protein, began to diminish after 126 dpi (Brown *et al.*, 2009). This ability to detect immune activity for longer time periods may be useful in providing a more accurate insight into the long term dynamics of PRRSv exposure within animals at both the individual and herd level.

A recent study by Langenhorst *et al*. (2012) reported the development of an immunological assay that combined the use of the nucleocapsid (N) and nsp7 antigen targets in a multiplex Fluorescent Microsphere Immunoassay (FMIA). The assay exploits micro beads that are coupled to the target antigen for sample antibody binding. The test plate is then read to measure the mean fluorescence intensity (MFI). The method was able to detect PRRSv seroconversion in porcine OF at 7 dpi following experimental infection, which is similar to ELISA methods currently being developed for use with OF (Kittawornrat *et al.*, 2012a). The FMIA specific to Type I PRRSv seemed to be slightly less sensitive than the Type II test, although the authors do acknowledge that this may be due to smaller sample sizes for Type I PRRSv. The study revealed that the pig antibody response towards N protein is higher than that for the non-structural proteins during early infection (14 dpi), and the opposite is true during later infection (>28 dpi) (Langenhorst *et al.*, 2012). These findings could provide a foundation upon which further work may build. The early antibody detection, and differential sensitivities to the antigenic targets, serve to support the FMIA method as a potential means of not only detecting seroconversion (i.e., the point at which antibody presence becomes detectable in the host blood serum), but potentially being able to distinguish recent conversions from those that occurred further in the past. Such knowledge could further deepen farmers' understanding of their herd health status, in terms of specific pathogens.

1.4 Introduction to laboratory-based molecular diagnostic methods

The detection of antibodies in a clinical sample is usually a good indicator of exposure to a given pathogen however the test is not capable of detecting viraemia in samples. Molecular methods target the aetiological agent itself as opposed to aspects of the host immune response, thus enabling the course of infection within an individual, or group of individuals to be tracked in situ, i.e. as

the infection occurs. This can be especially useful in terms of investigating the dynamics of a pathogen within a population. Such methods include isolation of the agent itself, or detection of genetic material pertaining to that agent, both of which are possible due to pathogen shedding by the host. The main focus of the current review will be the latter mentioned detection of genetic material from disease pathogens.

Deoxyribonucleic acid (DNA) is the basic functional genetic unit in humans, animals and some viruses and bacteria, upon which crucial processes are based both at the cellular level, e.g. mitosis (Halbrook, 1989), and up to the whole organism level, e.g. formulation of a diploid zygote in sexual reproduction (Janse *et al.*, 1986). Alternatively, some viruses utilise Ribonucleic acid (RNA) in this way. Both DNA and RNA have been shown to be detectable, and in some cases quantifiable in biological samples during the course of infection with a number of disease causing pathogens affecting humans and animals (Laure *et al.*, 1988; Ulrich *et al.*, 1990). The method by which this is achieved is called the Polymerase Chain Reaction (PCR), whereby a specific portion of target DNA is replicated many times through continuous cycling at pre-set conditions. The number of replicative cycles required for the quantity of DNA to reach a threshold level (Ct value) is the unit of measurement for the end result. Lower Ct values convey a larger quantity of starting point DNA, as fewer cycles are necessary to reach threshold, whereas higher Ct values are indicative of lower starting quantities. The first paper to document the successful application of PCR was published in 1985, involving the amplification of DNA pertaining to human beta-globin for the diagnosis of sickle cell anaemia using samples of amniotic fluid, and from the chorionic villus during pregnancy (Saiki *et al.*, 1985). From this initial discovery, the PCR has been developed for use with many biological samples in medical diagnostics, and additionally forensic analysis of criminal evidence. Further still, advancement of the technique has yielded the ability to detect and quantify RNA within a given biological sample. An additional step is required to first convert the target RNA into DNA for PCR amplification. The conversion of DNA to RNA (transcription) ordinarily takes place at the beginning of DNA replication. As the PCR reaction requires the opposite of this process, i.e. conversion of RNA to DNA, the first step is reverse transcription, catalysed by the enzyme Reverse Transcriptase. This type of PCR is known as

Reverse Transcription Polymerase Chain Reaction (RT-PCR), and is of particular significance to the swine industry, as one of the major endemic disease agents previously mentioned, PRRSv, is an RNA virus. To place the information previously presented into context with respect to swine disease diagnostics, the resultant Ct value of RT-PCR reflects the original starting amount of viral RNA (i.e., the lower the Ct, the greater was the starting level of RNA), as would be the case with PRRSv for example. From this, it is then possible to deduce the relative titre of virus that the individual subject or in this case, pig, is infected with at any given time point.

The application of PCR and RT-PCR to a wealth of biological sample types, including blood (Lanciotti *et al.*, 2000), many diverse tissue types (Kern *et al.*, 1995; DelaTorre *et al.*, 1996; Mace *et al.*, 1998), and also some cell free body fluids such as cerebrospinal fluid (Frisk *et al.*, 1999) is well characterised throughout the literature. The next portion of this review will focus on the application of molecular diagnostics to oral fluids (OF) initially in humans, with implications for public health. The importance of molecular OF diagnostics to the pig industry will then be highlighted and current advances within the field will be discussed.

1.4.1 *Human applications*

The basis for the application of molecular diagnostic techniques to oral fluids arose from the success of antibody detection which was previously discussed. The nature of molecular OF diagnostics would allow non-invasive analysis of pathogen titres during infection as opposed to following infection, as is the case with antibody testing.

Human Immunodeficiency Virus (HIV) is probably the most heavily investigated virus in relation to human OF diagnostics. Kits are now commercially available to screen human saliva samples for anti-HIV antibodies. The focus on molecular aspects of diagnosis came about almost immediately following the first application of PCR. Only five years after the first PCR publication in 1985, O'Shea and colleagues published a report which demonstrated the detection of HIV nucleic acids in saliva samples (O'Shea *et al.*, 1990). The group were looking at viral excretion routes and antibody responses, and analysed a number of bodily fluids including blood, semen and saliva. One of the indications from the experiments that was highlighted by the authors was the

fact that viral nucleic acids were detected in some of the saliva samples without the presence of infectious virus (O'Shea *et al.*, 1990). This was of considerable significance as a potential confounding factor to the new method, as it suggested that a positive PCR result may not necessarily confirm an infection *in situ* at the time of testing.

A wave of enthusiasm into PCR testing of OF ensued, with the reported detection of infection with Epstein-Barr Virus, Human Herpes Virus type 6 (Saito *et al.*, 1991), Cytomegalovirus (Greenfield *et al.*, 1991), Helicobacter pylori (Hammar *et al.*, 1992) and Herpes simplex virus type I (Robinson *et al.*, 1992) by PCR assay. The year of 1992 also brought about the first documented detection of viral RNA in saliva samples. Wang *et al*. (1992) carried out nested PCR on saliva samples from patients with clinically confirmed Hepatitis C infection following blood transfusion in 1992, with successful detection of viral RNA from 1 week to 38 months after the initial onset of Hepatitis ailment (Wang *et al.*, 1992). The technique of nested PCR is regarded as a slightly more refined approach, utilising two pairs of primers, the first to identify a specific sequence within the target DNA (or cDNA as is the case for reverse transcribed RNA). The second primer pair then targets a sequence of DNA within the amplicon that results from amplification by the first primers, thereby reducing the chance of amplification of the incorrect DNA sequence. In 1993, PCR was successfully performed on gingival fluid samples from human subjects in relation to the gum infection, periodontitis (Goncharoff *et al.*, 1993). Specific DNA sequences pertaining to the bacterium *Actinobacillus actinomycetemcomitans* were amplified from gingival fluids as part of a larger experiment looking to investigate one specific gene from the species, namely IktA (Goncharoff *et al.*, 1993).

The implications of PCR testing in human OF do not lie solely within the fields of medicine and public health. DNA fingerprinting is a widely used technique in forensic analysis of crime scenes and criminal evidence, for which PCR amplification of suspect DNA from hair, skin cells or bodily fluids forms the fundamental basis. Worth mentioning in particular are reports of using saliva traces from envelopes, stamps and cigarette butts for PCR amplification of target DNA (Allen *et al.*, 1994; Hochmeister *et al.*, 1998). Further to this, a report in 2000 demonstrated that it is possible to extract full DNA profiles from

saliva traces on stamps and envelope flaps using commercial DNA extraction kits (Sinclair and McKechnie, 2000).

More recent advances in molecular technology have led to the refinement of methods which are now able to target messenger (m)RNA transcripts of genes in tissues and fluids. Furthermore, techniques such as microarray allow the expression patterns of several genes and thus proteins to be investigated for diverse tissue types. Emerging evidence suggests that such assays may be applicable to OF and other bodily fluids. This ability to analyse extracellular DNA and RNA using fluids, most of which can be collected non-invasively could provide a novel platform for gene expression studies. This bears potential to benefit biomedical research into a number of diseases significant to public health. A review that places emphasis on this aspect of non-invasive molecular investigation is that by O'Driscoll in 2007. The article highlights the importance of DNA and mRNA as extracellular nucleic acids present in various bodily fluids including saliva, and their potential role in disease diagnosis and prognosis, as well as the implications for forensic investigations which have been briefly mentioned. Cancer was highlighted in particular as a candidate disease for which monitoring changes in expression patterns of specific target mRNAs via PCR and microarray analysis could assist in the early detection of the disease (O'Driscoll, 2007). For instance, one group found that upon investigation of the expression of four matrix metalloproteinase enzymes in oral epithelial tissues of oral cancer patients, two of these enzymes showed significantly higher expression levels in patient tissues compared to controls (Stott-Miller *et al.*, 2011). This result is one of many which have revealed changes in candidate gene expression patterns pertaining to various cancer types through the application of advanced molecular techniques. In addition to this, significant variation in the numbers of bacterial microflora species were found in the saliva pellets of patients diagnosed with pancreatic cancer compared to healthy control subjects (Farrell *et al.*, 2012). Initial profiling via Human Oral Microbe Identification Microarray (HOMIM), followed by quantitative PCR validation of species showed differential expression profiles of 56 microflora species belonging to 5 different bacterial phyla in pancreatic cancer patients (Farrell *et al.*, 2012).

1.4.2 *Pig applications*

The application of PCR to OF samples obtained from pigs is an idea that has emerged within the last decade. Before the discussion of how this was first achieved, and subsequent studies that resulted from this, it is important to mention the base from which the idea was built upon. Porcine Reproductive and Respiratory Syndrome (PRRS) virus was initially isolated in fluids obtained by scraping the oropharyngeal area of experimentally inoculated animals, and subsequently titrating the virus from this (Wills *et al.*, 1997b). Another report published in the same year also isolated the virus in oropharyngeal samples, as well as performing the first isolation of PRRS virus from saliva swab samples from experimentally inoculated pigs (Wills *et al.*, 1997a). These discoveries were acknowledged by Prickett et al. in 2008 in the first report of the detection of PRRS virus RNA in pig OF samples by Reverse Transcriptase-PCR (RT-PCR). The group inoculated three age groups of pigs at 4, 8, and 12 weeks old, and collected individual serum and buccal swab samples, as well as pen-based OF's twice per week for up to 63 days post inoculation (dpi). OF and serum samples were sent to two separate laboratories for comparative analysis of results. Laboratory A used a commercially available viral RNA extraction kit, following the manufacturer's instructions, and used a commercial RT-PCR assay for RNA quantification. Laboratory B used a different method to extract viral RNA, and slightly differently designed PCR primers and probe in their analysis (Prickett, 2008b). Full qualitative agreement was found for the RT-PCR results between the two laboratories, that is, the same samples were declared positive or negative by RT-PCR between the two laboratories. In general, viral RNA concentration in OF showed a similar pattern to that in serum, though at lower levels throughout the experimental duration. The mean diagnostic sensitivities for the first four weeks post inoculation in OF samples were 88% and 81% for Laboratories A and B, respectively. The mean sensitivity for serum testing from Laboratory A was 89%, which shows very good agreement with OF, thus acting to validate OF as a potential diagnostic medium for PRRSv testing by RT-PCR. No sensitivity value was reported for Laboratory B. This study was then supported by the group carrying out a field validation study, in which OF and serum samples were collected from commercial grower pens at three separate PRRSv infected pig finishing farms (Prickett, 2008a). Pen-based OF samples were collected using the rope method described earlier in the

review, and serum samples were obtained from 5 randomly selected animals per pen at regular intervals from site entry at 3 weeks of age, through to 16 weeks of age. Sera were tested for PRRSv RNA by qRT-PCR and anti-PRRSv antibodies by ELISA. OF samples were tested for PRRSv RNA by qRT-PCR, and also tested for DNA pertaining to Porcine Circovirus type 2 (PCV-2) by qPCR. A relative concordance was found between PRRSv qRT-PCR results for OF and serum, with 77% agreement overall (Prickett, 2008a). However, by considering the samples taken at 8 weeks of age, it was reported that on 2 of the 3 sites, the PRRSv-positive qRT-PCR data for OF and sera showed good agreement. This was not the case for the third site, where 25 of 30 pigs tested qRT-PCR positive for PRRSv in serum at 8 weeks of age, yet the corresponding OF's tested negative (Prickett, 2008a). The accompanying serological data for this site were also negative for anti-PRRSv antibodies. However, this may be expected, since seroconversion usually occurs some time (7-10 days) after the onset of viraemia in the blood. These data provided the first suggestion of inconsistency in the nature of PRRSv testing in OF by qRT-PCR. The qPCR data for PCV-2 in OF revealed infection at all 3 farms, with qPCR positive OF's occurring at two or more sampling time points for all sites (Prickett, 2008a). The authors state that sera were no longer available at the time to enable validation of PCV-2 testing in OF against that in serum.

Another virus that has been targeted for detection in porcine OF by RT-PCR is Influenza A virus (IAV). Fifteen of a total sixteen pen-based OF samples tested positive for IAV by RT-PCR following experimental inoculation of 4-week-old pigs (Detmer *et al.*, 2011). However all OF samples collected from both infected and control animals tested negative by virus isolation for IAV. Conversely, individual nasal swabs that were collected at the same time as OF tested positive for IAV by virus isolation (Detmer *et al.*, 2011). The group then applied their method of IAV analysis to field OF samples from commercial pig units. Around 51% of all OF samples yielded Ct values of less than 40, though it is worth noting that only samples yielding values less than 35 were deemed as positive for IAV, and of these RT-PCR positive samples, 50% tested IAV positive by virus isolation (Detmer *et al.*, 2011). A similar observation was reported by Romagosa *et al*. (2012) for pen-based OFs taken from pigs treated with one of two vaccine types (homologous or heterologous), or controls

(unvaccinated) and exposed to an IAV infected pig following experimental challenge. Here, the sensitivity of RT-PCR for IAV in pen based OF compared to individual nasal swabs was calculated to be 80.4%, with 100% specificity, and of those RT-PCR positive OF samples, 50% yielded positive IAV virus isolation results (Romagosa *et al.*, 2012). For the detection of IAV RNA by RT-PCR, good overall agreement was found between the IAV pen status, and pen-based OF samples, with kappa coefficient $\kappa = 0.82$ (Romagosa *et al.*, 2012). Pen status was declared positive following the occurrence of at least one RT-PCR positive nasal swab within that pen that did not correspond to the IAV challenged animal.

The use of OF to evaluate the distribution of a given pathogen within a population of pigs not directly infected but in contact with, or exposed to infected individuals has not been widely explored. This formed the foundation of the experimental design in the study by Romagosa *et al*. (2012) in order to specifically investigate IAV dynamics, particularly in relation to vaccine use. One additional report documents the use of such an approach to assess the distribution of Erysipelothrix species within groups of pigs. As part of a wider study, bacterial DNA was successfully detected by PCR in OF from pigs kept at the same site as clinically affected pigs at the time of Erysipelas outbreaks in the US (Bender *et al.*, 2010). As well as demonstrating that OF sampling can be used to monitor pathogen dynamics in all pigs, and not just those that are clinically infected, Bender et al. (2010) illustrates PCR confirmation of bacterial as opposed to viral infection, thus highlighting the multimodal potential of molecular OF diagnostics.

A second bacterium for which molecular diagnostics have been adapted for OF is *Actinobacillus pleuropneumoniae* (APP). Experimental infection with three out of seven different APP serovars yielded positive PCR results from OF samples collected from 0 to 7 weeks post inoculation (Costa *et al.*, 2012). Corresponding lung lesions characteristic of APP infection were identified post mortem for animals that were inoculated with two of the serovars which lead to PCR-positive OFs (Costa *et al.*, 2012). The evidence from these last two studies discussed clearly demonstrate the potential for molecular OF diagnostics to expand beyond viruses, and into bacterial disease diagnostics.

1.4.3 *Current state of the art of molecular methods and their application to pig oral fluid*

The most recent publication in relation to PRRSv diagnosis using OF described the collection of samples from pigs before weaning for monitoring of infection on breeding units. Cotton ropes were used to sample 600 litters of young pigs from four commercial sow herds across a four month period. Corresponding sera collected from the dams were used for comparison with the OF results. Of a total of 600 OF samples, only 9 tested positive by RT-PCR for PRRSv when tested at the authors' diagnostic facility, with all corresponding sera testing negative. These samples were then submitted to a second facility which confirmed the findings (Kittawornrat *et al.*, 2014). Six of the RT-PCR positive OF samples were then subjected to PRRSv ORF5 sequencing. This was successful for 2 of the samples, revealing that the pigs were infected with wild-type PRRSv strains that had been evident in the disease history of those units (Kittawornrat *et al.*, 2014). This is the first report of successful PRRSv sequencing from pen-based swine OF samples and thus demonstrates the latest achievement within the research field, with many implications for widespread screening and rapid diagnostic outcomes. The Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) now accepts OF sample submissions on a routine basis, and offers PCR testing for PCV-2, Porcine deltacoronavirus (PDCV), Porcine epidemic diarrhoea virus (PEDv), for which sequencing is also available, and PRRSv. In addition, virus isolation is offered for PRRSv and Swine Influenza virus (SIV). The work documented currently, from the first isolation of PRRSv from oropharyngeal swabs (Wills *et al.*, 1997b) to the most recently mentioned successful ORF5 sequencing (Kittawornrat *et al.*, 2014), illustrates the developmental process involved in OF diagnostic research. In the past year, groups have reported successful detection of Erysipelothrix rhusiopathiae (Giménez-Lirola et al., 2013) and Foot-and-Mouth Disease virus infection (Vosloo *et al.*, 2013) via PCR on OF samples. Furthermore, a study which investigated the probability of detecting Influenza A virus infection in individual nasal swabs compared to pen-based OF samples revealed that RT-PCR testing on OF performed equal to, or better than that on nasal swabs. Ct values yielded for OF were either equal to, or lower than those for the nasal swabs, which the authors propose is indicative of a higher virus concentration in the OF medium (Goodell *et al.*, 2013). Collectively these findings support the successful

development of molecular OF diagnostics for the range of pathogens affecting pigs, and its widespread application across the UK industry.

1.5 Application of oral fluid diagnostics within the UK context

The successful development of OF diagnostics in the US from a pioneering idea to routine surveillance testing has been outlined. Given the extent to which the field has advanced in the US, it may be logical to question the necessity for further developmental research specific to UK pigs. The present section will address this question by considering the differences between US and UK pig husbandry systems, and therefore highlight the need for specific research to assess the feasibility of adapting OF diagnostics for UK systems.

As previously mentioned, in the USA OF is recognised as a sample type for submission to diagnostic laboratories for the conduct of routine disease surveillance. Additionally, standardised protocol guidelines are available for the collection and handling of OF samples for diagnostic purposes (ISU, 2012). These protocols are based upon conventional indoor housed pigs on slatted floors. Pigs housed in this type of system are typically kept in group sizes of around 30 pigs per pen, for which the majority of OF developmental research has been carried out, and sample collection protocols validated (Seddon *et al.*, 2011). Furthermore, the slatted floor systems prevent the accumulation of manure and food waste upon the pen floor, resulting in a sample which should remain relatively “clean” from waste contaminants.

Conversely, in the UK there are a large number of pigs which are kept in straw yard systems which consist of large open-sided buildings, allowing good natural ventilation with the provision of deep straw bedding. Around two thirds of growing pigs in England are kept in such housing systems, according to the Pig and Poultry Farm Practices Survey conducted in 2009 (DEFRA, 2010). These systems allow farmers to operate on a large scale, with the ability to keep up to 400 pigs, and sometimes more, in a single “pen” or population, whilst maintaining high welfare standards via the environmental enrichment provided by the straw. It is a legislative requirement that pigs are provided with enrichment (EU Council Directive 91/630/EEC (as amended)). Often in the intensive conventional indoor systems this is achieved via hanging a toy in the pen for pigs to investigate and chew. Straw bedding however provides sufficient enrichment as pigs spend a large proportion of their active time exerting natural

rooting and nosing behaviours towards the straw. This rooting behaviour, combined with the manure that accumulates within this type of housing system, contributes to a greater potential for contamination of OF samples collected from pigs housed in this way. Straw-based systems are receiving increasing interest in the USA, however the extent to which large populations may affect OF sample collection has not been investigated.

The issue of large group sample representation, along with other knowledge gaps identified within this review, form the basis of the experimental work presented in this thesis, to further develop and optimise OF sample collection, handling and use for the diagnosis of infectious pig disease. Specifically, the aim to optimise OF sample collection in diverse housing systems will be addressed by the use of flavoured rope additives, and manipulation of the number of ropes offered to large groups to investigate the effects of these on OF sample yield and representation. The aim of establishing a sample handling system for shipment to the diagnostic facility will be addressed with the objective of investigating Flinders Technology Associates cards and their ability to store OF samples containing Porcine Reproductive and Respiratory Syndrome virus (PRRSv) RNA at ambient temperature for diagnostic recovery by RT-PCR. Finally, the use of pen OF will be validated against the current Gold Standard blood serum for assessment of PRRS status by collection and comparison of paired pen OF and blood sera from a range of commercial pig farms initially on a one-time basis. A longitudinal study will then provide further information on the suitability of OF as a medium for diagnostic surveillance and monitoring in UK pigs over time. .

Chapter 2: Oral fluid sample collection: approaches to optimise sample yield and representation

2.1 The effects of flavoured rope additives on commercial pen-based oral fluid yield in pigs

2.1.1 Introduction

The potential for the application of oral fluid (OF) diagnostics within the field of swine health is substantial (Prickett *et al.*, 2010). OF comprises a mixture of salivary gland and oral mucosal secretions, the latter component allowing substances to be transferred from the bloodstream into the oral cavity (Aravindha Babu *et al.*, 2012). These can include viruses, bacteria and antibodies raised within the host against infectious agents, and have been shown to be readily detected in swine OF by adapting existing experimental protocols designed for the analysis of blood serum (Kittawornrat *et al.*, 2012a). Increasing interest and finance are being invested in research to develop protocols that are diagnostically reliable, and relatively simple to implement for the farmer. Of particular significance to the pig industry is the use of OF diagnostics to screen large commercial populations of animals at the group level for key disease agents. One of the major advantages of this is the ability to test many animals at one cost, as opposed to the cumulative costs of individual testing. The method is based on collection of an OF sample generated by the group chewing of an offered cotton rope.

Pigs are naturally attracted to engage in chewing behaviour with cotton rope, the absorbent nature of which allows simple collection of OF for diagnostic purposes, which has been well documented (Prickett, 2008a; Prickett, 2008b). In humans it is possible to collect either unstimulated (Dawes, 1972) or stimulated (Neyraud *et al.*, 2011) OF samples. An unstimulated sample involves the subject allowing themselves to passively drool into a collection vessel with no swallowing, jaw or tongue movement, and cannot be reliably replicated in the pig. The OF samples obtained from pigs are therefore regarded as stimulated. Stimulation of the secretion of OF can be achieved either mechanically, by the action of chewing or mastication, or chemically, by introducing a flavour to the mouth. This latter mode of stimulation is also known as gustatory stimulation, as the taste sensation experienced by the subject triggers an increase in salivary flow. Various groups have researched the effects of salivary stimulants on flow

and composition in humans, where salivary flow rates have been reported to increase more than two-fold on stimulation over a 5 minute period (Neyraud *et al.*, 2009). To date, limited work has been done to investigate the potential effects this may have on animals. There are substantial differences between conducting such studies in humans, and in animals, the major barrier being the ability to communicate. Whereas a human can be asked to perform a task i.e. rinse the mouth out, chew an item uniformly for a time period etc. the same cannot be requested of an animal.

From research into human saliva, the strongest chemical stimulant of salivary flow has been found to be citric acid (Streckfus and Bigler, 2002). Furthermore, the effects of different flavours on salivary secretion and composition were investigated by Neyraud et al. in 2009. From this work, it was reported that the use of different flavours to stimulate salivary secretion was unlikely to affect the pH, but may affect the protein concentration in the increased flow (Neyraud *et al.*, 2009).The gustatory route of salivary stimulation in the pig has been briefly touched upon by a group in the US: Kittawornrat et al. (2010) used apple juice containing 50% sucrose, which was allowed to dry onto the rope before being presented to pigs for chewing (Kittawornrat *et al.*, 2010). However this group used flavour as an applied method, i.e. the effect of flavouring compared to no flavouring on salivary yield was not investigated during the study. Nevertheless, the use of flavour by the group to collect OF from pigs suggests that it may be worthwhile to explore the extent to which flavours might be able to influence both attraction to the rope and subsequent OF secretion in pigs. Therefore, the objective of the present study was to investigate the effect of different flavourings added to ropes on the collection yield of pen-based porcine OF samples.

2.1.2 Materials and Methods

2.1.2.1 *Ethical Statement*

Procedures performed on animals and which are documented within this thesis did not fall under the Animals (Scientific Procedures) Act 1986 (ASPA) because they were undertaken as part of clinical diagnostic disease investigation (blood serum samples), or constituted environmental samples (OF samples).

2.1.2.2 *Experimental Design*

This study comprised a replicated Latin Square design, testing 4 flavour treatments across two pig age groups. One replicate of each age group ran in parallel across 4 days. The groups studied were weaner pigs, aged 8 weeks, and finisher pigs, aged 18 weeks. Each replicate involved 4 pens in each of the weaner and finisher houses at Cockle Park Farm, Newcastle University. This was repeated twice using different animals, giving a total of 8 pens each for weaner and finisher pigs.

Table 2.1: Latin Square design illustrating the rope flavour treatment (A: apple, P: pineapple, S: 10% sucrose solution, N: no flavour) presented to each of four pig pens across four trial days

Pen → Day ↓	1	2	3	4
1	A	P	S	N
2	S	A	N	P
3	P	N	A	S
4	N	S	P	A

2.1.2.3 *Animals and Housing*

Commercial cross-bred ((Large White x Landrace) x (Duroc x Pietrain)) pigs (Hermitage Genetics, Kilkenny, Ireland) were used in the study. Entire male and female pigs were housed in pens of mixed, but not matched gender. Although human evidence suggests a difference in stimulated salivary flow rate between males and females, the parameter was also shown to be closely correlated to

body height and weight (Yamamoto *et al.*, 2009). The Latin Square design also removed any possible confound of gender ratio.

Weaner pens measured 3.42m x 1.84m with solid partitions and housed approximately 25 pigs each. Finisher pens measured 3.06m x 3.94m and were partitioned by barred fencing, such that pigs could have visual as well as nose to nose contact. Each pen in the finishing house contained approximately 17 pigs. Both housing types had fully slatted floors and were in temperature controlled rooms. The legislative requirement for enrichment was met by provision of a hanging plastic toy.

2.1.2.4 *Rope Treatment*

Three flavoured additives were investigated in the present study: apple juice (A); pineapple juice (P); and 10% mass by volume sucrose solution (S). The fourth treatment was a control rope with no flavour added (N). The fruit juice flavours used were ASDA “Chosen By You” apple juice from concentrate (1 litre), with acidity regulator (citric acid) and 11.3% sugars, and ASDA “Chosen By You” 100% pure pineapple juice from concentrate (1 litre) with 12.3% sugars, (Asda Stores Ltd, Leeds UK). The sucrose solution was prepared by dissolving 100 grams of granulated sugar into approximately 800 millilitres of water, and making up to 1 litre. Heating was not necessary, as the sugar dissolved relatively quickly with vigorous stirring. 18mm pure natural cotton rope (Outhwaites Ropemakers, Hawes UK) was cut into 4 x 60cm lengths for finishers, and 4 x 80cm lengths for weaners. One of each length of rope was soaked for 60 minutes in apple juice, pineapple juice, sucrose solution or water. Control ropes were soaked in water to control for any textural changes that may have occurred to ropes during the soaking process. After 60 minutes of soaking ropes were removed from their respective solutions and allowed to dry thoroughly overnight at room temperature.

2.1.2.5 *OF Collection*

On each sample collection day, ropes were presented to each pen in the replicate for a 30 minute period between 08:00 and 11:00 AM. Thirty minutes was selected as the sample collection time as previous research has shown that pigs elicit the maximal rope chewing response within the first 30 minutes of

presentation (Seddon *et al.*, 2011). Wooden bars were fixed to the top of each pen division, allowing ropes to be presented without the need for pen entry by the experimenter. Each bar had a hole drilled through the middle, where the non-flavoured end of the rope was threaded through, and secured by tying a knot which rested above the hole. The thickness of the rope ensured that when knotted, pigs could not pull the rope through the hole onto the pen floor. Ropes were positioned such that, when secured, the treatment tip of the rope was suspended approximately 20cm from the weaner pen floor, and approximately 40cm from the finisher pen floor. Timing of presentation commenced when the pigs began to interact with, and bite the rope, which in all cases occurred within a few minutes of presentation. After 30 minutes each rope was removed and placed immediately into a labelled sealable plastic bag and sealed to prevent evaporation losses. Bags were then refrigerated until processing to remove the OF. Most of the fluid was extracted by hand wringing the rope with gloves on, allowing the fluid to run into the sealable bag. One corner of the bag was then cut off, and the OF decanted into a 50ml centrifuge tube before being separated into 1.5ml aliquots using a pipette.

Following hand extraction, strong autoclave bags were opened out and cut into 10cm x 10cm squares. The hand wrung rope was cut into approximately 3cm pieces, and each piece was placed in the centre of a square made from the autoclave bags. The corners were gathered together and holes put into the bottom of the “pouch” using sharp forceps. Each pouch was placed into the top of a 50ml centrifuge tube with the gathered corners folded over the top of the tube and secured with an elastic band to ensure the rope piece remained suspended at the top of the tube during centrifugation. Ropes were spun for 10 minutes at 1500g (2000 rpm) and the OF spun out was transferred into graduated 1.5ml microcentrifuge tubes using a pipette. The rope pieces were sufficiently dry following this step, and were then discarded. The total volume of OF obtained from each rope was recorded.

2.1.2.6 *Rope Absorbance Test*

An experiment was conducted in order to assess the absorbance of the cotton rope used in the present study. Three 80cm lengths of 100% pure natural cotton rope (Outhwaites Ropemakers, Hawes UK) were cut and a portion of each rope

representative of that which pigs would be able to chew was allowed to soak in water for 3 hours with occasional deliberate disturbance of the ropes in order to separate the strands. This was done to simulate the process of the pigs separating the strands during chewing, thus giving the rope its maximal absorbing capacity. After 3 hours each replicate rope was removed from the water and allowed to drip until dripping stopped, then placed into a re-sealable plastic bag. Each rope was wrung out by hand, and the volume of water extracted by hand-wringing was recorded.

2.1.2.7 *Statistical Analysis*

Values for the OF volumes collected per pen and trial day were recorded. Analysis of Variance (ANOVA) was performed using Minitab version 16 (Minitab Ltd. Coventry UK). OF volumes, in millilitres, for weaners and finishers were selected as dependent variables, with rope treatment, pen number and trial day as fixed factors. A two sample t test using pen means from the four collection days was used to assess the effect of pig age, i.e. weaner versus finisher stage, on the volume of OF collected.

2.1.3 Results

All rope presentations in the study resulted in pig interaction, chewing and OF deposition onto the rope. Around 20ml of fluid was obtained from each pen for both weaner and finisher pigs upon each collection, with little variation. Rope treatment, i.e. flavour, was found to have no significant effect on the volume of OF deposited by weaner ($F_{(3,22)}$ =0.01, P=0.998) or finisher ($F_{(3,22)}$=0.53, P=0.668) pigs (Fig. 1).

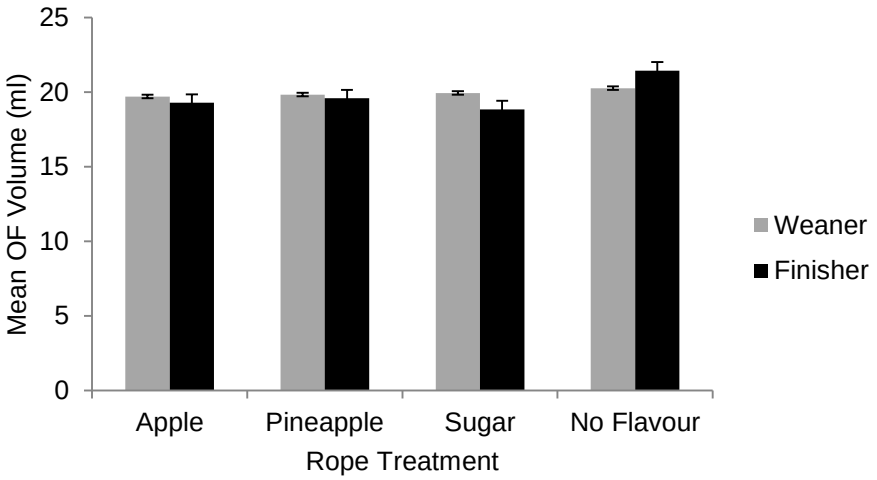

Figure 2.1: The effect of rope flavour treatment on the mean total OF volume ± SEM collected from groups of weaner and finisher pigs during a 30 minute presentation period across 2 replicates. Rope treatment had no effect on OF yield for weaned ($F_{(3,22)}$ =0.01, P=0.998) or finishing ($F_{(3,22)}$=0.53, P=0.668) pigs

Trial day number was found to have a significant effect on the volume of OF yielded from both weaner ($F_{(3,22)}$=3.67, P=0.028) and finisher ($F_{(3,22)}$=6.19, P=0.003) pigs. For the weaners, this corresponded to a decrease in volume collected after trial day 1, followed by consistent volumes being obtained for days 2, 3 and 4. Conversely, the finisher pigs showed a progressive increase in the OF volume collected across the trial days 1 to 4 (Fig. 2). There was no significant effect of pen number for weaner ($F_{(3,22)}$=0.62, P=0.611) or finisher ($F_{(3,22)}$=0.72, P=0.552) pigs.

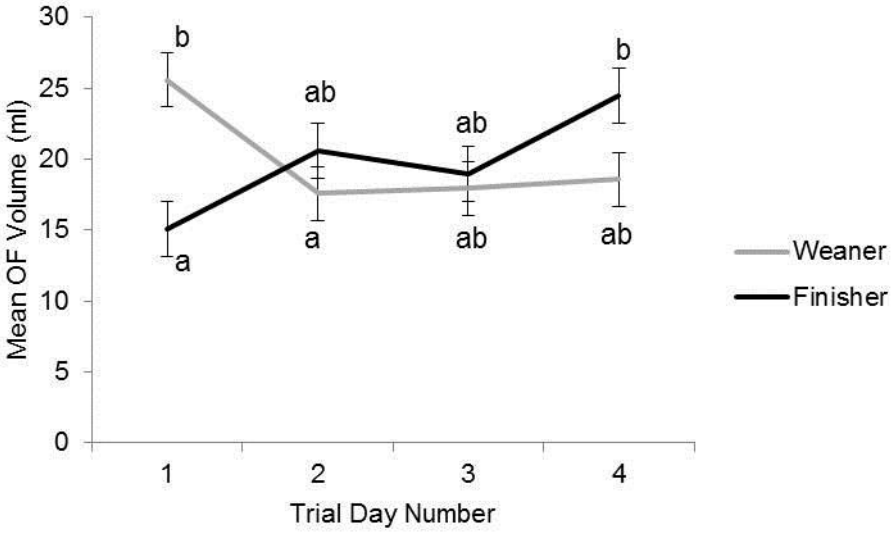

Figure 2.2: The mean total OF volume ± SEM collected from groups of weaner and finisher pigs during a 30 minute presentation period according to consecutive presentation day across 2 replicates. Trial day had a significant effect on OF yield for both weaned ($F_{(3,22)}$=3.67, P=0.028) and finishing ($F_{(3,22)}$=6.19, P=0.003) pigs. Means with different letters differ significantly (P<0.05).

The mean total volume of OF recovered was not found to differ significantly according to pig age (weaners 19.9ml, finishers 19.8ml, SEM 0.7ml, T=0.1, P=0.925).

Of the total volume collected, 68% was obtained by simple hand extraction, with the remaining 32% requiring centrifugation.

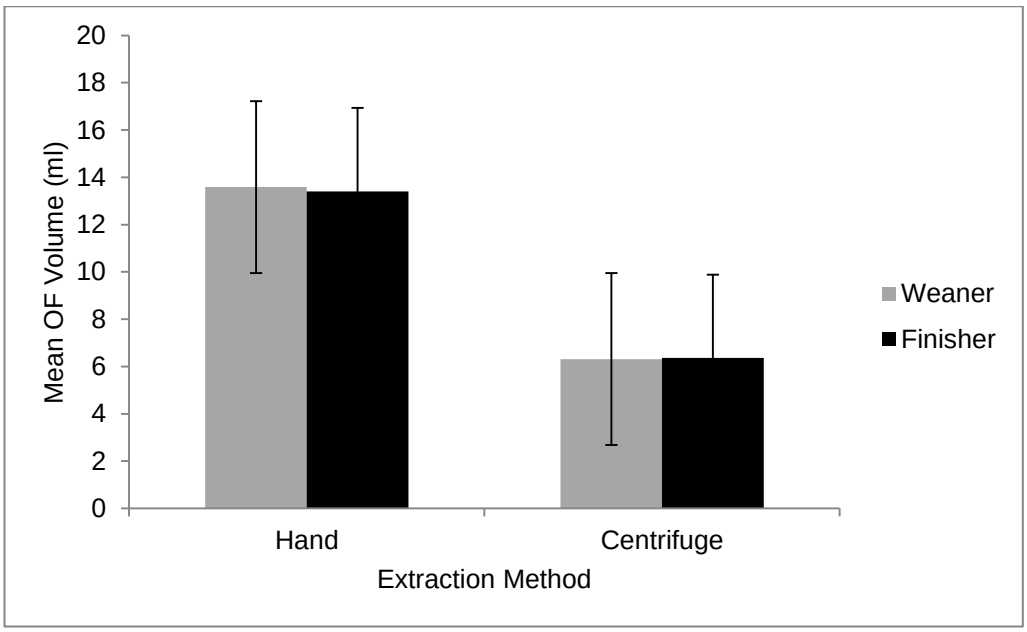

Figure 2.3: The mean OF volume extracted from ropes by either hand wringing or centrifugation after presentation to groups of weaner and finisher pigs for a 30 minute period

The experiment to investigate the absorbance of the rope used in the present study showed that the ropes were capable of absorbing up to 32ml fluid (Table 2.2). Ropes therefore did not become fully saturated during the trial, and so did not impose an artificial ceiling on the treatment comparison.

Table 2.2 Volume of water extracted from three replicate rope lengths following soaking for three hours to assess the maximum absorbance capacity of 100% pure natural cotton rope (Outhwaites Ropemakers, Hawes UK)

Replicate	Volume of water (ml)
1	30
2	28
3	32

2.1.4 Discussion

The collection of oral fluid (OF) samples from pigs involves the provision of absorbent cotton rope for chewing. These samples are deemed as stimulated, as the subjects actively engage with and chew the rope, during which mechanical jaw movements are the stimulus for OF secretion. The routes by

which the secretion of OF can be stimulated are mechanical and chemical / gustatory (Neyraud *et al.*, 2009). Current OF collection methods in pigs rely mainly on mechanical stimulation, however it may be possible for gustatory stimulation, by using flavoured additives, to increase OF yields obtained by the rope method. Additives may also increase the attractiveness of ropes to pigs for chewing, which will be discussed later. Increased OF yields could expand the scope for diagnostic testing, by delivering a larger volume which would allow more diagnostic tests to be carried out from one sample collection. The fruit juices used in the experiment were selected on the basis of their acidic content, combined with their sweet taste. Both acidic (citric acid) and sweet (sucrose) compounds have been shown to increase parotid salivary flow in humans (Neyraud *et al.*, 2009). The sucrose solution was chosen in the present study to measure the ability of sweetness to stimulate OF secretion alone, without citric acid.

2.1.4.1 *Effect of rope treatment on OF yield*

It was anticipated that gustatory stimulation by different flavours may lead to increased salivary secretion, measurable by total OF deposition during cotton rope chewing in pigs. However, the use of flavoured additives on ropes was shown to have no effect on the volume of OF obtained after 30 minute presentations to commercially housed weaner and finisher pigs. There are a number of factors which may contribute to the results obtained in the present study. Probably the most significant point to mention is the partial disagreement that has been found to exist between the neural taste responses in humans and pigs (Danilova *et al.*, 1999; Roura *et al.*, 2008). The neural taste response refers to the pathway by which stimulation of taste receptors on the tongue leads to a response, such as increased salivary flow in the oral cavity. There is evidence to suggest that pigs are able to distinguish between nutritive and non-nutritive sweet compounds. Day et al. (1996) reported that pigs were more inclined to chew on a length of tubing that dispensed sucrose solution than a tube that dispensed saccharin solution, or water (Day *et al.*, 1996). This paper shows that pigs are able to perceive sweet taste from both nutritive and non-nutritive compounds, and can distinguish between their rewarding, or non-rewarding properties, respectively. Pigs have been shown to be unable to perceive sweet taste when presented with other non-nutritive sweet compounds such as the

widely used artificial sweetener in human food and drink products, aspartame. On presentation of aspartame to the pig, no neural response associated with sweet taste perception could be observed (Danilova *et al.*, 1999). It has been proposed that taste receptor amino acid (AA) sequences are likely to differ between species (Roura *et al.*, 2008). Such diversity could account for the differential neural responses to the compound aspartame reported between pigs and humans, and also the lack of effect of the flavourings used in the present study on pig OF deposition. The number of pigs per pen differed for weaned (n=25) and finishing (n=17) pigs. It is possible that due to there being slightly fewer finishing pigs per pen than weaned, the larger volume of OF that is expected to be produced by finishing pigs could not be observed.

This study investigated the effects of sweet or palatable compounds on pig OF sampling ropes in line with evidence from human studies, proposing that salivary secretion may be stimulated by a gustatory pathway (Neyraud *et al.*, 2009). Other studies which have focussed on the behavioural issue of tail biting in pigs have compared chewing behaviour directed towards cotton ropes soaked in blood, sodium chloride (salt) solution or water (Fraser, 1987; McIntyre, 2002; Jankevicius and Widowski, 2003). Significantly larger chewing responses to blood soaked ropes as opposed to plain, salt or water soaked ropes have been consistently reported by all groups, indicating a taste preference for whole blood over salt solution. Although the purpose of these studies, i.e. investigation of tail biting, is not in line with that of the present study, they suggest that a salt flavour preference, and therefore increased attraction to ropes by salt in the pig is unlikely.

It may be that the pigs' motivation to interact with the rope is independent of any flavoured additives, and that the presentation of the rope itself to a group is a sufficient stimulus to attract animals to chew (Trickett *et al.*, 2009; van de Weerd and Day, 2009). This appears to be the case considering the results of the present study, which indicate that pigs motivated to chew ropes will do so irrespective of any additives used. There is also evidence to suggest an environmental influence on pig motivation to engage in rope chewing or not. Pigs housed in fully slatted floor pens were found to be more likely to chew ropes than those housed in straw kennels (Seddon *et al.*, 2011), most likely due

to the enrichment provided to pigs by straw bedding for expression of natural exploratory behaviours, resulting in less interest in cotton ropes.

2.1.4.2 *Effect of trial day on OF yield*

Trial day was found to have a significant effect on OF yield for both weaner and finisher pigs. The importance of novelty of enrichment objects for weaner pigs has been reported previously (Trickett *et al.*, 2009). Although the flavours of the ropes changed on each trial day, the object itself, i.e. the rope, was the same, and so decreasing novelty may explain the diminishing interest observed over the experimental period for the weaners in the present study. Additionally, the role of novelty for finisher pig enrichment objects has been illustrated. Significantly increased object interaction was recorded on the first observation day, compared to subsequent days with each of seven different enrichment items (Van De Perre *et al.*, 2011). The results obtained in the present study do not conform to this and, as mentioned, appear to reflect an increasing interest in the ropes as the 4 trial days progressed. One difference to note between the two studies is that Van De Perre et al. operated a chain system, where the chains were suspended into the pens for the entire experimental duration. The group would then add or remove the specific enrichment object for each presentation accordingly. The continuous chain presence might therefore detract from the novelty of the objects being added. Alternatively, the current study presented ropes for only 30 min per trial day, leaving no rope hanging between presentations and the daily removal of the ropes following presentation may have provided sufficient novelty to elicit increasing interest over the four days.

A combination of extraction techniques were used in the study to yield the OF from the cotton rope following chewing. Wringing the rope by hand was implemented initially to extract OF, in line with other studies that have used the cotton rope method of OF sampling in pigs (Prickett, 2008a; Prickett, 2008b; Prickett *et al.*, 2011). The method was successful in extracting the majority of OF from the rope. The centrifugation method was then able to extract the remaining fluid that could not be removed by the force of hand wringing to provide a more objective final total for OF deposition. This was an effective method, able to extract approximately a further 50% of the hand-wrung volume

out of the rope following the wringing process. However, it is anticipated that for the practical application of the rope method of OF collection for disease diagnostics on farm, centrifugation of ropes is unnecessary. The addition of fruit juice / sucrose flavouring to cotton ropes did not appear to influence OF yield or attraction of pigs to chew. It is important to note that any potential effects of rope additives on the recovery of diagnostic targets would need to be investigated before including flavoured additives in sampling protocols for disease monitoring.

2.1.5 Conclusion

The results obtained in the present study suggest that adding flavours to ropes for porcine OF sampling will not affect OF yield via greater attraction to chew or salivary stimulation. They concur with other studies to support the ease of collection and extraction of diagnostically relevant sample volumes of porcine OF.

2.2 The provision of multiple ropes to large pig populations and the effects on oral fluid sample representation

2.2.1 Introduction

The numerous advantages of oral fluid (OF) as an alternative diagnostic medium to blood serum are driving increasing research interest into the field. OF can be easily collected non-invasively by providing pigs with a length of absorbent cotton rope to chew and thus deposit their OF (Prickett, 2008b). The fluid can then be wrung out of the rope by hand for subsequent laboratory analysis for a range of diagnostic targets. At present in the US, OF is used for the routine diagnosis and monitoring of a number of key infectious pig agents such as Porcine Reproductive and Respiratory Syndrome virus (PRRSv), Porcine Circovirus Type 2 (PCV-2), *Mycoplasma hyopneumoniae* and Swine influenza virus, and is well recognised by both producers and veterinary practitioners. Additionally, an OF assay was successfully developed for the detection of Porcine Epidemic Diarrhoea virus (PEDv) during the US outbreak in 2012 (Burrough, 2014), demonstrating the potential for the use of OF with new emerging diseases as well as those which are well characterised.

The collection of an OF sample consists of the provision of a length of cotton rope to the group of pigs in order to gain a sample that is representative of that population by allowing as many pigs as possible to willingly approach the rope and engage in chewing behaviours. Ropes can be suspended from customised steel brackets designed to fix to the pen side, with a hole to allow the provision of the rope. Standardised protocols for the collection of OF for diagnostic purposes have been developed and published in the US, stating that 30 minutes of rope presentation to a group of 30 or fewer growing pigs is sufficient to collect a representative pen-based sample (ISU, 2012). This was then confirmed by a UK study which manipulated the rope presentation time to pigs kept on fully slatted floor systems or in straw kennels. 30 minutes was found to be the time period required to generate maximal pig chewing behaviour with no significant change in the pigs' response elicited by extending the presentation period (Seddon *et al.*, 2011). The study did however highlight a significant difference in pigs' chewing behaviour when kept in straw kennels as opposed to fully slatted floor accommodation. Pen representation, expressed as the percentage of pigs in the population to engage in rope chewing behaviour for more than 20 seconds within a 60 minute period was found to be 95% for fully slatted floor housing, whereas this figure was found to be 79% for pigs kept in straw kennels. The environmental enrichment provided by the straw in the kennel housing was postulated as the likely cause for the decreased response observed (Seddon *et al.*, 2011). The information yielded by this study supports the protocols currently in practice in the US, but only when population sizes are 30 pigs per pen or fewer.

In the UK there are a large number of outdoor and straw-based pig production systems (DEFRA, 2010). The latter in particular allow commercial scale production (up to 400 weaned / 100 finishing pigs per pen) whilst maintaining high animal welfare standards. Controlled ventilation and heating, and their associated costs, are exchanged for large open-sided buildings and deep straw bedding. Such systems comprise around 65% of the feeder pigs in England (DEFRA, 2010), and interest towards these extensive, as opposed to intensive, pig husbandry methods is growing in the US (Honeyman, 2005). These diverse housing types must be taken into account, firstly for the successful initial

application of OF diagnostics within the UK and, secondly, for the improvement of existing US protocols as its pig industry evolves.

In the present study, representation will be assessed in terms of the number of pigs to contribute to the pen-based OF sample. This is highly important for diagnostic application, as disease prevalence varies widely and therefore the number of animals sampled is crucial in the detection of a given pathogen. Ideally, biological sampling protocols should be adaptable to different circulating disease prevalence and thus investigations into ways in which OF sampling might be suitable for this are necessary.

OF sampling from large populations, i.e. more than 30 pigs per pen, has not been investigated. This, plus the straw enrichment issues mentioned earlier, form the basis of the questions to be addressed in the present study. The experiment was designed to determine: 1) how representative of a large population is an OF sample collected using only one rope?, and 2) can the level of pen representation be manipulated by offering pigs more than one rope to engage in chewing behaviour? More specifically, the study aimed to produce recommendations for optimal rope:pig ratios for effective OF sampling for disease diagnostics in commercial practice using large group, straw-based housing systems.

2.2.2 Materials and Methods

2.2.2.1 *Experimental Design*

This study presented either 1, 2, 4 or 8 cotton ropes to each of 4 pens of commercial pigs according to a Latin Square design (Table 2.3). The trial was conducted using finishing pigs initially and then repeated using weaned pigs.

Table 2.3: Latin Square design showing the number of ropes to be presented to each of four pens of pigs on each of four separate occasions

Pen → Presentation ↓	1	2	3	4
1	1	2	4	8
2	8	4	2	1
3	2	8	1	4
4	4	1	8	2

2.2.2.2 *Animals and Housing*

The study was conducted at Grange Hill Farm, Bishop Auckland, UK. Groups of 10 week old weaned, or 24 week old finisher, commercial crossbred pigs (Large White x Landrace x Pietrain) of mixed but not matched gender were used. Pigs were housed in large open-sided sheds (n= 150-200 per group for weaners, n=80-100 per group for finishers) with automatic feeders and drinking troughs. Deep straw bedding was provided for the pigs and replenished each day.

A subset (~25%) of the population of each pen used for the study was selected at random and individual pigs marked using coloured spray markers. Each marked pig had an individual coloured marking code, allowing clear identification and distinction from all other marked pigs. Pigs were marked on the day before the first rope presentation to remove any potential influence of handling stress. A labelling code was established for marked pigs to enable quick recording during the observation of rope presentations. Chewing behaviours were then recorded for the marked pigs to serve as a representation of the total population.

2.2.2.3 *Experimental Protocol*

100% pure natural cotton rope (Outhwaites Ropemakers, Hawes UK) was cut into 80cm lengths for presentation to the pigs. Ropes were suspended from custom-made steel brackets designed to fix onto pen dividers with a hole large enough to thread the rope through and tie a knot to prevent the pigs from pulling it completely through. When multiple ropes were being provided to pens, approximately 40cm space was allowed between each rope. Presentations

were timed for 30 minutes from the first pig-rope chewing interaction, during which time a team of three observers recorded the colour code of each marked pig to chew a rope. At the end of the presentation period, ropes were removed and placed into re-sealable plastic bags. The oral fluid (OF) deposited onto the ropes by the pigs was extracted by hand-wringing, allowing the fluids to collect at the bottom of the bag, then cutting the corner off to decant the fluid into a 50ml centrifuge tube. The approximate volume of OF in millilitres (ml) was recorded for each rope.

2.2.2.4 *Statistical Analysis*

Minitab version 16 software was used to investigate the effects of providing multiple ropes on the number of marked pigs to engage in rope chewing behaviour. OF yield was used as the dependent variable in an Analysis of Variance (ANOVA), with rope number, day and pen number as factors. Pairwise comparisons of the different rope treatments using Tukey's test were performed to investigate the significance of each step increase in the number of ropes offered to pigs.

2.2.3 Results

All rope presentations resulted in pigs engaging in chewing activity and in most cases where multiple ropes were presented, all ropes were investigated by the pigs such that a sample of oral fluid could be extracted.

The provision of a single rope to weaned pigs led to a mean of 42% of the marked population chewing that rope in a 30 minute period. This increased slightly to 46% with the provision of a second rope for chewing. Four ropes generated 58% of the marked pigs chewing and, finally, the provision of 8 ropes lead to 74% marked pigs engaging in chewing behaviour towards ropes within a 30 minute period (Fig 2.4). The effect of increasing the number of ropes on the number of pigs to engage in rope chewing was confirmed statistically significant by ANOVA ($F_{(3,6)}$ =13.93, P=0.004). Conversely the same effect could not be shown for the finishing pigs. One rope resulted in a mean of 50% marked pigs chewing, 45% when 2 ropes were provided, 54% with four ropes, and 45% when 8 ropes were presented. Therefore, providing multiple ropes to finishers

had no effect on the number of pigs to engage in rope chewing ($F_{(3,6)}$ = 0.16, P = 0.916).

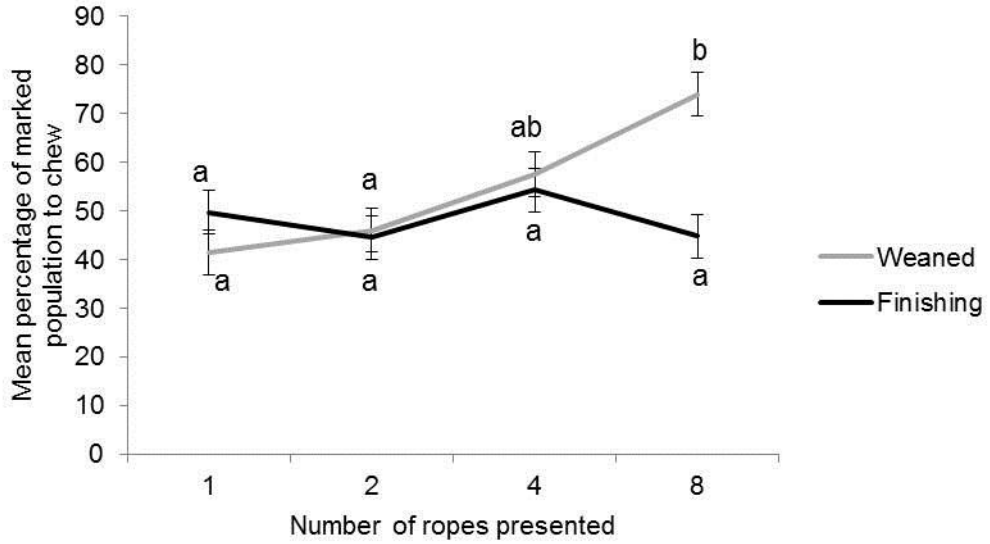

Figure 2.4 Mean percentage of the marked population (±SEM) of weaned and finishing pigs to engage in rope chewing when 1, 2, 4 or 8 cotton ropes were offered. Provision of multiple ropes increased the number of weaned ($F_{(3,6)}$ =13.93, P=0.004) but not finishing ($F_{(3,6)}$ = 0.16, P = 0.916) pigs to chew ropes. Means with different letters differ significantly (P<0.05).

The total volume of OF extracted from ropes presented to pigs in the study increased when more ropes were offered. ANOVA confirmed this to be true for both weaned ($F_{(3,6)}$ = 13.54, P = 0.004) and finishing ($F_{(3,6)}$ = 10.47, P = 0.008) pigs. Mean ± SEM total volumes extracted when only a single rope was offered to weaned and finishing pigs were 14 ± 5.04ml and 21 ± 4.13ml, respectively. This increased to 70 ± 11.6ml for weaned and 53 ± 11.7ml for finishing pigs when the maximum 8 ropes were offered (Fig 2.5).

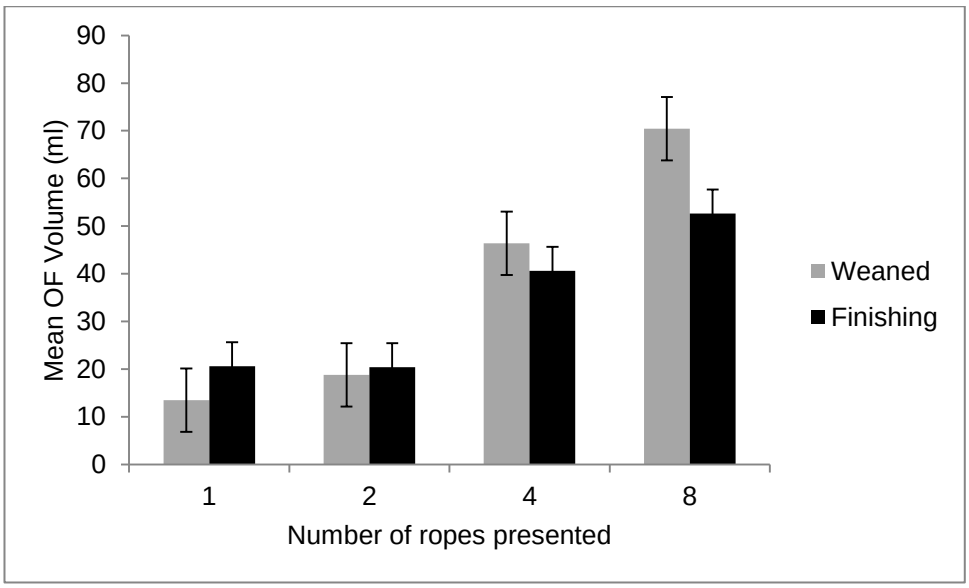

Figure 2.5 Mean volume of OF collected (±SEM) from weaned and finishing pigs when 1, 2, 4 or 8 cotton ropes were offered. Increasing the number of ropes offered lead to significantly increased OF yields in weaned ($F_{(3,6)}$ = 13.54, P = 0.004) and finishing ($F_{(3,6)}$ = 10.47, P = 0.008) pigs

For weaned pigs, the offering of a single rope generated the greatest variability in the volume of OF extracted from a rope compared to when 2, 4 or 8 were offered (Figure 2.6). The greatest variability in the volume of OF extracted from individual ropes presented to finisher pigs was recorded when 4 ropes were offered (Figure 2.7).

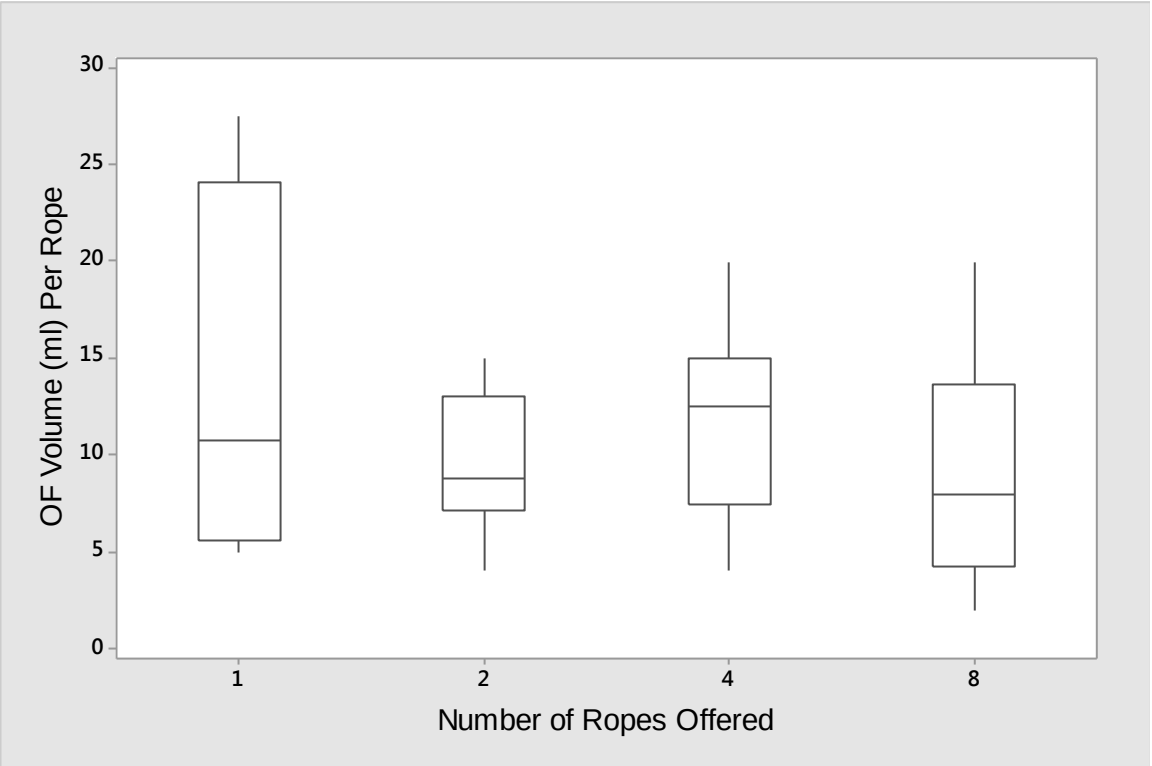

Figure 2.6: Box and whisker plot showing the variation in OF volume extracted from individual ropes according to the number of ropes offered to weaned pigs

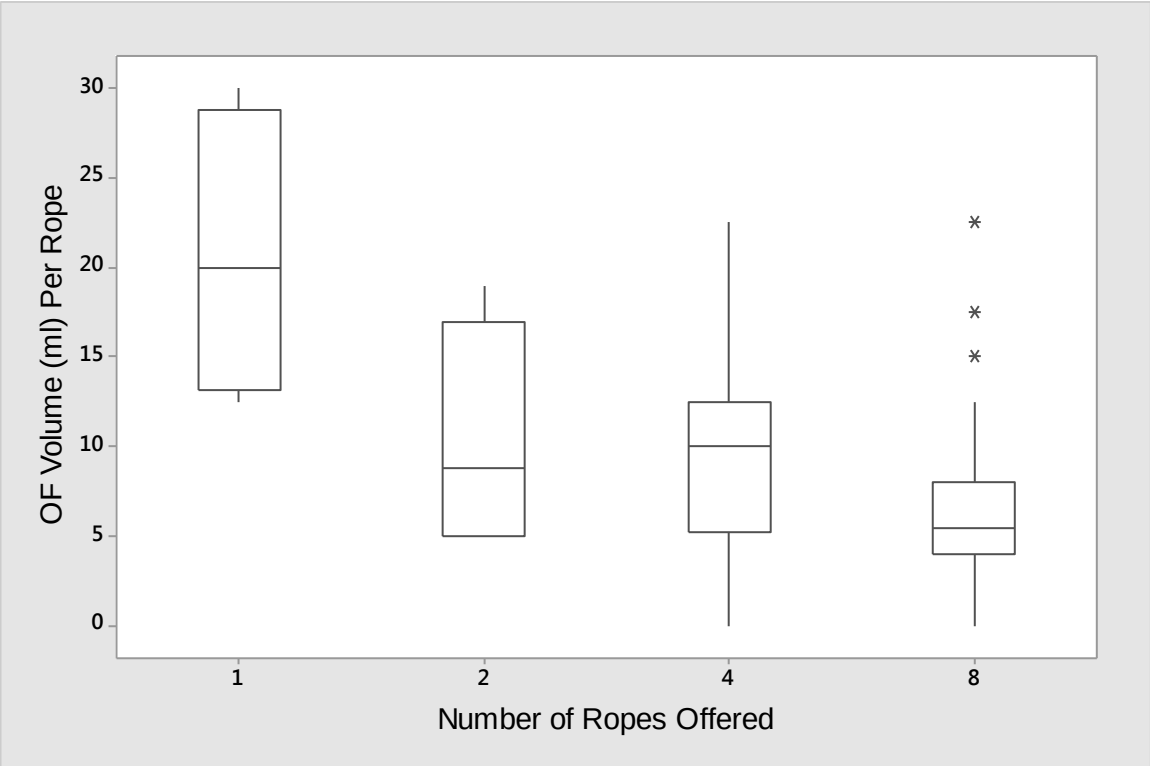

Figure 2.7: Box and whisker plot showing the variation in OF volume extracted from individual ropes according to the number of ropes offered to finisher pigs

The greatest chewing response observed for weaned pigs occurred when 8 ropes were offered, resulting in 74% of the marked pigs chewing. Tukey's

pairwise comparisons of the mean numbers of marked pigs to chew according to the number of ropes offered revealed that the representation achieved by providing 8 ropes was significantly higher than that when 1 or 2 ropes were provided, but not when 4 ropes were provided. In finishing pigs the greatest chewing response occurred when 4 ropes were offered, however the number of ropes offered to pigs did not significantly affect sample representation when pairwise comparisons using Tukey's method were performed.

2.2.4 Discussion

The present study aimed to investigate the representativeness of OF samples collected from large pig populations kept in straw-based housing and the potential manipulation of this by changing the number of ropes offered to pigs for chewing. The establishment of an optimal rope:pig ratio for use in diverse housing systems would allow efficient and representative sample collection, thus aiding the application of OF diagnostics to UK pigs.

2.2.4.1 *Effect of multiple ropes on the number of pigs to engage in chewing behaviour*

In the present study, the number of ropes offered to pigs had a significant effect on weaned, but not finishing pig chewing behaviour. Increased numbers of weaned pigs engaging with ropes were observed as the number of ropes provided was increased. Seventy four percent of marked weaned pigs engaged in rope chewing when the maximum 8 ropes were offered. This response might be expected from pigs of this age, as weaned pigs show the lowest levels of pen inactivity compared to suckling and growing pigs (Docking *et al.*, 2008), suggesting that they may have increased motivation to explore and engage in rope chewing. This study also monitored the synchronisation of behaviour in pigs towards different objects provided as enrichment "toys", and found synchrony of behaviour to be higher in younger pigs, diminishing with age (Docking *et al.*, 2008). This finding could aid the explanation of the different responses to multiple ropes observed in the present study for the weaned and finishing pigs. Synchrony, also known as social facilitation refers to the tendency of pigs to engage in certain behaviours simultaneously, often giving rise to social competition. This has been well characterised by studies which have investigated feeding behaviour within groups of pigs (Young and

Lawrence, 1994; Nyachoti *et al.*, 2004). It may be that increased behavioural synchronisation in weaned pigs could lead to increased competition, which can be relieved by the provision of multiple ropes, allowing less dominant pigs to chew rather than their access to ropes being prevented by more dominant pigs.

Another aspect of the study which must be considered, particularly for the finishers, is the use of straw bedding. Finishing pigs have been found to show behavioural preferences towards straw bedding when either singular (Scott *et al.*, 2006), or multiple chewable hanging toys have been provided (Scott *et al.*, 2007). Furthermore, increased chewing interactions with straw have been reported for finishing pigs kept in slatted floor pens when multiple racks of straw have been provided, compared to a single rack (Zwicker *et al.*, 2012). This study shows that finishing pigs can respond positively to increased access to enrichment objects, but in the case of chewable hanging toys, straw bedding may confound the behavioural effects of providing such objects.

2.2.4.2 *Effect of multiple ropes on OF yield*

Provision of multiple ropes resulted in increased OF yields for both weaned and finishing pigs. All OF volumes recorded from each rope in the study did not exceed the maximum rope absorbing capacity of 32ml (Section 2.1.3 of this Chapter), therefore the use of this method to measure OF yield was valid.

For weaned pigs, as the number of ropes offered increased, the number of pigs to chew ropes increased, as did the resultant OF yield. From this, the casual assumption that OF yield may be dependent upon the number of animals to contribute to that sample could be derived. However, the finisher pig data contradict this assumption. The percentage of the marked pig population to chew ropes when 8 were offered was lower than that when 4 ropes were offered, yet the volume of OF yielded from the offering of 8 ropes was higher than that for 4 ropes. This is indicative of yield not depending upon the number of animals to contribute. The increased opportunity to chew ropes presented to pigs via the provision of increased numbers of ropes may result in pigs being able to spend more time chewing, it could therefore be the duration of chewing that determines OF sample yield as opposed to the number of contributing pigs. A study mentioned previously found that the provision of up to 4 ropes per small pen (n<30 pigs) did not affect the mean percentage of pigs to chew ropes, but

significantly affected the time spent chewing per pig (Seddon *et al.*, 2011). This finding supports the hypothesis that the time spent chewing rope per pig is a stronger determinant of OF yield than the number of pigs to contribute to a sample.

2.2.4.3 *Interpretation within the diagnostic context and implications*

The extent to which a biological sample represents the population or system from which it is taken is crucial for the diagnosis of infectious disease. In the present study, pen-based OF sample representation was assessed by considering the percentage of the given pen population that contributed to the OF sample. At present in the UK, the Gold Standard medium for diagnostic testing is blood serum. Standardised blood sample collection protocols exist for the detection of specific disease markers and these protocols can be modified for diseases which circulate at different prevalence. However, in most cases the representation of a population does not exceed 10% when blood sampling is used for disease diagnosis on farm based upon 95% confidence of detecting disease when prevalence is $\leq$40%.

The data presented in this study show that pen-based OF sampling allows much greater representation of a population than normal blood sampling protocols, in a simple to implement and non-invasive manner. The lowest mean percentage of marked pigs to engage in rope chewing was 42%: this exceeds the representation given by typical blood sampling protocols by four times. It could therefore be postulated that the increased number of individuals that can be sampled by OF testing may give greater potential for the detection of pathogens that may be circulating at low prevalence within populations. This would need to be further validated before OF testing could be applied to larger pig populations. Replicated studies of similar design to that presented here are necessary, ideally with a large scale individual identification method such as radio frequency identification (RFID) tagging for exact quantitation of the numbers of pigs engaging with ropes. This could also be accompanied by video recordings to take into account the time spent chewing rope, as this seems to be involved in OF sample yield. Finally, experimental infections would need to be conducted in order to gauge the sensitivity of pen-based OF sampling in

larger populations, particularly using the multiple rope approach at different disease prevalence.

2.2.5 Conclusion

For large populations (n=150-200 pigs per pen) of weaned pigs housed in straw-based systems, a ratio of 1 rope per 18--50 pigs generated optimal pen representation. Large populations (n=80-100 pigs per pen) of finishing pigs housed in straw-based systems generated maximal pen representation when 4 ropes were presented. This equates to a ratio of 1 rope per 20-25 pigs, yet representation was not found to differ significantly when rope:pig ratio was reduced to 1:80-100.

Although the number of pigs to contribute to an OF sample does not seem to affect sample yield, it is crucial for obtaining representative samples of diagnostic quality. A multiple rope approach should therefore be considered for OF sampling in larger pig populations, however, the sensitivity of this sampling method for disease diagnostics, in terms of the number of ropes required to detect disease circulating at variable prevalence, remains to be tested.

Chapter 3: Ambient temperature storage of pig oral fluid samples for the diagnosis of Porcine Reproductive and Respiratory Syndrome virus (PRRSv)

3.1 Introduction

Increasing global demands for food are continuously exerting pressure on farmers to maximise productivity. Herd health status has a pivotal role in the productivity of a livestock system, and can have significant financial implications for the farmer. Accurate, early diagnosis of infectious disease in livestock is imperative in the effective control of pathogens and, in particular, pathogens that exist endemically within herds causing subclinical disease, which can substantially decrease productivity (Jacobson *et al.*, 1999; Alarcon *et al.*, 2013). In UK pigs, Porcine Reproductive and Respiratory Syndrome virus (PRRSv) is one such endemic disease responsible for premature abortion in gestating sows as well as respiratory ailment in growing pigs. Diagnostic tests for PRRSv at present usually involve blood sample collection from individual animals by veterinarians, followed by laboratory analysis for confirmation of disease status, which can be costly to the farmer in terms of veterinary costs, laboratory costs and labour. The potential of oral fluid (OF), collected by providing cotton ropes to groups of pigs to chew, as a diagnostic medium has been well documented (Prickett, 2008b; Prickett, 2008a; Prickett *et al.*, 2011; Romagosa *et al.*, 2011; Ramirez *et al.*, 2012). The non-invasive nature of OF collection offers farmers the ability to collect samples themselves to give a group/herd level disease status.

OF is a complex matrix composed of salivary secretions and mucosal transudates such as gingival crevicular fluid (De Almeida *et al.*, 2008), as well as a high digestive enzyme content. The degradation of viral RNA of PRRS in swine OF stored at ambient temperatures has been previously demonstrated (Prickett *et al.*, 2010). In the same study, RNA was successfully recovered from samples that had been stored at lower temperatures, and thus farmers are advised to chill OF samples immediately following extraction from rope and maintain a cold chain during shipment to the diagnostic laboratory. Although this is an improvement from the veterinary visits / individual bleeding process, OF samples must be transported under chilled packaging conditions, and in most cases sent by courier to guarantee next day delivery to the lab facility, thereby incurring a significant cost and some inconvenience. A method to store OF

samples at ambient temperature whilst maintaining diagnostic integrity would remove the requirement for chilling during shipping and facilitate prolonged storage intervals, thus improving the practicality of farmer-driven diagnostic sample collection and disease surveillance.

Flinders Technology Associates (FTA) cards (GE Healthcare Life Sciences, Buckinghamshire, UK) are filter paper cards treated with a patented formula for safe storage of biological samples for molecular applications at ambient temperatures. The cards offer a safe means of collecting and transporting biological samples whilst maintaining diagnostic integrity. When a sample becomes embedded into the FTA card matrix it is rendered non-infectious, therefore allowing shipment through the standard postal system. At present, the majority of applications involving FTA cards are within the human medical field in countries with hot climates and/or limited sample processing facilities (Kato *et al.*, 2011; Phongsavan *et al.*, 2012). The potential for veterinary applications has not been widely explored, but could provide a means for livestock farmers to begin to collect and safely transport their own biological samples for disease diagnostics. Furthermore, samples stored on the cards can be analysed by molecular methods such as RT-PCR for identification of viraemic animals in real time. The manufacturer's guidelines for FTA cards state that animal blood / tissue samples can be stored at ambient temperatures for reliable RNA recovery by RT-PCR for up to 8 weeks (GE, 2010). Such storing capacity could be of great benefit during investigation of infection at either clinical or sub-clinical levels, allowing farmers and vets to track pathogen dynamics throughout pig herds and flows without the need for extensive cross sectional bleeding.

The present study aimed to investigate the ability of FTA cards to store swine OF spiked with PRRS virus at ambient temperature, in line with the manufacturer's claim of long term sample storage for successful RNA recovery. Four weeks was selected as a suitable test period for OF due to the known RNA stability issues associated with ambient temperature storage (Prickett *et al.*, 2010). The use of the whole sample spot for downstream processing was introduced and compared with the original punch protocol, where only 2 x 2mm punches are taken from the card sample spot for processing (Inoue *et al.*, 2007) in order to investigate the possibility to increase the sensitivity of the method.

3.2 Materials and Methods

3.2.1 *Experimental Design*

The present study was a 2 x 3 x 7 factorial design. Two FTA card extraction methods, i.e. 2 x 2mm card punches as currently recommended by the manufacturer and utilised for extraction of viral RNA from tissue FTA samples, versus the whole sample area, were assessed for optimal PRRSv RNA recovery as a function of time (Figure 3.1). Three PRRSv concentrations ($10^{3.5}$, $10^{2.5}$ and $10^{1.5}$ $TCID_{50}$/ml based on a ten-fold dilution series) were investigated based on previous work conducted by the Animal Health and Veterinary Laboratories Agency (AHVLA, UK) (Strugnell, 2010). Samples were removed from room temperature storage for RNA extraction at each of seven time points across a period of 28 days, in order to test the ambient temperature storage of RNA for up to 4 weeks. Each experimental condition was run in triplicate.

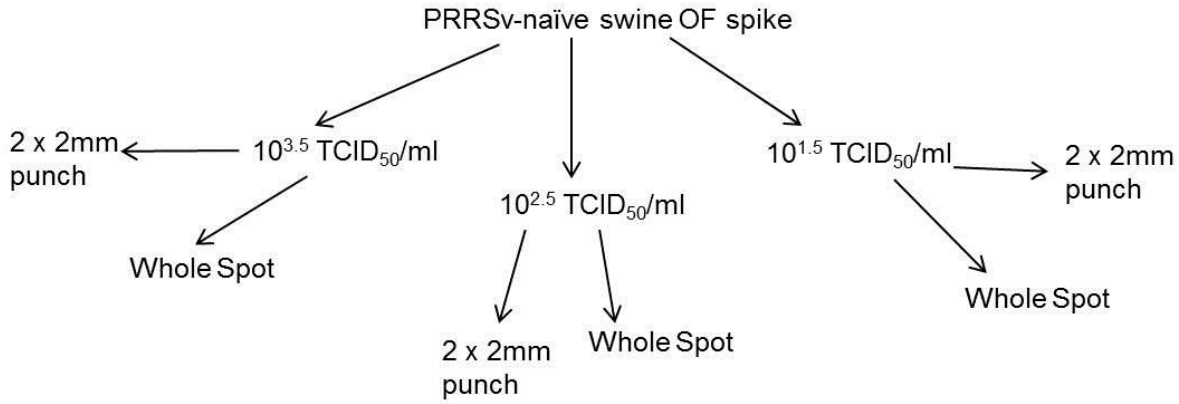

Figure 3.1: Diagram to illustrate the virus concentrations and FTA card processing methods used in the experimental design

3.2.2 *Oral Fluid Collection*

Oral fluid (OF) for the present study was collected by presentation for 30 minutes of an 80cm length of natural cotton rope (Outhwaites Ropemakers, Hawes, UK) to a pen of 25 eight-week old commercial weaned pigs. The pigs were PRRSv-negative and all OF samples tested negative for PRRSv by RT-PCR prior to spiking with the virus agent. Timing of the presentation period commenced when the first biting interaction with the rope was observed and, at

the end of the period, the wet portion of rope was cut off and sealed into a plastic bag. OF was extracted by hand wringing the wet rope inside the bag, allowing the fluid to drain to the bottom. A corner was then cut from the plastic bag to decant approximately 25ml of OF into a 50ml centrifuge tube.

3.2.3 *PRRSv Dilution Series*

A 10-fold serial dilution was set up using the unprocessed OF to dilute a European strain (H2) of PRRSv culture supernatant, $TCID_{50}$ = $1x10^{5.5}$/ml (Drew, 1995). 600µl virus culture supernatant was mixed with 5400µl swine OF to give the virus concentration $1x10^{4.5}$/ml, 600µl was then taken from this and added to 5400µl swine OF to give the $1x10^{3.5}$/ml dilution. The process was repeated until the final tube of swine OF had been spiked at the virus concentration $TCID_{50}$ = $1x10^{1.5}$/ml, as this was the dilution at which PRRSv sensitivity was lost in the previous UK work and thus demonstrating the assay's limit of detection (Strugnell, 2010).

3.2.4 *FTA Card inoculation and storage*

FTA Mini cards (GE Healthcare Life Sciences, Buckinghamshire, UK) with two sample spots were inoculated with the PRRSv OF dilutions. Both sample spots were inoculated with 100µl PRRSv OF dilution, and the cards were then labelled with the dilution factor. A total of 21 FTA Mini cards were inoculated for each of the PRRSv OF dilutions. In addition, 100µl swine OF that had not been spiked with PRRSv was inoculated onto one sample spot of each of a further 7 FTA Mini cards. These cards were labelled as no template control (NTC) and negative card control (NCC) and would confirm 1) the PRRS-negative status of the pigs used for OF sample collection and 2) that the FTA cards were not contaminated prior to inoculation. Cards were allowed to dry thoroughly at room temperature (18-25°C), and were then stored in a cardboard box at the same temperature until removal for RNA extraction.

3.2.5 *FTA Card processing and RNA extraction*

On each of 0, 1, 3, 7, 14, 21 and 28 days post card inoculation (DPCI) a total of 10 FTA cards were removed from storage. Three cards were taken for each PRRS spike concentration ($1x10^{3.5}$, $1x10^{2.5}$, $1x10^{1.5}$), plus one negative control card embedded with non-spiked OF. For each of the nine cards embedded with

PRRS-spiked OF, a 2mm Harris Micropunch (GE Healthcare Life Sciences, Buckinghamshire, UK) was used to take 2 punches from the centre of the left-hand sample spot, and these were placed immediately into labelled 1.5ml microcentrifuge tubes. The right-hand sample spot was cut entirely from the card using a pair of scissors and placed into a single well of a 6-well tissue culture plate. The Harris micropunch, scissors and the forceps used to transfer the cut sample spots into the tissue culture plates were washed using 70% ethanol and dried thoroughly between samples. For the negative control cards, only whole cut sample spots were processed and analysed in order to ensure that no part of the card sample areas could be contaminated and to give the greatest chance of detecting any PRRSv in the non-spiked OF.

For the 2mm punch samples, 675µl RNA Rapid Extraction Solution (Life Technologies Ltd, Carlsbad, USA) was added to the punches in the microcentrifuge tube. Tubes were then vortexed for 15 seconds, ensuring that the punches stayed within the liquid buffer, and incubated at room temperature for 30 minutes. For the whole sample spots, 1ml RNA Rapid Extraction Solution was added to each well of the tissue culture plate containing a sample spot, and the plates were placed into a shaking incubator set at a temperature of 12°C on shaking speed 50, for 30 minutes. The buffer samples were then transferred into new vials for processing using the QIAamp Viral RNA Mini Kit (Qiagen Ltd, Venlo, Limburg Netherlands) with the following protocol modifications. The volumes of buffer AVL and ethanol added to each sample were increased to 2700µl and, once these were added to the buffer sample, the full volume of liquid was passed through the QIAamp spin column in volumes of 700µl per spin. Additionally, two elution steps using 50µl elution buffer per step were performed at the end of the protocol giving a final elution volume of 100µl RNA extract. These samples were separated into aliquots of 25µl and stored at -80°C until analysis by RT-PCR.

3.2.6 *RT-PCR*

RNA extractions were analysed by RT-PCR according to Kleiboeker et al. (2005). The method had been adapted by the Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge, UK by using double the recommended RT volume and additional HotStarTaq DNA Polymerase to that

which is already present in the RT-PCR kit (Strugnell, 2010). The North American primers and probe were omitted in place of a known European strain (H2) of PRRSv. Primer sequences were: PRRSv EU Forward: 5' GAT GAC RTC CGG CAY C 3'; PRRSv EU Reverse: 5' CAG TTC CTG CGC CTT GAT 3'; PRRSv EU Probe 5' Fam-TGC AAT CGA TCC AGA CGG CTT-Tamra 3' where R= A+G (Sigma Aldrich, St Louis USA). Total reaction volume was 25µl with a sample volume of 2µl. The Quantitect Probe RT-PCR kit (Qiagen Ltd, Venlo, Limburg, Netherlands) was used, and the reaction was carried out using an Eppendorf ep Realplex cycler according to the following cycling conditions: a 30 minute reverse transcription step at 50°C and 15 minute activation step at 95°C, followed by 40-45 two-step cycles of denaturing for 15 seconds at 94°C and combined annealing and extension for 60 seconds at 60°C (Kleiboeker, 2005). Results were recorded using the Eppendorf Realplex software.

3.2.7 *Statistical Analysis*

As each replicate sample was analysed by RT-PCR in duplicate, the mean Ct values generated by the duplicates were used to perform Kruskall Wallis tests using Minitab Version 16 (Minitab Ltd, Coventry, United Kingdom). Means were calculated where RT-PCR yielded Ct values, and for the non-parametric analysis, all cases where no Ct value was generated were given a value of 41. The coefficient of variation as a percentage (CV%) was calculated for all 6 Ct values (i.e. 3 replicates x 2 RT-PCR duplicates) obtained for each PRRSv spike concentration tested using the whole sample spot method, in order to assess the concordance of the results.

3.3 Results

PRRSv RNA was successfully recovered by RT-PCR from samples with PRRSv concentration $1x10^{3.5}$ and $1x10^{2.5}$ $TCID_{50}$/ml using the whole sample spot method. The punch method was not successful, with only three samples successfully detecting PRRSv RNA (see Table 3.1). Furthermore these values were above 39, which would not be deemed as a positive result within the clinical setting (Prickett, 2008b).

Table 3.1: Mean Ct values yielded by swine OF spiked with PRRSv stored at ambient temperature and using different FTA card processing methods

Day	PRRSv Concentration ($TCID_{50}$/ml)	Punch method	Whole spot method
0	$1x10^{3.5}$	39.50	26.83
	$1x10^{2.5}$	No Ct	31.34
	$1x10^{1.5}$	No Ct	38.45
1	$1x10^{3.5}$	No Ct	27.39
	$1x10^{2.5}$	No Ct	31.38
	$1x10^{1.5}$	No Ct	No Ct
3	$1x10^{3.5}$	41.67	26.79
	$1x10^{2.5}$	40.24	34.20
	$1x10^{1.5}$	No Ct	No Ct
7	$1x10^{3.5}$	No Ct	27.32
	$1x10^{2.5}$	No Ct	32.42
	$1x10^{1.5}$	No Ct	No Ct
14	$1x10^{3.5}$	No Ct	27.45
	$1x10^{2.5}$	No Ct	32.76
	$1x10^{1.5}$	No Ct	No Ct
21	$1x10^{3.5}$	No Ct	27.80
	$1x10^{2.5}$	No Ct	32.85
	$1x10^{1.5}$	No Ct	No Ct
28	$1x10^{3.5}$	No Ct	28.51
	$1x10^{2.5}$	No Ct	33.06
	$1x10^{1.5}$	No Ct	No Ct

The limit of detection for the successful method using the whole sample spot processing method for RNA extraction was $1x10^{2.5}$ $TCID_{50}$/ml. PRRSv RNA failed to be consistently detected after this spike concentration, therefore the assay reached its limit of detection in the present study.

A significant difference ($H(1)=14.92$, $P<0.001$) was found between the mean Ct vales generated by the two FTA card processing methods, indicating that the

use of the whole sample spot for PRRSv RNA extraction and RT-PCR analysis is a more effective and reliable means of detecting virus present in swine OF samples than the existing 2mm punch protocols.

PRRSv RNA was detected consistently in swine OF stored at ambient temperature throughout the 28 day test period when extracted using the whole sample spot (Figure 3.2). Ct values were higher for the $1x10^{2.5}TCID_{50}$/ml PRRSv concentration compared to those for the $1x10^{3.5}$ $TCID_{50}$/ml ($H(2)=12.72$, $P=0.002$), however the Ct values obtained all remained below 39 and therefore would be deemed as positive diagnostic results. The number of days stored at ambient temperature was found to have no effect on Ct value ($H(6)=2.66$, $P=0.851$).

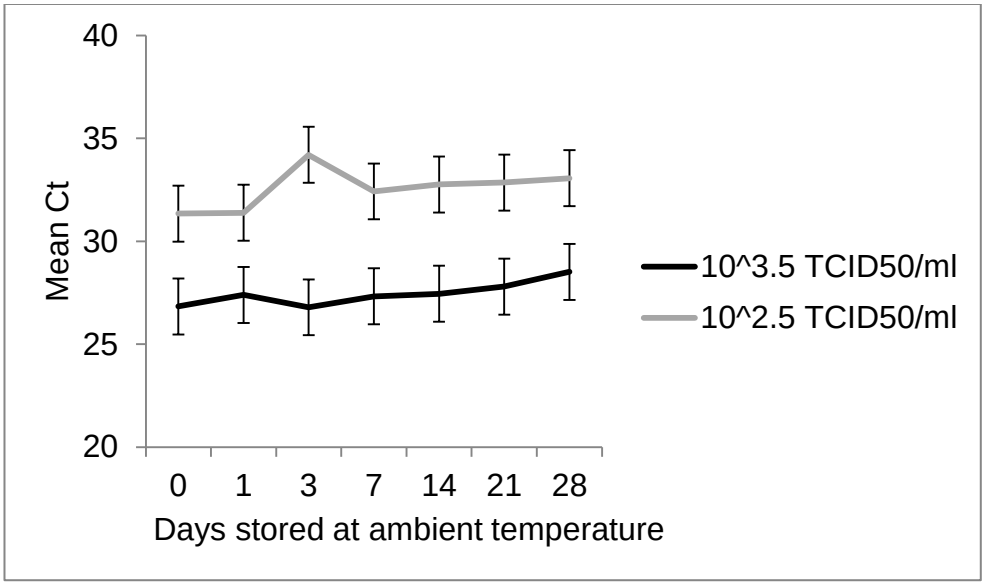

Figure 3.2: Mean Ct values ± SEM for spiked swine OF stored at ambient temperature across a 28 day period with RNA extracted from the FTA whole sample spot

The coefficient of variation (CV%) was calculated for each of the $10^{3.5}$ and $10^{2.5}$ $TCID_{50}$/ml PRRSv spike concentrations processed using the whole sample spot method in order to assess the concordance of the replications in the study. All six Ct values obtained for each condition i.e. duplicate RT-PCR reactions x three replicates were included in the calculation. The mean ± SEM CV across the whole time period was 2.34% ± 0.44 showing that the method yields consistent positive RT-PCR results (Table 3.2).

Table 3.2: Coefficient of variation (CV%) for whole sample spot method where positive Ct values were yielded.

Day	PRRSv Concentration ($TCID_{50}$/ml)	CV%
0	$1x10^{3.5}$	0.55
	$1x10^{2.5}$	2.85
1	$1x10^{3.5}$	1.95
	$1x10^{2.5}$	1.82
3	$1x10^{3.5}$	1.12
	$1x10^{2.5}$	6.83
7	$1x10^{3.5}$	2.09
	$1x10^{2.5}$	3.81
14	$1x10^{3.5}$	2.35
	$1x10^{2.5}$	3.89
21	$1x10^{3.5}$	1.18
	$1x10^{2.5}$	2.04
28	$1x10^{3.5}$	0.53
	$1x10^{2.5}$	1.76

3.4 Discussion

Previous work has reported a loss of sensitivity when swine OF stored on FTA cards was analysed by RT-PCR for PRRSv RNA, with particular losses after ambient temperature storage for 24 hours and 14 days (Linhares *et al.*, 2012). The present study aimed to investigate the ability of FTA cards to store swine OF samples spiked with PRRSv at ambient temperature, and to assess different card processing approaches to improve sensitivity.

3.4.1 *Storage of pig oral fluid at ambient temperature for the detection of Porcine Reproductive and Respiratory Syndrome virus by RT-PCR*

In the present study, FTA cards were able to store PRRSv RNA in swine OF embedded on cards for 28 days at ambient temperature when the whole sample spot was used for RNA extraction. This is in agreement with the manufacturer's guidelines which state that FTA cards can store RNA for up to 8 weeks at

ambient temperature. Consistent RT-PCR results were yielded for both the $1x10^{3.5}$ and $1x10^{2.5}$ $TCID_{50}$ /ml virus concentrations in OF throughout the whole time period. The ability to collect and store biological samples over a period of time could be advantageous to farmers, allowing them to build diagnostic archives in cases where infection is suspected, for bulk shipping and subsequent testing after 2 or 3 weeks. The non-invasive nature of OF sample collection enables the collection of multiple samples from animal populations over time with minimal stress being caused to those animals. FTA cards render biological samples non-infectious upon embedding into the card matrix (Bankamp *et al.*, 2013), thus making storage of samples from potentially infected animals safe. Furthermore, samples are dried onto the cards, which can then be folded, allowing easy storage of large numbers of samples.

3.4.2 *FTA card processing: whole sample spot versus 2mm punch method*

The results of the present study suggest that the use of the whole sample spot area for FTA card processing may be more beneficial than the original 2mm card punch methods used currently (Inoue *et al.*, 2007), especially for sample types where the concentration of the diagnostic target is known to be low, such as OF. Very few samples yielded Ct values when cards were processed using the 2mm punch method, which could potentially be explained by the antiviral activity in salivary secretions. Mucins are large protein molecules which are capable of binding to the surface of virus particles, numerous events of which can lead to “entrapment” of the virus in a mucin cage as part of the antiviral function of OF (Shine *et al.*, 1997). It is possible that these protein cages could make it difficult for virus to become evenly distributed within the FTA matrix during sample application. The result of this is the sample spot containing “hot spots” of mucin-entrapped virus within the OF, which could be easily missed when taking only two small punches of 2mm in diameter. One study did investigate the use of a larger punch diameter (6mm), however sensitivity was low for FTA card-embedded OF samples compared to the fresh equivalent (Linhares *et al.*, 2012). The degradation of viral RNA in swine OF stored at ambient temperatures has been reported previously (Prickett *et al.*, 2010), the current study therefore did not investigate RNA recovery from fresh PRRSv-spiked OF samples. The whole sample spot processing method was introduced in the present study to ensure that any PRRSv present within the OF embedded

onto the card would be released from the card for the RNA extraction procedure. This method was more successful than the 2mm punch protocol for the recovery of PRRSv RNA in the pig OF samples, delivering consistent RT-PCR results across the experimental time period.

3.4.3 *Test sensitivity and considerations for field application*

We report a limit of detection of 1 x $10^{2.5}$ $TCID_{50}$ /ml, as this was the lowest virus concentration for which consistent positive RT-PCR results were yielded. Data from a pilot study to investigate PRRSv monitoring in UK pig farms using OF (Strugnell, 2010) has been used to derive an estimation of the relative PRRSv concentration in swine OF. These concentrations ranged from $1x10^{-0.6}$ to $1x10^{2.1}$ $TCID_{50}$/ml and, although the limit of detection for the FTA card storage method presented here lies just above this range, it is possible that the method will be able to detect disease when pigs are highly viraemic.

It is important to consider that the results obtained and reported were for OF samples that had been experimentally spiked with PRRSv, and the field OF PRRSv concentration estimates were derived from previous work conducted in one region within the UK. Future work is required to fully validate this method using field OF samples collected from herds of pigs actively shedding PRRSv in order to gauge exactly how sensitive the test is under field conditions.

3.5 Conclusion

The present study has proved that Whatman FTA cards are capable of storing swine OF samples for up to one month at ambient temperatures for recovery of PRRSv when virus concentrations are relatively high, and that the use of the whole sample spot for viral RNA extraction yields consistent results by RT-PCR testing. However, validation using field samples with unknown virus concentrations is required before this sample storage method could be applied fully within the clinical setting.

Chapter 4: Detection of anti-Salmonella antibodies in porcine oral fluid samples using a commercial serum antibody ELISA

4.1 Introduction

Salmonellosis is a significant issue affecting both animal and public health. The pig industry has been highlighted as a major contributor to the problem due to the isolation of multiple *Salmonella* species within pig meat products (Harvey *et al.*, 2000; Evangelopoulou *et al.*, 2014; Lai *et al.*, 2014). There were 127 isolations of Salmonella from pigs submitted for disease diagnosis to AHVLA laboratories throughout 2013, and this represented a 31% decrease in isolations compared to 2012 (AHVLA, 2014). Though this may initially deflect attention from pigs as one of the key culprits in the investigation of any causes of the disease, the pig industry must not be omitted from consideration. One of the most prominent *Salmonella* serovars in human infection is *S. Typhimurium*, which is closely associated with the consumption of contaminated pig meat, as well as bovine products(European Food Safety Authority, 2014)..

The Zoonoses National Control Programme (ZNCP) for Salmonella in pigs (formerly known as the Zoonoses Action Plan (ZAP) Salmonella Monitoring Programme) was initiated by the British Pig Executive (BPEX) in order to encourage farmers to perform risk based assessment of Salmonella prevalence on farm for better health management and control (BPEX, 2011d). As part of this programme, Salmonella status of individual animals is assessed by taking a sample of muscle transudate (meat juice) post-slaughter for anti-Salmonella antibody testing by Enzyme Linked Immunosorbent Assay (ELISA). This method of testing provides an indication of Salmonella exposure at the individual carcass level, thus reflecting pathogen prevalence within the farm system. However, such endpoint-type analysis post mortem has certain limitations due to the animal being deceased at the point of testing and gives no indication of infection dynamics within the production process on farm. One area for which this method may not be effective is the assessment of the effects of disease intervention methods, for instance. Current testing in the live pig is bacteriological, relying heavily upon large numbers of faeces samples to be taken from pig populations for analysis by bacterial culture. This is costly to farmers in terms of time, labour and finance due to the requirement of veterinary attendance for blood sample collection, as well as labour for pig snare capture

and restraint. Serum antibody ELISA testing provides rapid screening of live pig populations for exposure to *Salmonella,* although concerns about the accuracy of serological tests have been raised. Also, the costs involved in conducting such tests on farm lead to the post-mortem meat juice testing being more widely used.

Oral fluid (OF) is a rapidly emerging diagnostic medium with an increasing number of pathogens being successfully detected, and assays subsequently developed for use with this sample type. The collection of OF is a non-invasive procedure, exploiting pigs' natural behavioural tendency to chew a piece of sterile, absorbent cotton rope provided to the pen (Prickett, 2008a). The fluid can then be easily wrung out by hand for subsequent work-up and analysis in the laboratory. It is possible to use existing commercial serum ELISA kit reagents to test for antibodies in OF samples by adapting the experimental protocols detailed by the manufacturer (Kittawornrat *et al.*, 2012a; Panyasing *et al.*, 2014). The sample incubation time period and temperature in particular seem to be key aspects which, when manipulated, can greatly affect the performance of the diagnostic assay when the test medium differs to that which the test kit was originally designed for.

At present, protocols exist for the analysis of porcine serum and meat juice samples for anti-Salmonella antibodies by commercial ELISA test kits. These detect Salmonella serogroups B and C1, which contain Salmonella typhimurium and Salmonella cholerasuis, the major pathogenic serovars. The current study aimed firstly to adapt a commercial Salmonella ELISA kit (IDEXX Laboratories, Westbrook, ME) for use with porcine OF samples via the modification of the existing kit protocol. Secondly, based upon the assay performance, one of the adapted protocols was then used to test field pen-OF samples collected from 29 different commercial UK pig units for comparison with corresponding blood sera, as well as pooled faeces samples.

4.2 Materials and Methods

4.2.1 *Experimental Design*

This study comprised an initial assay optimisation phase involving a 5 x 3 factorial design (5 experimental protocols x 3 sample dilution factors) which was

conducted using a total of 9 pen-based OF samples. The experimental protocol that showed the greatest detection of anti-Salmonella antibody in OF samples was then selected to test 190 pen-based pig OF samples collected from the field.

4.2.2 *Biological sample collection*

For the experimental protocol optimisation, blood samples (representing 24% of the pen population) were collected by jugular venepuncture from 13 pens of pigs ranging from nursery to finishing stages, in a cross sectional bleed on a commercial farm operating a continuous flow system, with a known high prevalence of Salmonella. The samples were allowed to clot for a minimum of 1 hour before centrifugation at 1500 RCF for 10 minutes to separate the serum for storage in 1ml aliquots at -80°C. Samples of pen-based oral fluid (OF) were collected from each of the 13 pens as well as blood sera Eighty centimetre lengths of 100% natural cotton rope (Outhwaites Ropemakers, Hawes UK) were provided to pens for pigs to chew. Ropes were presented for a minimum period of 30 minutes from observation of the first biting interaction, after which time the rope became saturated with OF. OF was extracted from individual ropes by cutting the wet portion of the rope and allowing it to fall into a labelled re-sealable plastic bag, wringing the rope by hand, and then allowing the OF to drain to the bottom of the bag. The bottom corner of the bag was then cut off, and the OF decanted into a 50ml centrifuge tube. Tubes were placed on ice for transport back to the University laboratory where samples were separated into 1ml aliquots for storage at -80°C.

Following serum testing, pen-based OF samples were selected for the assay optimisation based on the corresponding pen serum results: 3 OF samples were expected to be strong ELISA positive samples (defined by at least 50% pen sera testing ELISA-positive S:P > 0.25), 3 were expected to be intermediate positive (defined by <50% pen sera testing ELISA-positive), and 3 expected to be negative (defined by no pen sera testing ELISA-positive). A total of 9 paired pen OF and sera sample sets were therefore used for this assay optimisation step.

Field samples of pen-based OF were collected as described above from 29 different commercial pig units across the North of England along with

corresponding blood samples from each pen as part of a separate study (see Chapter 5). In brief, the number of blood samples taken per pen ranged from 7-14, and the number of pigs per pen ranged from 25-200 (therefore blood sample representation of each pen ranged from 4-56%). A statistical approach for sample size calculations was adopted, to ensure 95% certainty of detecting disease at an assumed seroprevalence (Thrusfield, 2007). All OF and serum samples were treated and stored as described. Pooled faecal samples were collected from each pen of pigs for Salmonella bacterial culture testing. Samples were kept chilled from the point of collection and shipped to the Animal Health and Veterinary Laboratories Agency (AHVLA Bury St. Edmunds, UK) for analysis.

4.2.3 *Antibody ELISA Testing*

4.2.3.1 *Oral fluid assay optimisation*

The present study used the commercially available Swine Salmonella Antibody Test Kit (IDEXX Laboratories, Westbrook, ME), designed for testing serum, plasma and meat juice samples, in order to test the porcine OF for anti-Salmonella antibodies. OF samples were applied to the antigen-coated plate either undiluted, or diluted 1:2 or 1:5. Details of the other protocol modifications from the serum protocol described in Section 4.2.3.2.1 are shown in Table 4.1.

Table 4.1: Experimental protocol modifications for the use of porcine OF as a test medium with the IDEXX Swine Salmonella Antibody Test Kit

Modified Condition → Protocol ↓	OF Sample Volume (µl)	Incubation Time	Incubation Temperature (°C)
1	200	18 hrs	4
2	100	30 mins	23
3	200	2 hrs	23
4	200	18 hrs	23
5	200	30 mins	23

For each test protocol, 1ml aliquots of pen-based OF were removed from -80°C storage and allowed to defrost thoroughly. ELISA kit reagents were also removed from the cold store (4°C) and allowed to equilibrate to room temperature prior to testing. With the exception of the modifications shown in Table 4.1, samples were tested in duplicate according to the manufacturer's protocol provided with the kit. In brief, following sample incubation, plates were washed three times with 1:10 diluted kit wash buffer and incubated for 30 minutes with kit antibody conjugate. Plates were washed a further three times, incubated for 15 minutes with kit TMB substrate and treated with an acid stop solution at the end. Plates were then read at 650nm absorbance. Mean optical density (OD) values were used to calculate the sample to positive (S:P) ratio.

4.2.3.2 *Field sample testing*

4.2.3.2.1 *Serum*

Individual pig serum samples were tested using the Swine Salmonella Antibody Test Kit (IDEXX Laboratories, Westbrook, ME) according to the manufacturer's protocol for analysis of serum and plasma samples, including the use of internal kit positive and negative controls. 100µl serum was incubated in the antigen-coated ELISA plate for 30 minutes, plates were washed three times with kit wash buffer diluted 1:10, incubated for 30 minutes with 100µl antibody conjugate and then washed three times again. 100µl TMB substrate was then added for 15 minutes and a final 100µl acid solution was added to stop the colour changing reaction. Plates were read at 650nm absorbance and the OD values converted into S:P ratios.

4.2.3.2.2 *Oral Fluid*

Pen-based OF samples were tested in duplicate using the Swine Salmonella Antibody Test Kit (IDEXX) according to protocol 4, which involved incubating 200µl undiluted OF in the antigen-coated ELISA plate for 18 hours (overnight) in a controlled temperature room set at 23°C. Plates were removed from incubation and washed three times with kit wash buffer diluted 1:10. From this point onwards, the manufacturer's protocol was followed for completion of the assay. The mean OD values were used to calculate the sample S:P ratios.

4.2.4 *Statistical Analysis*

All pen-OF and corresponding serum sample S:P ratios were compared according to the manufacturer's guideline of S:P ratio $\geq$ 0.25 being deemed as positive. Sensitivity and specificity tables were constructed to assess the performance of the anti-Salmonella antibody ELISA with OF.

4.3 Results

4.3.1 *Oral fluid assay optimisation*

The experiment to investigate the manipulation of the IDEXX Swine Salmonella Antibody test kit protocol for use with OF yielded varied results. Two out of six expected strong positive pen-based OFs (3 undiluted pen samples x 2 duplicates) tested ELISA-positive with S:P $\geq$ 0.25 and this occurred only when tested according to protocol 4. (Figure 4.1). Protocols 1 and 3 yielded single positive results, however since protocol 4 detected anti-Salmonella antibody within two distinct samples and additionally detected antibody at the dilution 1:2 for one of these, this was the assay which offered the most sensitive performance, and therefore was selected as the working protocol for use with the field samples. All expected negative, as well as expected intermediate OFs tested negative for all experimental protocols investigated.

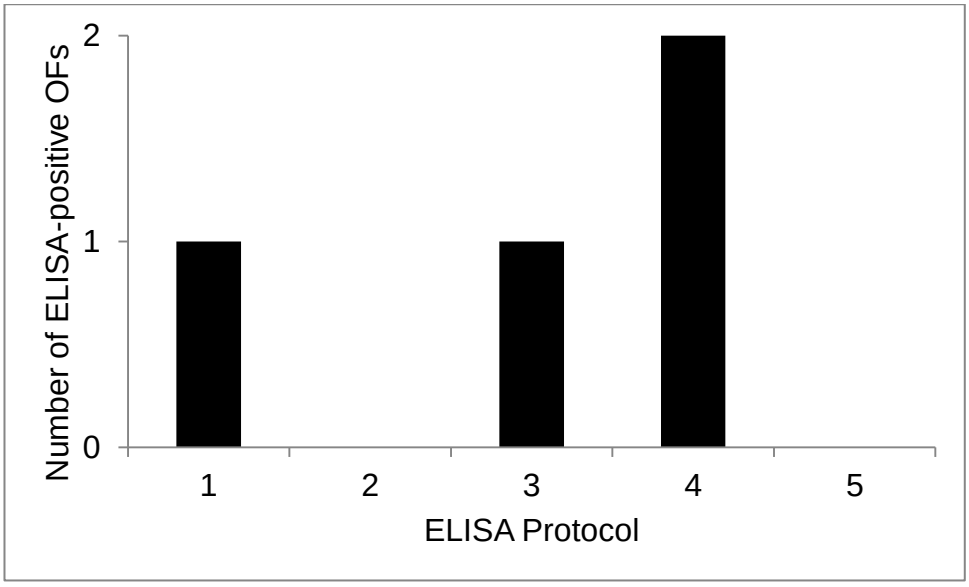

Figure 4.1: The number of anti-Salmonella antibody-positive OF samples, from 2 duplicates of 3 seropositive pens, yielded by each of the 5 modified ELISA protocols as described in Table 4.1

4.3.2 *Field sample testing*

In order to assess the performance of the anti-Salmonella antibody ELISA for OF, the results must be compared with a Gold Standard, which in this case was the blood serum test. Table 4.2 shows the proportion of positive/negative results by pen for the field OF and serum ELISA testing. Using the data from the table, when a positive pen was defined as that which yielded one or more positive sera, the sensitivity and specificity of the OF antibody test were estimated to be 29% and 98%, respectively.

When ELISA-positive pen OF results were compared with the results from pooled faecal culture, agreement was found for 12.5% (13 pens out of 104) of the total seropositive pens, where agreement was defined by a pen showing positive OF antibody test and successful culture and identification of Salmonella species'. One may not expect pens to be faecal culture-positive and serologically positive at the same time with the latter remaining positive after pigs have ceased shedding Salmonella in faeces.

Table 4.2: Table showing the number of porcine serum and OF samples to test positive and negative for anti-Salmonella antibody by ELISA when a positive pen was defined by one or more ELISA-positive sera

Serum → OF ↓	Positive	Negative	Totals
Positive	31	1	32
Negative	73	85	158
Totals	104	86	190

Data from the present study are also reanalysed according to the criteria that a pen was defined as positive if 50% or more of the sera taken from that pen tested ELISA-positive (Table 4.3). Taking this criterion for the determination of a "true" positive pen, the sensitivity and specificity of the OF assay for anti-Salmonella antibodies were 56% and 97%, respectively.

According to these criteria, the proportion of pen-based OF results that agreed with corresponding pooled faecal culture data was 26%.

Table 4.3: Table showing the number of porcine serum and OF samples to test positive and negative for anti-Salmonella antibody by ELISA when a positive pen was defined by 50% or more ELISA-positive sera

Serum → OF ↓	Positive	Negative	Totals
Positive	28	3	31
Negative	22	137	159
Totals	50	140	190

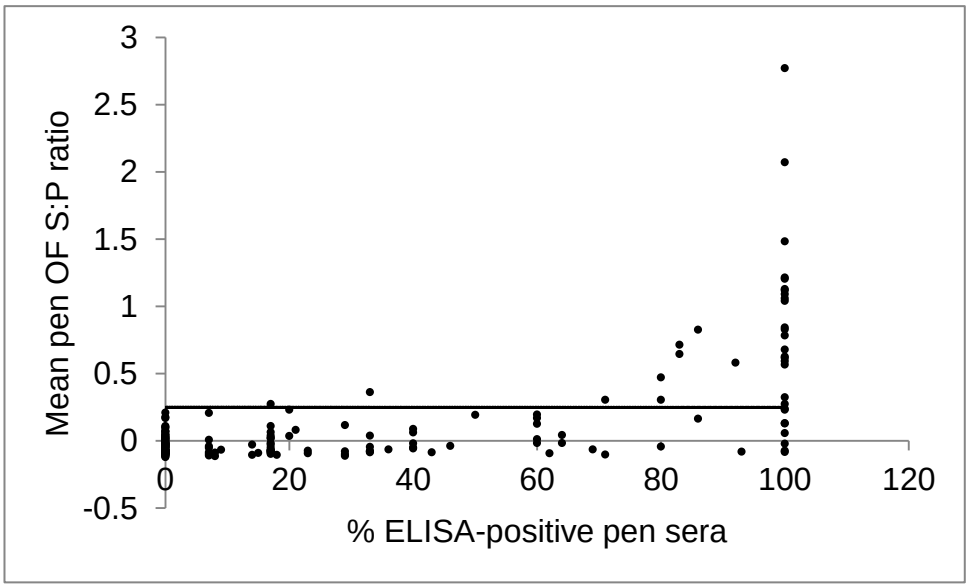

Figure 4.2: Dot plot showing the mean OF S:P ratio for anti-Salmonella antibody ELISA according to the proportion of positive corresponding pen sera. Line shows positive cut-off S:P ratio, 0.25

Pen-based OFs that tested ELISA-positive for anti-Salmonella antibody corresponded to pens showing a high proportion (>50%) of ELISA-positive sera (Figure 4.2). However in some cases pen-OFs which tested negative also corresponded to pens showing a high proportion of positive sera.

4.4 Discussion

Interest in OF as a diagnostic tool is mounting globally, and in particular, the potential to broaden the scope to screen for many key diseases using this non-invasive sample type. Work to date has indicated that OF could be both a

robust and versatile sample medium for widespread disease monitoring, however extensive protocol modifications and validation against current Gold Standard methods are required for each disease agent tested for. The present study aimed to investigate possibilities to apply OF diagnostics to Salmonella testing via the modification of an existing commercial ELISA test kit protocol and application of this to pen-OFs for comparison with corresponding blood sera.

4.4.1 *Adaptation of a commercial serum antibody assay for the detection of anti-Salmonella antibodies in porcine OF*

The experimental approach adopted in the present study has been used widely by research groups in the US, where OF is now recognised officially as a biological sample type for the diagnosis and monitoring of a number of key pig diseases (ISU, 2012). Studies have highlighted aspects of the ELISA protocol which may influence antibody detection in sample types where the titre or concentration may be lower than in blood serum. Sample dilution, incubation time and incubation temperature are the areas which have been manipulated, (Panyasing *et al.*, 2014) along with more complex approaches including modification of the test kit reagents themselves (Kittawornrat *et al.*, 2012a). It is for these reasons that the protocol modifications used in the present study were selected. Protocol 4 offered the highest level of detection for the undiluted samples, and also yielded a positive result when one of the strong positive samples was diluted 1:2. This involved using a large sample volume of 200µl, which is double the volume used in the serum assay, and this was incubated overnight at 23°C, compared to 30 minutes for serum. Even with this protocol, detection ability was not optimal, with only 2 out of 3 expected strong positive pens detected and none of the weak positive pens. However, since it gave the highest level of detection, it was subsequently used to test the field samples. The field OF samples were tested undiluted, ensuring that antibody could not be diluted within the sample and thus offering the optimal chance for assay detection.

4.4.2 *Detection of anti-Salmonella antibody in field pig OF samples using a commercial serum ELISA with modified protocol*

The field sample testing using an extended incubation time and elevated temperature yielded results which confirm that it is possible to detect anti-

Salmonella antibodies in porcine OF samples by modifying an existing commercial kit protocol. The protocol detected antibody in OFs from a range of different farms and showed good agreement with the corresponding blood serum results when prevalence was high (Figure 4.2). Sensitivity and specificity were calculated initially by classifying a pen as positive if one blood sample or more tested ELISA-positive. These were 29% sensitivity and 98% specificity, calculated using the data from Table 4.2. The latter value is good, showing a strong likelihood that positive results are in fact anti-Salmonella antibody, as opposed to a non-specific protein binding to the ELISA plate and triggering a positive result. The sensitivity of the assay however is very low at 29%.

The definition of a positive pen of pigs in terms of disease diagnostics may vary depending upon the causative agent and its nature. This particularly applies to disease agents such as Salmonella, which form part of the normal gut flora of the pig, as well as diseases which can be vaccinated against. Either of these circumstances can result in ELISA-positive diagnostic results which do not pertain to clinical disease breakdown (clinical salmonellosis). Additionally, the persistence of maternal antibody within the bloodstream and OF may be sufficient to trigger ELISA-positive test results where no clinical disease is evident. Great care must therefore be taken when interpreting data, and defining diagnostic thresholds for such agents. Data from the present study were therefore reanalysed according to the criteria that a pen was defined as positive if 50% or more of the sera taken from that pen tested ELISA-positive. A higher proportion of positive sera are likely to be more indicative of a true disease breakdown and thus, should provide a more accurate interpretation of the OF assay performance for detection of disease when prevalence is high.

This criterion change for the definition of positive pen status resulted in sensitivity and specificity of 56% and 97%, respectively, calculated using the data from Table 4.3. This reflected the test's more accurate performance when disease seroprevalence was high on farm. The effect can be observed in Figure 4.2, where pens with OF S:P ratios above the positive cut-off value of 0.25 corresponded largely with high proportions of positive sera. However the figure also shows that anti-Salmonella antibody was not detected in OFs from some pens with 100% positive sera, i.e. all samples taken tested ELISA-positive. Such sensitivity issues are to be expected initially when adapting diagnostic

assays for OF due to the lower concentration of the target present in OF compared to blood serum. The concentration of IgG antibody in finishing pig serum has been reported to be around 18mg/ml when blood samples were collected at slaughter (Curtis and Bourne, 1971). A recent study which investigated antibody concentrations in porcine OF following vaccination of young pigs with a commercial PRRSv attenuated vaccine (Porcilis PRRS, Intervet) yielded an IgG concentration of 20µg/ml in OF (Decorte *et al.*, 2014). This demonstrates a thousand-fold difference in the relative concentrations of IgG antibody found in porcine serum and OF, and therefore serves to explain the sensitivity issues encountered within the present study.

4.4.3 *Limitations and considerations for further development*

Although the present study did successfully address and fulfil its intended aims, the work was not without limitations. The investigation of protocol modifications was constrained by both time and finance, as the work formed a small portion of a wider project. Despite this, a protocol was established and used to test a large number of commercial field samples, with moderately successful results. The information gained from the study will provide a platform upon which further research and investigations can build.

4.5 Conclusion

The present study has demonstrated that it is possible to detect anti-Salmonella antibody in porcine OF samples via the use of a commercial serum test kit and modification of the test protocol. The protocol used in the present study to test the field samples was effective when Salmonella prevalence was high, showing reasonable agreement between OF and blood serum. However, further work is required to investigate ways to increase the sensitivity of the OF assay when disease prevalence is low. The detection of anti-Salmonella antibody in pen-based OF would provide a more historic and comprehensive picture of Salmonella exposure compared to faecal bacterial culture which is used currently, and which tends to provide only a snapshot in time of Salmonella shedding.

Chapter 5: Validation of pen-based oral fluid for the assessment of Porcine Reproductive and Respiratory Syndrome (PRRS) status in UK pigs

5.1 Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is a clinical condition affecting pigs worldwide. The causative agent, PRRS virus (PRRSv), is an RNA virus which exists endemically within the UK, as well as many other countries. As the name suggests, the clinical manifestation presents with two major symptom categories. During infection, breeding sows often suffer premature abortion, or deliver mummified piglets (Baysinger *et al.*, 1997). Younger pigs present with typical respiratory symptoms such as coughing, which can in some cases linger through the growing and finishing stages (Xing *et al.*, 2014). Clinical PRRS therefore poses significant welfare implications for pigs. The disease can also be financially devastating for farmers, from the loss of entire litters at the level of the breeding herd, to poor performance of growing animals due to respiratory ailment and the costs of veterinary treatment interventions. The cost of treatment interventions for PRRS in the UK has not been widely investigated however a Canadian study revealed that producers could pay up to $12.80/sow/outbreak in PRRS medication expenses (Poljak, 2011).

The clinical disease problems highlighted above serve to demonstrate the importance of PRRS to both the farmer and animals. The virus can also infect pigs sub-clinically, with no presentation of the characteristic symptoms (Jones-Lang *et al.*, 1997) however, pigs perform sub-optimally due to heavy energy investment into the immune response against the invading pathogen. This results in reduced average daily gain (ADG) (Regula *et al.*, 2000), meaning pigs require longer finishing periods in order to reach slaughter weight, thus increasing production costs for farmers in terms of building overhead, feed, water and (in cases where straw or equivalent substrate is provided) bedding per pig. These problems, associated with the sub-clinical stage of PRRS infection emphasise the necessity for routine surveillance and monitoring of pig populations in order to firstly confirm that the agent is present on a farm and secondly, formulate strategies for effective treatment.

Current methods to conduct routine surveillance for PRRSv involve laboratory testing of blood serum (Takikawa *et al.*, 1996; Spagnuolo-Weaver *et al.*, 1998).

The collection of blood for such testing requires the capture and restraint of pigs using a snare device, followed by jugular venepuncture. The bleeding procedure must be performed by a veterinarian, with an additional person being required to restrain the animal during the sample collection. This is costly and laborious, as multiple samples must be collected from a number of different pens on farm in order to detect the presence and/or dynamics of any circulating pathogens. Current blood sampling protocols are based upon the 95% confidence interval to be able to detect a disease agent circulating at an assumed prevalence, from which the number of animals to bleed from each pen/unit can be calculated.

The offering of a cotton rope to a group of pigs results in a proportion of that group exhibiting natural chewing behaviour on the rope (Prickett, 2008b; Prickett, 2008a; Prickett *et al.*, 2011), thereby depositing oral fluid (OF), a mixture of salivary secretions and gingival crevicular fluid (De Almeida *et al.*, 2008). The latter originates in the bloodstream, allowing agents such as viral DNA/RNA and antibodies circulating within the blood to be passed into the oral cavity and thus present in OF (Streckfus and Bigler, 2002). The concentration of these potential diagnostic targets within OF is lower than that observed in blood, however a number of serum-based assays have been successfully adapted for use with OF (Kittawornrat *et al.*, 2012a; Panyasing *et al.*, 2014).

The collection of an OF sample allows pigs to freely engage with the rope within a 30 minute period and is therefore non-invasive to the animals. The chewed portion of the rope can then be wrung out by hand to remove the OF for subsequent laboratory testing. The voluntary nature of OF sampling may also result in better diagnostic representation of the total population than current blood sampling protocols. One study has shown that in fully slatted floored accommodation, over 80% of the population actively chewed a cotton rope within a 30 minute presentation period (Seddon *et al.*, 2011). Current blood sampling protocols usually result in approximately 10% representation depending upon the assumed disease prevalence. The ease of sample collection and wider pen representation that could potentially be achieved using OF as an alternative to blood serum for disease diagnostics have triggered keen interest in application within the UK.

Previous work has revealed the ability to detect both viral RNA directly pertaining to, and host antibodies raised against, Porcine Reproductive and Respiratory Syndrome virus (PRRSv) within pen-based swine OF samples using adapted protocols for serum (Prickett, 2008b; Prickett, 2008a). Commercial test kit manufacturers are now beginning to develop test kits that are specifically designed for use with OF samples. The first such kit is the PRRS X3 Antibody Test Kit for Oral Fluids (IDEXX Laboratories, Westbrook ME). Although the performance of the adapted serum protocols is well characterised throughout the literature (Kittawornrat *et al.*, 2012a; Kittawornrat *et al.*, 2012b), limited field data exist for the recently launched commercial OF kit. The aim of the current study was therefore to generate the first UK field data using the commercial ELISA for swine OF and to compare the performance of this test to the current UK Gold Standard method of blood serum testing.

5.2 Materials and Methods

A total of 33 visits to commercial pig units across the North of England were conducted in 2 phases. Phase I took place between January and May 2013 and Phase II between September and December 2013. Blood samples were taken by veterinarians in line with their current diagnostic protocols for routine PRRSv surveillance. The serum anti-PRRSv antibody results from Phase I were suggestive of lower seroprevalence than that assumed initially, and feedback of these results to the veterinarians resulted in modification of the blood sampling protocols. This resulted in an increased number of blood samples collected per pen during the visits in Phase II, in order to increase the chance of disease detection at lower prevalence (20% versus 40% in Phase I (Thrusfield, 2007)).

5.2.1 *Blood Sample Collection: Phase I*

Samples were taken from 18 farms. The number of blood samples taken per pen ranged from 5-8. This was the number chosen to be 95% certain of detecting at least one seropositive pig at an assumed seroprevalence of 40% (Thrusfield, 2007). The total number of pigs per pen for each farm visit varied according to the husbandry system and stage of production at which pigs were tested (Appendix C). A standard BD Vacutainer system was used to perform jugular venepuncture to obtain whole blood samples. These were allowed to clot for a minimum of 1 hour before centrifugation at 1500 RCF for 10 minutes.

Serum was then separated into 1ml aliquots for storage at -80°C. Samples were labelled using a numerical code for randomisation.

5.2.2 *Blood Sample Collection: Phase II*

Whole blood samples were collected from a further 11 farms in the same way as described in Phase I, however the number of blood samples collected per pen of pigs ranged from 7 to 14. This increased sample size was chosen to be 95% certain of detecting at least one seropositive pig at an assumed seroprevalence of >20% (Thrusfield, 2007). As for Phase I, a range of husbandry systems were included in the study (Appendix A).

5.2.3 *Oral Fluid Sample Collection: All visits*

Each pen of pigs from which blood samples were collected was also presented with a length of 18mm 100% pure natural cotton rope (Outhwaites Ropemakers, Hawes UK) suspended such that the end of the rope hung at pig shoulder height. The position of the rope within the pen was adjusted for each unit according to the age/size of pigs being tested and so that ropes were not immediately close to feeders or drinkers. The 30 minute presentation period was timed from the first biting interaction observed by pigs, which occurred within 1-3 minutes in the majority of cases. Presentation time was increased by a further 10 minutes for pens of younger pigs (<8 weeks old) in order to allow ropes to become sufficiently wet for removal of an adequate OF sample.

At the end of the presentation period, with the operator wearing sterile gloves, the wet chewed portion of rope was cut off and placed into a re-sealable plastic bag, and then wrung out by hand allowing the fluid to drain to the bottom of the plastic bag. The bottom corner of the bag was cut off, and the OF decanted into a sterile pre-labelled 50ml centrifuge tube for transport to Newcastle University on ice. Some ropes from younger pigs (<8 weeks old), when kept in outdoor systems in particular, did not yield a sample from hand wringing and had to be cut into smaller pieces for removal of the OF using centrifugation. Approximately 3cm pieces of rope were placed onto 10cm x 10cm squares cut from autoclave bags, and the corners gathered together to create a pouch containing the rope piece. Scissors were used to make holes in the bottom of the pouch and this was then positioned at the top of a 50ml centrifuge tube and

secured into place using an elastic band. Tubes containing rope pieces were centrifuged at 1500 RCF for 10 minutes, and in all cases this removed all of the fluid that was present on the rope pieces.

All OF samples were separated into 1ml aliquots and frozen at -80°C. Pen-based OF sample labels corresponded with their respective blood serum labels in order to allow comparison of the diagnostic results for each test matrix.

5.2.4 *Anti-PRRSv antibody testing*

Serum samples were analysed for the presence of anti-PRRSv antibody using the commercially available PRRS X3 Antibody Test Kit (IDEXX Laboratories, Westbrook ME). The sera were tested in singular according to the ELISA kit manufacturer's protocol and the sample to positive ratio (S:P) calculated for each sample by entering the assay results as optical density (OD) values into a pre-programmed spreadsheet. Singular sample testing was deemed adequate as the kit validation data report showed very low intra-plate variability for PRRS-negative, PRRS-positive (low) and PRRS-positive (high) samples tested repeatedly on a 96-well ELISA plate (IDEXX Laboratories Inc., 2011).

The PRRS X3 Antibody Test Kit for Oral Fluids (IDEXX Laboratories, Westbrook ME), now also commercially available, was used to test the pen-based OF samples for anti-PRRSv antibody presence. Samples were not processed or treated in any way prior to ELISA testing and were tested according to the kit manufacturer's protocol (Appendix B). Phase I samples were tested in singular and Phase II samples in duplicate. For those samples tested in duplicate, mean OD values were used to calculate a sample S:P ratio for each OF using a pre-programmed spreadsheet.

5.2.5 *Statistical Analysis*

Pens with one or more ELISA positive serum sample(s) (i.e. S:P ratio greater than 0.4) were deemed as true PRRS positive pens and those with no serum S:P ratios greater than 0.4 were deemed as PRRS negative. The same S:P cut-off value applied to OF results. Tables were constructed to show the proportion of ELISA positive and negative samples for serum and OF, and the sensitivity and specificity calculated for the use of pen-based OF compared to serum as the Gold Standard.

A chi squared test was used to investigate any potential effects of 1) production stage, or 2) housing system on the agreement between OF and serum ELISA results.

Bland-Altman plots were made using the data for true-positive and true-negative samples in order to investigate the correlation between OF and serum ELISA results.

5.3 Results

Pen-based OF showed general agreement with serum for the detection of anti-PRRSv antibody using commercial ELISA kits. The results from Phase I did, however, yield a high proportion (16%) of positive OF samples where the corresponding sera were negative (Table 5.1). Following the advice to the veterinarians to collect a greater number of blood samples per pen in Phase II, the proportion of incidences where OF samples tested antibody positive with negative corresponding sera decreased (4%).

Table 5.1: Comparison of pen-based OF with serum ELISA results by pen for anti-PRRSv antibody

Samples	Serum → OF ↓	Positive	Negative	Total
Phase I	Positive	49	20	69
	Negative	3	48	51
	Total	52	68	120
Phase II	Positive	39	3	42
	Negative	7	26	33
	Total	46	29	75
All Pens	Positive	88	23	111
	Negative	10	74	84
	Total	98	97	195

The pen-based OF tests from Phase I of the study showed 94% sensitivity and 70% specificity at pen level when compared with the current Gold Standard, blood serum for detection of anti-PRRSv antibody. These values were 85% and

90%, respectively for the samples collected during Phase II, and the values for all pen samples were 90% sensitivity and 76% specificity.

Data were then reanalysed by farm, where any farm having at least one sero-positive pen was defined as a positive farm (Table 5.2).

Table 5.2: Comparison of pen-based OF with serum ELISA results by farm visit for anti-PRRSv antibody

Samples	Serum → OF ↓	Positive	Negative	Total
Phase I	Positive	10	2	12
	Negative	1	5	6
	Total	11	7	18
Phase II	Positive	11	1	12
	Negative	1	2	3
	Total	12	3	15
All Visits	Positive	21	3	24
	Negative	2	7	9
	Total	23	10	33

Pen-based OF showed 91% sensitivity and 71% specificity when ELISA results for serum and OF from Phase I were compared at the whole-farm level. These values were 92% and 67%, respectively for Phase II, and when all farm visits were included the sensitivity was 91%, and specificity was 70%.

Of a total of 195 pens, 86 nursery/grower, and 73 finisher pens showed full agreement between OF and serum ELISA results, giving 81% agreement overall between the 2 sample types. The proportion of agreement with corresponding sera (i.e. true positive and true negative OF results) was higher in finishing pigs than nursery/growers (Figure 5.1). A Chi squared test confirmed this finding (X^2=15.63, p<0.001). Housing system did not affect the agreement between OF and serum ELISA results (X^2=0.63, p=0.426).

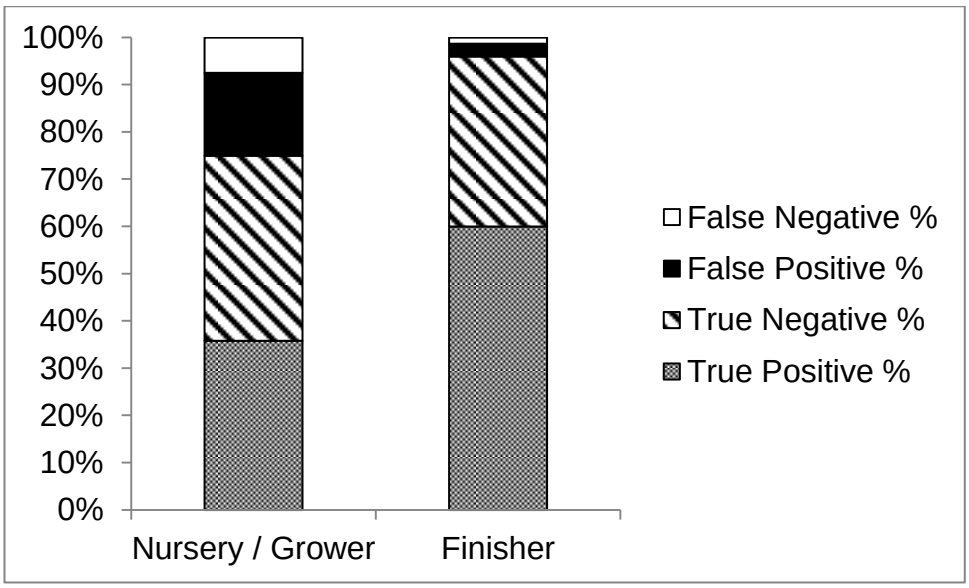

Figure 5.1: The proportion of OF ELISA result outcomes for anti-PRRSv antibody according to the stage of production

The OF samples that were classed as false positives, i.e. positive OF with negative corresponding pen sera, yielded a greater number of S:P ratios that were close to the positive cut-off value of 0.4 than true positive OFs where sera were also ELISA-positive (Figure 5.2).

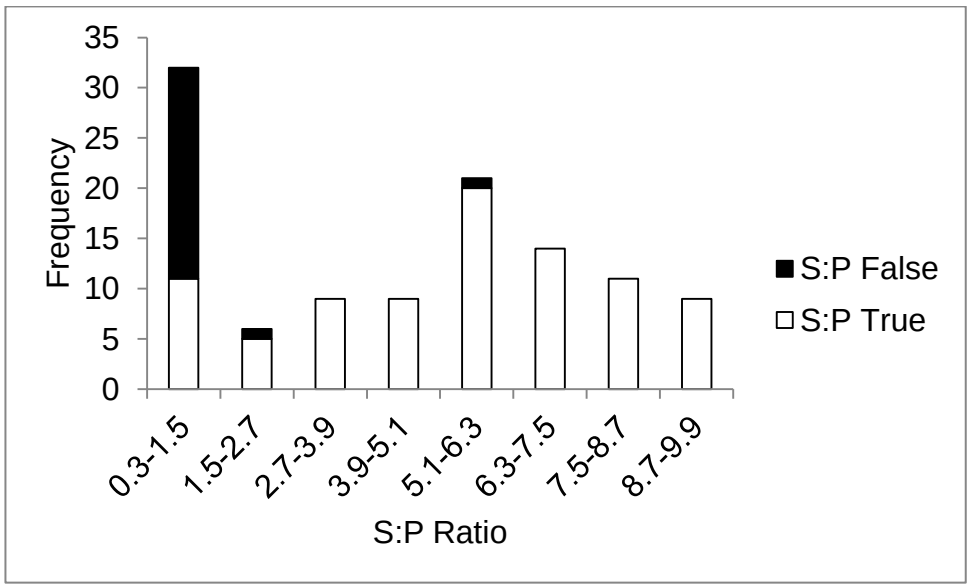

Figure 5.2: Stacked histogram to show the distribution of S:P ratios for true and false positive OF ELISA results

Bland-Altman plots of the true-positive (Figure 5.3) and true-negative (Figure 5.4) pen-based OF and corresponding serum data revealed significant correlations between the mean of both methods and the difference between methods for both positive ($r=0.911$, $P<0.01$) and negative ($r=0.493$, $P<0.01$) OF

outcomes. For the true-positive samples, only 3 data points lie outside the 95% confidence interval, 2 of these are below the mean difference and 1 is above. The difference between the OF and serum antibody detection methods increases with increasing mean, showing a divergence in test performance. The OF test seems to offer better anti-PRRSv antibody detection than serum. No data points lie outside the 95% confidence interval for the true-negative sample data.

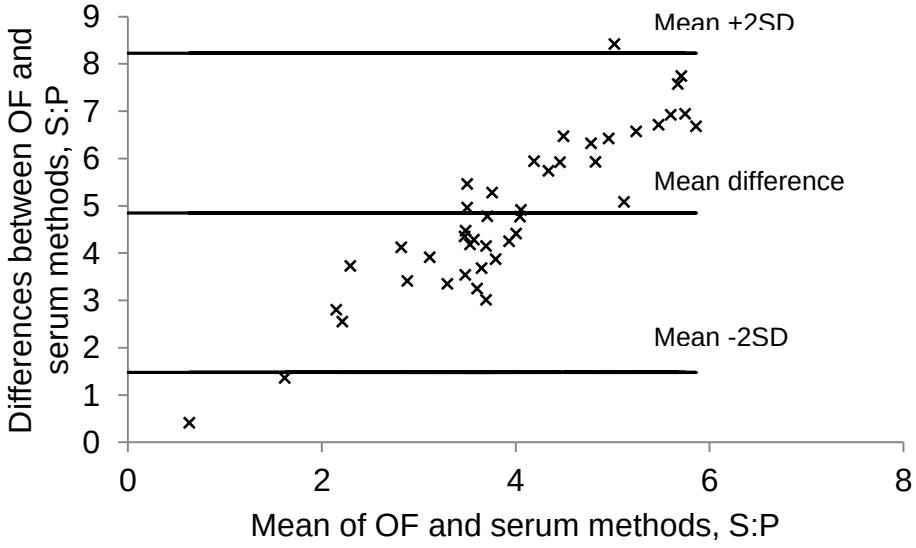

Figure 5.3: Bland-Altman plot of the mean pen-based OF and serum ELISA S:P ratios against the difference between the OF and serum ELISA S:P ratios for the true PRRS-positive samples collected during the study. Correlation R=0.911, P<0.01. Solid lines show the mean difference between methods,, mean +2SD and mean -2SD

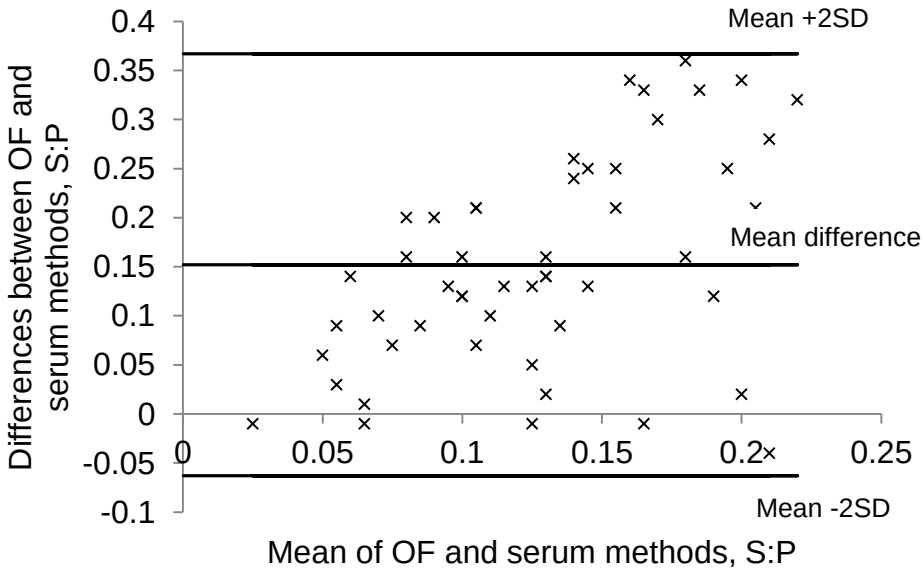

Figure 5.4: Bland-Altman plot of the mean pen-based OF and serum ELISA S:P ratios against the difference between the OF and serum ELISA S:P ratios for the true PRRS-negative samples collected during the study. Correlation R=0.493, P<0.01. Solid lines show the mean difference between methods, mean +2SD and mean -2SD.

5.4 Discussion

The present study aimed to validate the use of swine OF as a non-invasive tool for the detection of anti-PRRSv antibody in commercial UK pigs. Work previously conducted in the US developed a working diagnostic assay based on an original protocol for serum (Kittawornrat *et al.*, 2012a). A commercial test kit specially designed for use with the OF matrix is now available, although no data exist on the performance of this assay in comparison to the current Gold Standard diagnostic test which uses blood serum, especially in UK pig populations. Good agreement was found between the serum and OF ELISA results, with approximately 90% sensitivity when data for all pens from all farms visited were compared. We show that the OF assay is capable of reliably detecting anti-PRRSv antibody in samples from UK pig farms when animals are seropositive. There were however a considerable number of cases where pen-OFs tested positive and the corresponding sera were all ELISA-negative. This was particularly prominent in younger pigs sampled from nursery units, and during Phase I of the study. Potential explanations for these results will now be discussed.

5.4.1 Pen-OF may offer greater pen representation and thus more sensitive disease detection than blood serum

A previously published study (Seddon *et al.*, 2011) and further work described in Chapter 2 indicate that the OF collection method used here with a single rope is likely to sample 50-88% of the pigs present in the pen. The number of blood samples collected per pen represented approximately 6% of the pigs in a pen, and was calculated based upon the probability of detecting disease at a prevalence of 40% (Thrusfield, 2007). A decision by the veterinarians performing the bleeds on the farms after receiving feedback on Phase I results led to the number of blood samples collected per pen increasing for Phase II to approximately 18%. The effect of this on the representation of each pig population can be seen in Appendix A. The number of ELISA positive OF samples with negative corresponding sera from Phase II was lower than for Phase I, indicating that the blood sampling protocols utilised in Phase I may not have been sufficient to detect disease if seroprevalence was particularly low. These observations from Phase II therefore suggest that the positive OF results seen in Phase I may have been truly positive, and that the OF test may have been more capable of detecting anti-PRRSv antibody where disease seroprevalence was low. This would probably be due to the greater representation of the population allowed by OF sampling compared to blood, as it is well known that the concentration of diagnostic targets such as antibodies is much lower in OF than serum (Prickett and Zimmerman, 2010). Bland-Altman plot statistics for true positive and negative samples revealed that as the mean S:P values yielded by the OF and sera increased, the difference between the S:P values also increased significantly. Semi-quantitative interpretation of these data may suggest that the OF ELISA test is more sensitive than the serum test for detection of anti-PRRSv antibody due to this correlation. However further investigation of the OF test compared with serum is required in order to fully assess and evaluate the sensitivity of the assay.

Around 5% of pens yielded serum-positive, OF-negative results, where the proportion of ELISA-positive pen sera was 25% (i.e. 2 positive sera from a pen total of 8) or less, with S:P ratios ranging from 0.4 to 0.9. Although these data may suggest a sensitivity issue with the OF test when disease prevalence is low, the lower rate of occurrence overall within the present study indicates that the dilution of anti-PRRSv antibody within pen-OF as a result of many

individuals contributing to the sample is unlikely to compromise the method to an unacceptable extent.

5.4.2 "False positive" ELISA results triggered by porcine OF

The data presented are the first that have been generated for commercial UK pigs using the IDEXX PRRS X3 Antibody Test for Oral Fluids. In light of this, the possibility of false positive results being triggered by non-specific agents within the porcine OF samples cannot be disregarded. Cross reactivity is an issue that can confound ELISA test results from any sample type, however porcine OF may be likely to contain more cross reacting factors, such as antibodies raised against other pathogens or protein-rich feed contaminants. Research into human OF antibody testing has found that assay specificity can be highly variable when cross reacting contaminants (i.e. antibodies raised in response to infection with other disease agents) are likely to be present in OF at the time of testing (Scalioni *et al.*, 2014). Housing system was found to have no significant effect on the agreement between OF and serum ELISA results for anti-PRRSv antibody in the present study. This suggests that contamination of OF samples by faecal matter, which is more likely to to occur in straw-based and outdoor systems, does not affect antibody detection. The effects of other potential contaminants such as feed have not been looked at. One way in which this could be tested might be to include a centrifugation treatment of the OF samples prior to ELISA testing in order to remove large debris and thus some of the potential cross-reacting contaminants. Not all of these could be removed e.g. antibodies raised against other pathogens, however investigation of the removal of larger debris may aid the explanation of whether or not contamination is causing false positive ELISA results.

5.4.3 Maternally-derived antibodies may remain detectable in OF for extended periods

The vertical transmission of maternal immunity to offspring via colostrum is well documented (Nechvatalova *et al.*, 2011; Bandrick *et al.*, 2014). Ninety-one percent of cases in the present study, where pen-OFs tested ELISA-positive, with negative corresponding sera were pens of young pigs from nursery units. Additionally, the S:P ratios for the OFs were close to the positive cut-off value of 0.4 (Figure 5.2). These cases therefore raised suspicion of maternal antibody lingering within the serum of the younger pigs and being detected by the OF

ELISA. Maternally-derived antibody has been found to remain in the serum of weaned pigs for between 3 to 6 weeks, and in one case up to 12 weeks, and the duration of maternally-derived antibody presence was correlated to antibody titre within the dam (García-Bocanegra *et al.*, 2010). The pigs with serum showing presence of maternal antibody for the longest periods also had high antibody titres (García-Bocanegra *et al.*, 2010). This study shows that it is possible for maternally-derived antibody to linger within the serum of pigs when maternal titres are high. This may be especially true where sows are vaccinated against PRRSv, as most were in this study. It might be postulated that this could not be the case, as the appearance of maternal antibody in OF should be reflected in the corresponding serum ELISA results. However, taking into account the nature of OF sample collection, it is possible that some pigs within a pen had high maternal antibody titres where some did not, and these pigs' contribution to the pen-based OF sample lead to the OF positive result. The corresponding sera were collected from a smaller number of pigs selected at random within the pen, which may or may not include those individuals with the highest maternal antibody titres.

5.5 Conclusion

The present study has shown that results obtained from pen-based OF show good agreement (94% for finishing and 72% for weaned pigs) with the current Gold Standard of blood serum sampling for the detection of anti-PRRSv antibody.

Test sensitivity was high at pen and farm levels (90% and 91%, respectively), however the specificity of the assay was lower due to the occurrence of pen OF-positive, sera-negative cases, and is an area which requires further investigation.

Chapter 6: Longitudinal monitoring of anti-Porcine Reproductive and Respiratory Syndrome virus (PRRSv) antibody using pen-based porcine oral fluids

6.1 Introduction

Research has demonstrated the potential of porcine oral fluid (OF) as an alternative diagnostic medium to blood serum, which is the current UK Gold Standard for routine disease surveillance (Prickett, 2008b; Prickett, 2008a; Prickett *et al.*, 2011). Testing by both molecular and serological methods is now being successfully performed on porcine OF for the monitoring of a number of key disease agents in the US. The nature of OF sample collection allows the capacity to screen large numbers of animals at a fixed cost, and the large sample volumes that can be obtained allow many diagnostic tests to be performed using one sample.

At present, pig herd health monitoring is based upon visits by veterinarians, where blood samples are collected from pigs and sent to the diagnostic facility for testing and feedback of results. Blood sampling is an invasive procedure requiring capture and restraint of pigs by a snare and is therefore generally restricted to veterinary visits. The costs and logistics of this direct sampling towards a one-time collection basis, providing accurate disease information pertaining only to a single point in time. In contrast, the non-invasive collection of OF, involving the provision of a length of absorbent cotton rope for pigs to chew voluntarily and deposit their OF, facilitates more frequent collection by farm staff.

Most of the diseases which affect the pig industry are endemic, e.g. Porcine Reproductive and Respiratory Syndrome (PRRS), Enzootic pneumonia, PCV-2 related disease, and thus do not follow the typical epidemic pattern of breakdown – eradication – prevention. These can be present within herds at any time, often showing no clinical symptoms despite causing decreased productivity due to immune challenge (Regula *et al.*, 2000). The unpredictable dynamics of such disease agents are less well characterised than those which infect epidemically, e.g. Classical Swine Fever (CSF). Emphasis must then be placed upon stringent surveillance within farms where endemic disease is present, in order to gauge the extent to which pigs and their performance are affected.

Longitudinal-type monitoring of pigs can provide accurate disease information in real-time, potentially allowing the farmer and veterinarian to visualise disease dynamics within the herd over time. The non-invasive nature of the OF sampling method for diagnostics could bridge the gap between current one-time-collection methods using blood serum, and collecting multiple samples from the same animals over a given time period for a more detailed understanding of the disease situation on farm.

The aim of the current study was to investigate the suitability of pen-based OF for longitudinal monitoring of anti-PRRSv antibody in growing pigs of undetermined PRRS status.

6.2 Materials and Methods

6.2.1 Experimental Design

This study involved longitudinal collection of OF samples from the same groups of pigs over a 10 week period. The first samples were collected when pigs were between 3 and 6 weeks of age, depending on the practice of populating the individual nursery units. Samples were subsequently collected at 2 week intervals and a total of 5 collections were performed for each of eight pig flows. Pigs were between 11 and 14 weeks of age at the final sampling. The time period and stage of production during sampling were selected in order to investigate the dynamics of anti-PRRSv antibody throughout the nursery and growing stages, since a lack of diagnostic accuracy, i.e. high false positive rate in anti-PRRSv antibody detection was expected for pen OF from younger pigs (see Chapter 5).

6.2.2 Farms

Eight commercial pig production flows operating on an all-in-all-out by batch system, and populating their nursery units over a period of 1-3 weeks were included in the current study (see Figure 6.1). Pigs originated from four different breeding sources, but all animals present in a pig flow were from the same original source. All sites comprised straw-based housing, with the exception of one site (Flow 3) where pigs were kept in both straw-based and slatted floored housing. Pen sizes ranged from 25-300 pigs per pen, and the number of pens sampled for each pig flow ranged from 4-7. Some of the pigs were moved to finishing accommodation on separate sites to those where sampling started ,

and where this was the case, only the final sample collection was performed on the finishing site(s).

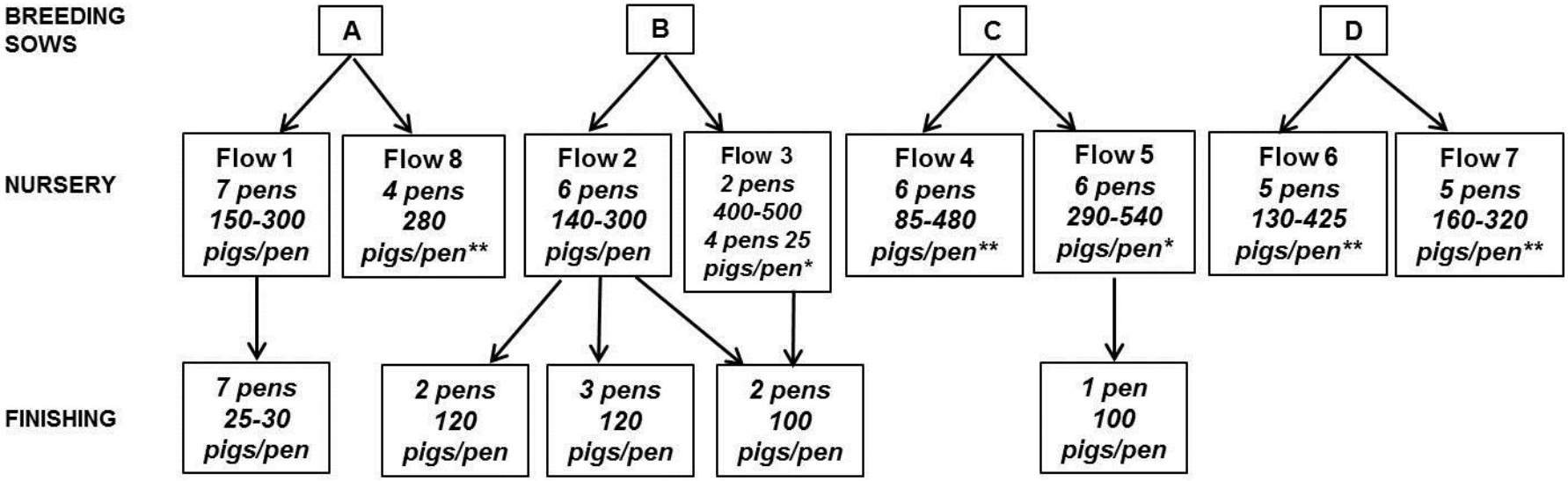

*Figure 6.1: Map of the breeding source and distribution of pigs in each of eight pig production flows used in the longitudinal OF study to monitor anti- PRRS antibody during the 10 week sampling period. Different letters and numbers denote different sites / flows. * shown in cases where only one pen of pigs was moved to the finishing site. ** shown in cases where pigs remained on a single site for the entire study period*

6.2.3 OF Collection

On each of the five sampling visits, OF samples were collected from the same pens of pigs in each pig flow. 100% pure natural cotton rope (Outhwaites Ropemakers, Hawes, UK) was used as described in Chapters 2, 3 and 5 to collect pen-based samples. Each rope was presented for 30 minutes from observation of the first biting interaction, then removed wearing gloves and placed into a re-sealable plastic bag. OF was removed from the rope by hand wringing, and then decanted into a 50ml centrifuge tube for transport on ice back to the University on ice, where each sample was separated into 1ml aliquots and stored at -80°C.

6.2.4 Blood Sample Collection

For each pen included in the study a single set of corresponding blood samples were collected for veterinary diagnostic purposes. Pig ages at bleeding ranged from 10-14 weeks of age. The number of blood samples collected per pen was 14 in all cases, based upon the 95% confidence interval to be able to detect disease at an assumed prevalence of 20%. Some pigs had moved on to finishing sites when the blood samples were collected. Where this was the case, bleeding was performed as close to the movement time as possible to avoid the detection of seroconversion as a result of challenge at the new site.

Pigs were restrained using a snare and blood samples were collected by jugular venepuncture using a BD Vacutainer system. Following collection, blood samples were allowed to clot at room temperature for a minimum of 1 hour, then centrifuged at 1500 RCF for 10 minutes. Each serum sample was separated into 1ml aliquots and stored at -80°C.

6.2.5 Anti-PRRSv antibody testing

Serum and OF samples were all analysed, using the IDEXX PRRS X3 Antibody test kit (IDEXX Laboratories, Westbrook, ME) and the IDEXX PRRS X3 Antibody test kit for oral fluids (IDEXX Laboratories, Westbrook, ME) respectively, at the end of the sample collection period in order to minimise variation between test days. Samples were allowed to equilibrate to room temperature, and were tested in singular according to the manufacturer's protocol for both sample types / test kits. In brief, serum samples were diluted 1:40, OF samples diluted 1:2 using kit sample diluent and 100µl of each diluted sample was incubated for 30 minutes at room temperature in the PRRS X3

antigen-coated ELISA plate. Plates were washed 3 times using kit wash buffer diluted 1:10 before incubation for 30 minutes at room temperature in 100µl of the kit antibody conjugate. Plates were washed 3 times using the diluted kit wash buffer, and 100µl TMB substrate added to the plate wells for 15 minutes at room temperature. The reaction was stopped by the addition of 100µl acid stop solution, serumplates were read at 650nm absorbance to obtain the Optical Density (OD) values. For measurement of OD values for OF plates, see Appendix B.

6.2.6 Statistical Analysis

OD values were used to calculate the sample-to-positive (S:P) ratio for each sample using a pre-programmed spreadsheet (Microsoft Excel 2010). The cut-off used for determination of a positive sample was 0.4, as defined in the kit manufacturer's protocol for both the serum and OF tests. A linear mixed effects model was used to investigate whether antibody patterns differed significantly 1) over time, or 2) between pens of pigs within the same pig flow. Visit was included as a fixed effect, with pen nested within pig flow as random effects.

6.3 Results

All pens included in the study engaged with ropes on all presentations, and produced adequate OF samples for analysis by ELISA.

6.3.1 Longitudinal OF samples

All pig flows included in the study yielded OF-ELISA-positive results on at least two of the five consecutive sampling visits. Pen-based OFs from 50% of all flows (1, 2, 3 and 8) tested positive for anti-PRRSv antibody throughout the entire study period (Table 6.1). Pigs from Flow 4 tested positive at the beginning of the study period, until 8 weeks of age, then the subsequent two samplings at 10 and 12 weeks of age yielded negative results. This was also the case for the pigs tested in Flow 6, where pen-OFs tested positive at 4 and 6 weeks of age, then antibody was not detected from 8 weeks until the final sampling at 12 weeks.

Two of the flows where pigs of different ages were tested (represented by different letters following the flow number) yielded different patterns of anti-PRRSv antibody detection across the sampling period. Younger pigs from Flow 5 (initially sampled at 4 weeks of age) tested positive until the final sampling

visit at 12 weeks, whereas pigs in the same flow which were one week older remained negative for anti-PRRSv antibody throughout. Pigs from Flow 7 which were initially sampled at 4 weeks, or 6 weeks of age tested OF-ELISA-positive until 10 weeks of age where antibody was no longer detected. Pigs from the same flow which were initially tested at 5 weeks of age yielded positive OF ELISA results throughout the entire study period.

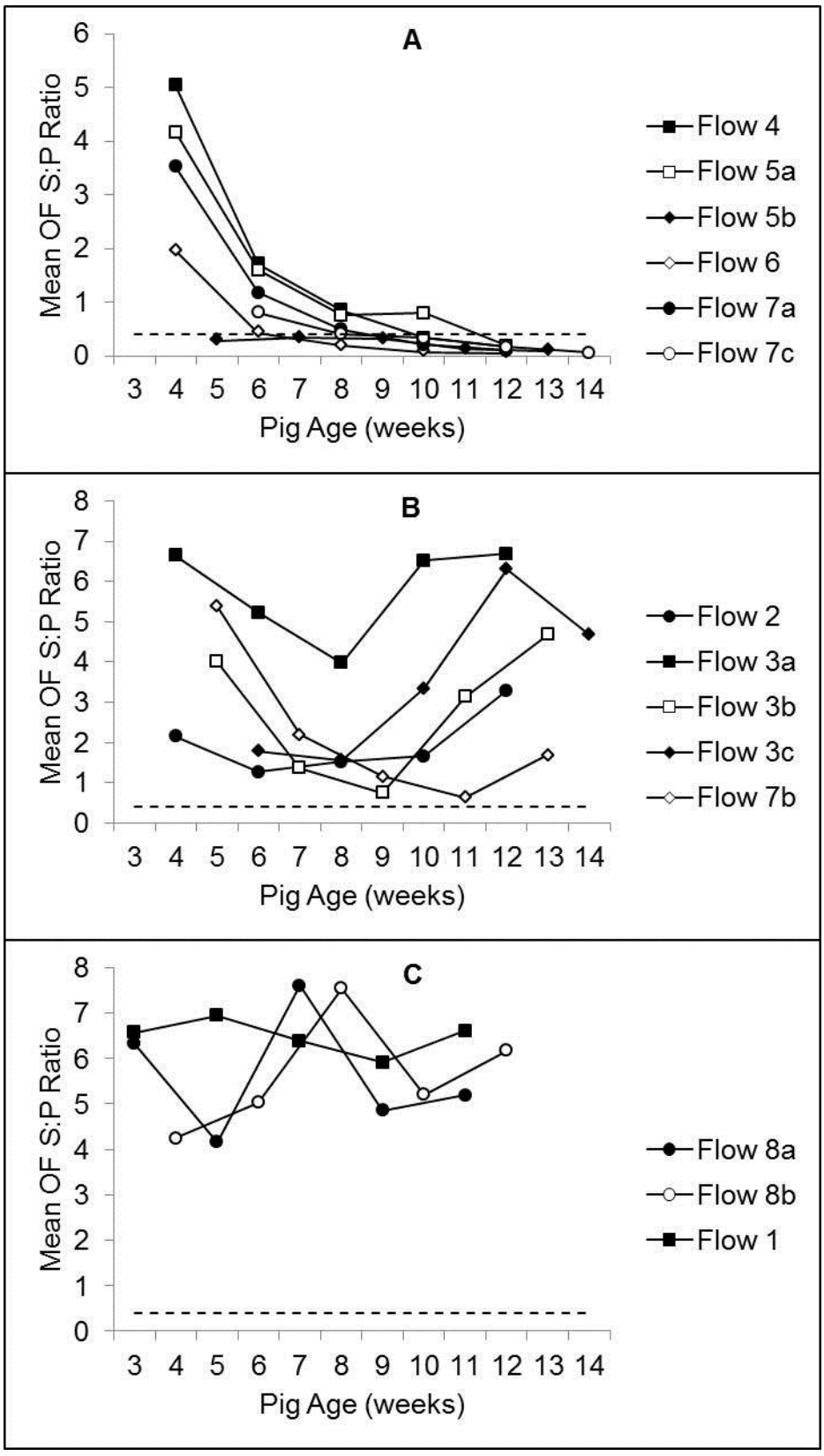

Figure 6.2: Mean OF S:P ratios according to pig age across the 10 week study period for eight different pig flows and age at first sampling. Panels A, B and C show PRRS antibody patterns: A: initially high, then declining ; B: declining, followed by spiking suggestive of re-infection; or C: continuously high. Dotted line represents the positive cut-off S:P value of 0.4

Analysis of the data using a linear mixed effects model revealed a significant effect of visit ($F_{(4,45)}$=7.93, P<0.01) on the detection of anti-PRRSv antibody in the pen-OF. Additionally, the different antibody patterns observed for pens from the same pig flows were found to be statistically significant (Wald Z=4.43, P<0.01).

Table 6.1 Mean OF S:P ratios for pigs on each of the eight pig production flows studied according to age. Boxes highlighted in pink illustrate PRRS-positive results according to the IDEXX PRRS X3 kit cut-off level of 0.4

Pig Age (weeks)	Unit 1	Unit 2	Unit 3a	Unit 3b	Unit 3c	Unit 4	Unit 5a	Unit 5b	Unit 6	Unit 7a	Unit 7b	Unit 7c	Unit 8a	Unit 8b
3													6.33	
4	6.57	2.15	6.64			5.05	4.17		1.96	3.53				4.25
5				4.01				0.29			5.38		4.16	
6	6.94	1.27	5.22		1.78	1.72	1.6		0.43	1.18		0.81		5.03
7				1.36				0.34			2.19		7.6	
8	6.39	1.52	3.97		1.56	0.85	0.76		0.19	0.5		0.41		7.54
9				0.74				0.32			1.15		4.86	
10	5.91	1.66	6.52		3.35	0.34	0.8		0.08	0.21		0.34		5.21
11				3.13				0.13			0.63		5.19	
12	6.61	3.29	6.69		6.31	0.17	0.19		0.06	0.11		0.17		6.18
13				4.68				0.1			1.69			
14					4.68							0.07		

6.3.2 One-time blood serum samples

Corresponding blood serum samples showed good agreement with the pen-based OFs collected on the same visit (Figure 6.3). Although there were some outlying data points, there was a significant correlation between the mean pen serum S:P ratio and the pen OF S:P ratio (r=0.939, P<0.01).

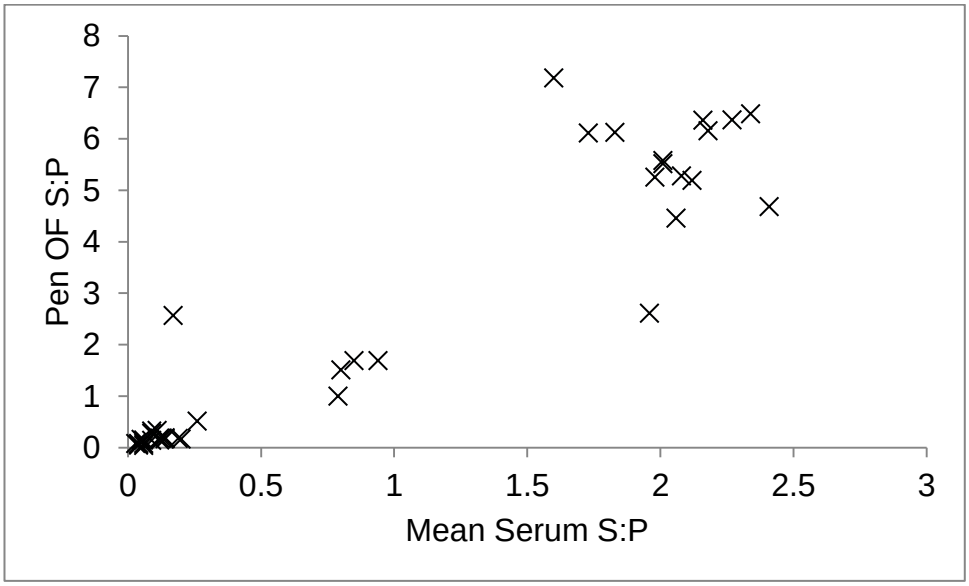

Figure 6.3: Relationship between the pen-OF S:P ratio and the mean pen serum S:P ratio for each pen on the sampling day when both pen-OF and blood samples were collected (r=0.939, P<0.01)

Table 6.2: OF antibody pattern, with corresponding pen serum mean S:P ratio and proportion of pen sera to test ELISA-positive (S:P ≥ 0.4). Highlighted boxes represent ELISA-positive S:P ratio

Flow	Pen	OF antibody pattern	Mean serum S:P	% positive pen sera
1	1	High	2.01	100
	2	High	2.01	100
	3	High	1.83	93
	4	High	2.18	93
	5	High	2.08	100
	6	High	2.27	100
	7	High	2.16	100
2	1	Decline - re-infect	1.96	100
	2	Decline - re-infect	2.06	100
	3	Decline - re-infect	0.79	57
	4	Decline - re-infect	0.13	0
	5	Decline - re-infect	0.06	0
3	1	Decline - re-infect	2.41	100
	2	Decline - re-infect	0.8	64
	3	Decline - re-infect	2.34	100
4	1	Decline	0.14	0
	2	Decline	0.12	0
	3	Decline	0.19	14
	4	Decline	0.12	0
	5	Decline	0.14	14
	6	Decline	0.05	0
5	1	Decline	0.17	7
	2	Decline	0.09	0
	3	Decline	0.11	7
	4	Decline	0.26	29
	5	Decline	0.09	7
6	1	Decline	0.03	0
	2	Decline	0.04	0
	3	Decline	0.09	0
	4	Decline	0.06	0
	5	Decline	0.04	0
7	1	Decline	0.04	0
	2	Decline	0.2	7
	3	Decline	0.06	0
	4	Decline – re-infect	0.94	57
	5	Decline – re-infect	0.85	57
8	1	High	1.73	100
	2	High	1.6	100
	3	High	1.98	100
	4	High	2.12	100

6.4 Discussion

This study introduced a temporal aspect to the use of OF diagnostics in order to gauge whether OF could track changes in anti-PRRSv antibody in the same groups of pigs over time. This was achieved, and the temporal pattern varied between different pig flows.

6.4.1 Interpretation of farm data according to anti-PRRSv antibody pattern

Anti-PRRSv antibody was consistently detected at every sampling in the pen OFs throughout the study period for 4 pig flows (1, 2, 3, 8). Corresponding blood serum samples also tested strongly positive (Table 6.2). These results suggest that pigs experienced a PRRSv challenge during the time of the study. The flows can therefore be classified as “positive”, which is useful information for the producer. The majority of these pigs were tested from 4 until 12 weeks of age, with one set of pigs being aged 5 weeks old at first test, and therefore 13 weeks old at the final sampling. Despite the difference in age at first sampling, pigs initially tested positive, and antibody continued to be strongly detected throughout.

One pen of pigs from Flow 5 remained OF negative from first to last sampling, although other pigs from the same flow showed detection of anti-PRRSv antibody in the pen-OFs. However, for these pens, only 1 and 4 out of the 14 corresponding pen sera tested ELISA-positive (7% and 29%, respectively, see Table 6.2), these low numbers may suggest that it is maternally-derived antibody being detected as opposed to an immunological response to infection. As discussed in Chapter 5, maternally derived antibody can remain in the serum of young pigs for up to 12 weeks (García-Bocanegra *et al.*, 2010). The diminishing antibody detection seen in the pen-OF, combined with the low numbers of ELISA-positive serum samples both support the postulation that maternally derived antibody remains detectable within pig OF for a considerable time post-weaning.

A similar situation occurred for pigs from Flow 7, where pen OFs collected from pigs aged 4 or 6 weeks at first sampling tested positive for anti-PRRSv antibody until 10 weeks of age where antibody was no longer detected. As with Flow 5,

only 1 or 0 of a total of 14 pen sera tested ELISA-positive (Table 6.2), suggesting that the pen-OF positives were due to the presence of maternally-derived antibody as opposed to infectious challenge. However, other pens in this pig flow which were tested initially at 5 weeks old tested strongly positive in the OFs throughout the entire study period. Eight out of 14 (57%) pen blood sera tested ELISA-positive for anti-PRRSv antibody in each of these 2 pens, indicating the possibility of infectious challenge with PRRSv. One point which may be worthy to note in this case is that the building where the pigs initially sampled at 5 weeks old were kept was separated from those which housed the other pigs initially sampled at different ages by another building which did not contain pigs. The approach can therefore inform conclusions about within-farm PRRSv epidemiology as well as between-farm observations.

6.4.2 Implications of maternally-derived antibody detection in disease monitoring

In Chapter 5, a question was raised as to the possibility of maternally derived antibody remaining in young pigs post-weaning in a way that could be detectable in pen-based OF. The current study targeted young pigs, following them through the growing stage in order to further investigate this question.

The data presented are supportive of maternal antibody presence and detection in pen-based porcine OF, but in a way that may be distinguishable from antibody produced in response to infection by accurate interpretation. In the present study, the suspected maternal antibody-positive samples showed a pattern of diminishing detection by 12 weeks of age at the latest, with little or no detection of antibody within the corresponding pen sera at this time. In contrast, in cases where infectious challenge with PRRSv was suspected, antibody was strongly detected throughout the entire sampling period, i.e. up to 14 weeks old in one pen’s case, along with strong positive serum ELISA results.

It is therefore possible to successfully monitor PRRSv antibody over time, as well as assess the status of a population or herd using pen-based OF. However, results must be carefully interpreted, in particular for younger animals, due to the potential for detection of maternally-derived antibody and therefore misdiagnosis of disease. The same considerations must be made when interpreting data pertaining to blood serum from young pigs, and so the application of such interpretational precautions to pen-OF data is feasible.

6.4.3 Scope for longitudinal disease monitoring in pig health management

The present study has looked at longitudinal antibody detection for disease monitoring in commercial pig flows. The information obtained from this investigation, and the method by which it was gathered, will assist in developing our understanding of the dynamics of PRRS on pig farms. Longitudinal antibody monitoring allows the producer/veterinary team to generate a “footprint” of where a pathogen has been on a farm, due to the antibody’s production and role in response to infection of the host with a given pathogen. In addition, molecular testing methods such as reverse transcription polymerase chain reaction (RT-PCR) offer the ability to detect causative agents directly, effectively offering a two-pronged approach for potential disease investigations. This could be particularly useful for sub-clinical infection, providing farmers with the ability to visualise disease progression on their unit(s).

There are advantages of longitudinal disease monitoring for both single- and multi-site pig producers. Firstly, in single-site production, the use of longitudinal disease monitoring, i.e. via antibody detection in OF, would allow farmers to easily collect samples themselves as frequently as desired over a period of time to track pathogen exposure throughout livestock.

As mentioned, the units in this study were all part of a large multi-site production system, where pigs weaned at the breeding units were moved to the nursery sites and then, in addition, some of the units had a separate finishing site. Longitudinal disease monitoring could be advantageous to farmers operating such systems as a means of assessing the health status of the different sites, and pinpointing potential infectious challenges animals may face as they progress through the production stages, thus enabling optimisation of pig flows in the network.

6.5 Conclusion

This study has shown that pen-based OF can be used to monitor anti-PRRSv antibody in pigs through the nursery and grower stages of production. The data also suggest that PRRSv infection may be distinguishable from maternal antibody in porcine OF by consideration of the pattern of antibody detection according to pig age.

Chapter 7: General Discussion

This thesis has addressed some of the key questions surrounding the application of OF diagnostics to commercial pig populations. The collection of the sample itself, the treatment of this once collected and validation of the approach as compared to current Gold Standard diagnostic methods have been investigated.

As a method which has received a great deal of focussed research within the US, the use of OF diagnostics has been validated for typical pig housing types utilised across the US. This thesis looked at expanding this to pigs kept in straw-based housing systems which feature large group sizes (>30 pigs per pen), to develop optimised OF collection protocols for detection of disease in pigs kept in such systems where the attraction to ropes might be more difficult (Chapter 2). This included investigation of a method to improve sample yield via the use of flavoured additives on cotton ropes, which might increase OF yield via salivary stimulation or generation of increased interest in ropes (Chapter 2).

To give more flexibility in sample handling and transport, a practical method to store porcine OF at ambient temperatures for subsequent testing by molecular methods was developed (Chapter 3). Additionally, to expand the possible uses of the sample once collected, a commercial serum-based antibody ELISA assay (IDEXX Laboratories, Westbrook, ME) was modified for the detection of anti-Salmonella antibodies in OF samples (Chapter 4).

Validation studies compared the use of OF with the current Gold Standard method involving blood serum collected from commercial UK pig farms. Initially, a series of one-time sampling visits were performed and the detection of anti-PRRSv antibody in the two sample media was compared (Chapter 5). A longitudinal study then focussed on serial OF samples collected from selected pig populations throughout the nursery and grower stages to assess the suitability of OF as a means of tracking anti-PRRSv antibody dynamics within the same groups of pigs over time (Chapter 6).

The findings of this thesis will contribute to a growing knowledge base moving toward the eventual application of OF diagnostics as an accurate, flexible and

reliable alternative to blood serum testing for the routine surveillance of a number of key pig diseases.

7.1 Effectiveness of OF collection methods to improve sample yield and representation

The use of OF as a diagnostic medium, including the sample collection method, has been well documented and is being used in routine practice across the US (Prickett, 2008a; Prickett and Zimmerman, 2010; Ramirez *et al.*, 2012; Kittawornrat *et al.*, 2014). Sampling protocols exist for pigs kept in conventional indoor housing systems with slatted floors, however many UK farmers operate straw-based systems with large group sizes (100-400 pigs per pen). The reduced capital cost, economies of scale in working routines and consumer perception of improvement in welfare resulting from environmental enrichment for the pigs offered by these systems have led to many farmers adopting the method of husbandry in recent years. Additionally, these systems do not require the specialist building adaptations associated with more conventional indoor pig production, i.e. controlled heating and ventilation facilities, making basic pig production accessible to more farmers with large, general purpose buildings. These straw-based systems account for around 65% of the current feeder pigs in England (DEFRA, 2010), and therefore must be considered as part of the development of OF diagnostics for successful application within the UK pig industry. Previous work has suggested that the provision of straw bedding may impair OF sampling via the rope chewing method, as pigs may be less interested in chewing ropes when environmental enrichment such as straw is provided (Seddon *et al.*, 2011). The large spatial areas and pig numbers may also make it more difficult to achieve representative samples.

The work in Chapter 2 of this thesis aimed to investigate ways in which OF sample yield may be increased, and secondly to develop and optimise OF collection protocols for large groups of pigs housed in straw-based systems. The use of apple juice, pineapple juice or 10% sucrose solution as rope flavourings did not affect the volume of OF produced by pens of weaned (25 pigs/pen) or finishing (17 pigs/pen) pigs. This disproved the hypothesis that the flavours might stimulate salivary secretion via the neural gustatory pathway and/or generate increased interest in the pigs to chew ropes. Other studies

which have looked into the use of flavoured rope additives have not done so to investigate the potential effects on OF yield, but as an applied method, e.g. apple juice on ropes to collect OF from individually housed boars (Kittawornrat *et al.*, 2010) and salt solution on ropes (to simulate blood) in studies looking at the issue of tail biting (Fraser, 1987; McIntyre, 2002; Jankevicius and Widowski, 2003). Although flavourings did not yield a positive benefit in this critical evaluation, the data generated could provide a useful starting point for potential future studies focussing on optimising OF yield. One area which has not yet been explored is olfactory stimulation, and whether or not the use of olfactory stimuli, e.g. banana aroma as chewing rope additives could generate increased pig interest and thus OF yield. This could be considered as a future research area.

Ways in which OF sample representation could be optimised were also investigated in Chapter 2 via the provision of multiple ropes to pigs housed in large groups in straw-based systems. Greater sample representation, i.e. up to 74% of pigs chewing ropes, was achieved for weaner pigs by the provision of multiple ropes, compared to 42% when a single rope was offered. This was likely to be due to synchrony of chewing behaviour in the younger pigs, whereby the multiple ropes provided increased opportunity for less dominant pigs to access ropes and thus contribute to the OF sample. Conversely, the provision of multiple ropes to finisher pigs did not increase representation above 50% of the pigs in a pen. A study which compared the rope chewing behaviour of smaller groups of pigs kept in fully-slatted floor versus straw-based housing gave representation of 79% of animals in the straw-based system after 60 minutes. This was lower than the chewing response observed for pigs in fully-slatted floored housing (95%), probably due to the environmental enrichment provided by the straw bedding (Seddon *et al.*, 2011). In contrast, current blood sampling protocols usually result in around 10% pen representation. The cost of veterinary time, labour to assist collection and stress to pigs involved in blood sampling all place a downward pressure on the pen sample size, however there is also the requirement for confidence in detection of disease when the prevalence is low. Alternatively, the OF approach offers more representative testing, with the capacity to screen larger numbers of pigs at low labour input and minimal stress to the animals. The data presented in Chapter 2

demonstrate that OF collection protocols can be successfully modified for diverse pig husbandry systems by considering factors such as pig age and housing type. Based upon the findings of this research, we recommend a rope:pig ratio of 1:25 for large populations of weaned pigs (up to 200 pigs per pen), and 1:100 for finisher (up to 100 pigs per pen) pigs.

7.2 Practical storage of porcine OF samples for easy application on farm

Molecular diagnostic testing methods such as reverse-transcription polymerase chain reaction (RT-PCR) target the RNA of the causative agent directly, and are therefore highly specific and sensitive methods. Viral RNA, and in particular that of PRRS virus, has been shown to be highly unstable within porcine OF when samples are stored at ambient temperature over time (Prickett *et al.*, 2010). It is therefore recommended that a cold chain must be maintained from the point of collection, and throughout transport of OF samples to the diagnostic test facility. This may not be a feasible protocol for all pig producers to adopt due to limited chilling facilities on farm and the inconvenience of cold sample shipment, which requires specialist packaging and therefore additional cost.

Chapter 3 of this thesis documents the development of a method to store porcine OF at ambient temperature which preserves viral RNA for accurate detection by RT-PCR. Flinders Technology Associates (FTA) cards stored PRRSv-spiked OF for up to 4 weeks at ambient temperature, offering consistent PRRSv RNA detection by RT-PCR throughout the entire period. Other research groups have investigated the use of RNA preservatives with OF samples stored at ambient temperatures with mixed results (Decorte *et al.*, 2014; Jones and Muehlhauser, 2014). These products were in the form of solutions which were added to, and then mixed with the OF for efficient nucleic acid preservation and storage. The major advantage of FTA cards above these solutions is that they are a dry storage method, making them easier to handle for producers, and more compact to store in the form of thin cards as opposed to liquid vials as would be the case with the wet preservative solutions.

Previous attempts to use FTA cards for porcine OF storage have found a significant loss in test sensitivity compared with results from blood serum or untreated OF testing (Linhares *et al.*, 2012; Steinrigl *et al.*, 2014). One of the

aims of the study in Chapter 2 was therefore to investigate methodologies to increase test sensitivity for FTA cards. This was achieved by taking the entire sample spot, i.e. the whole area of the card where the sample has become embedded, as opposed to a small punch from within this area which is recommended by the card manufacturer and was used previously in other studies (Inoue *et al.*, 2007; Linhares *et al.*, 2012). The whole sample spot method ensures that all virus particles that are present within the OF that is embedded in the FTA card matrix are processed during RNA extraction, thus optimising the chances of RNA detection by RT-PCR. The method must now be trialled using field samples collected from commercial pig units of unknown PRRS status in order to assess the performance of the FTA cards within the real veterinary clinical setting.

7.3 Broadening the spectrum: detection of anti-Salmonella antibodies in pen-based OF

A number of disease agents can now be tested for using porcine OF such as PRRSv, PCV-2, Influenza A virus, PEDV, and these tests are being used in the US for routine disease monitoring (Prickett, 2008a; Goodell *et al.*, 2013; Burrough, 2014; Panyasing *et al.*, 2014). Current research is continually aiming to expand this list of disease agents. The origin of the diagnostic targets sought in OF is the bloodstream, and therefore it may be hypothesised that any virus or bacterium, or alternative markers of infection, e.g. antibodies or Acute Phase Proteins, circulating within the blood could also be present and thus detectable within OF, via the modification of the laboratory test.

Salmonella was selected as the disease of focus for Chapter 4 in this thesis due to the widespread impacts that it exerts upon the pig industry, as well as human public health. Salmonellosis can cause severe ailment in pigs, and can sometimes be fatal (Wilcock *et al.*, 1976). Typically, clinical signs include enteric symptoms such as diarrhoea, and fever during septicaemia, which can follow acute infection if left untreated (Bauer and Hörmansdorfer, 1996; Van Der Wolf *et al.*, 2001). Conversely, the disease may not show any clinical symptoms in the pigs, as animals have been shown to shed Salmonella bacteria, as well as generate an immune response, in the absence of any typical clinical presentation (Rostagno *et al.*, 2011).

The highly variable nature of Salmonellosis in pigs, plus the zoonotic transmission of the Salmonella bacteria to humans which can result in infection and moderate / severe illness, mean that heavy emphasis must be placed on surveillance of the pathogen throughout pig production. The British Pig Health Scheme (BPHS), which is coordinated by the British Pig Executive (BPEX), aims to control Salmonellosis on pig farms in England (BPEX, 2011c). Current surveillance strategies involve the analysis of meat juice samples collected from the abattoir, and blood samples collected from the live pig throughout production (BPEX, 2011c). The development of an OF test would enable the screening of live pigs to occur with much greater ease than is possible at present, thus providing the rationale behind the work conducted in Chapter 4.

An assay to detect anti-Salmonella antibody in porcine OF using a commercially available test kit designed for use with serum and meat juice samples was developed. Various protocol aspects were experimentally manipulated, including sample dilution and incubation time, in line with other studies which have used similar approaches in the development of OF diagnostic assays for different disease agents (Kittawornrat *et al.*, 2012a; Panyasing *et al.*, 2014). The assay showing the highest level of antibody detection was then used to test field samples with corresponding blood sera for comparison / assessment of the OF test performance. When compared with the corresponding pen sera as the Gold Standard method of testing, the OF assay developed in Chapter 4 was found to have 56% sensitivity and 97% specificity. Interpretation of data in a zoonotic context must be approached with caution, as anti-Salmonella antibody presence did not always pertain to infection with problematic strains in pigs.

The relatively low sensitivity indicates that further optimisation of the assay will be required for introduction as a routine surveillance-type test. However, the findings show that it is possible to detect anti-Salmonella antibodies within pen-based pig OF. This therefore serves to support the hypothesis that markers of disease detectable in serum can be detected in OF via modification of the experimental test protocol.

7.4 OF as a non-invasive sample medium for the assessment of PRRS status

Porcine Reproductive and Respiratory Syndrome virus (PRRSv) is a disease which affects pig welfare and has severe economic impacts. Clinical symptoms can be separated into two categories: reproductive and respiratory. Reproductive failure is characterised by premature abortion, as well as the delivery of mummified piglets (Lewis *et al.*, 2009). The estimated cost of PRRSv-related reproductive problems in the UK is up to £107 per sow per year, based on survey results from 12 veterinary practitioners (Richardson, 2011). The same survey also addressed the respiratory symptoms, i.e. coughing associated with PRRS, reporting an estimated cost of £104 per sow per year when a PRRS vaccination programme is not implemented (Richardson, 2011). These figures illustrate the economic impacts of the disease, and must be associated with the presentation of clinical signs, linking together the economic and animal welfare impacts posed by clinical PRRSv infection.

Animals can also become infected with PRRSv without showing any clinical signs. However the immune challenge and energy investment into raising a response to infection causes pigs to perform sub-optimally. Decreased average daily gain (ADG) is often recognised in pigs with sub-clinical infection (Regula *et al.*, 2000), which leads to longer finishing periods and therefore increased feed and overhead costs. This “silent” nature of sub-clinical PRRSv infection emphasises the importance of diagnostic surveillance and monitoring of the pathogen on pig farms. Chapter 5 of this thesis investigated the suitability of pen-based OF samples for the determination of PRRS status in commercial UK pigs. These were the first UK data generated using the IDEXX PRRS X3 Antibody test kit for oral fluids (IDEXX Laboratories, Westbrook. ME).

Overall, a reasonable level of agreement was found between antibody detection in OF and the current Gold Standard of blood serum. Sensitivity and specificity values at pen level were calculated to be 90% and 76%, respectively. The specificity value was particularly low, due to the detection of anti-PRRSv antibody in pen-based OF samples but not in the corresponding pen sera. This occurred in samples taken from nursery units only and therefore raised the suspicion that maternally-derived antibody could be being detected in the OF. Confirmation of this would require repeated sequential bleeding visits in order to

eliminate the possibility of clinical infection, however the blood samples collected for this study were for diagnostic, rather than experimental purposes and thus repeated collections were not possible. Another potential explanation was that, due to the larger representation of the pen allowed by OF sampling (see Chapter 2), disease which may have been circulating at low prevalence was being detected in the pen-OFs, but not in the more limited sampling of pen sera. In order to investigate this, the number of blood samples taken per pen was increased, by assuming a disease prevalence of 20% as opposed to 40% which was the assumption for the initial farm visits. This action did result in fewer cases of antibody detection in OF but not in sera, however overall specificity remained relatively low.

An ELISA assay for the detection of anti-PRRSv antibody in porcine OF has been successfully developed based upon an original serum assay (IDEXX Laboratories, Westbrook, ME) (Kittawornrat *et al.*, 2012a). Sufficient protocol modification and reconfiguration of the test reagents has led to an OF assay which performs with 94.7% sensitivity and 100% specificity (Kittawornrat *et al.*, 2012a). The same assay has also been shown to be adequately reproducible through ring testing involving different laboratories (Kittawornrat *et al.*, 2012b). No such ring test data are available for the IDEXX PRRS X3 antibody test kit for oral fluids, therefore the data presented in Chapter 5 of this thesis must be interpreted with care. It was due to this, and the questions raised by these results, that the longitudinal study detailed in Chapter 6 was conducted. The data from the single farm visits show that it is possible to assess PRRS status in finisher pigs using pen-based OFs , however further work is required in order to explain the detection of antibody in OF, but not in blood serum in nursery pigs.

7.5 Use of OF as a longitudinal monitoring tool for tracking of disease dynamics

The longitudinal style of disease monitoring allows producers to collect detailed information about the health status of animals over time. This information could provide the basis of a better understanding of how disease agents circulate within herds. The method also presents a more realistic reflection of health on farm as opposed to one-time sample collection and analysis. Samples collected longitudinally can take into account changes occurring over time, e.g. seasonal

variation and the effects that these may have on production and herd health, and the cycles of immune suppression and resurgence of disease within production systems.

The advantages of OF sampling for diagnostics have been highlighted. The non-invasive nature of this sampling method means that it could be utilised as a tool for longitudinal monitoring as opposed to blood sampling, which is much more labour intensive and stressful to pigs. The large volume of sample obtained when OF is collected also allows many tests to be performed from that one sample, thus potentially allowing the capacity to screen longitudinally for many diseases over time. To date OF has been used to monitor PRRSv in pigs longitudinally via RT-PCR detection of PRRSv RNA (Prickett, 2008b) and this was following experimental infection of pigs with the virus. Chapter 6 of this thesis investigated the use of pen-based OF for longitudinal monitoring of anti-PRRSv antibody on commercial pig farms.

The results show that groups could be separated into one of three categories depending upon the pattern of anti-PRRSv antibody detection. Pen OFs either 1) tested strongly positive throughout the entire experimental period without diminishing, 2) tested positive followed by a decrease in S:P ratio, then became strongly positive again towards the end of the sampling period, or 3) tested positive at the beginning, then turned and remained negative during the experimental period. From these different patterns it may be possible to distinguish the detection of maternally-derived antibody from that which is the result of an actual infection with PRRSv. The cases where antibody was detected at the beginning of the sampling period and then diminished during the course of the study were likely to be due to maternally derived antibody lingering for prolonged periods within pigs. This information supports the suspicion of maternal antibody being detected in the OFs of nursery pigs as the reason for “false positives” in Chapter 5, as the corresponding blood serum samples for these cases showed either no or very few positive sera which were close to the positive cut-off value. It is likely that where anti-PRRSv antibody was consistently detected in the pen-OFs there was PRRSv infection originating from the breeding unit, which then continued by continuous reinfection throughout the study period, as the corresponding pen sera for these pigs all tested strongly positive. The third pattern of antibody detection observed

involved OFs testing positive at the beginning, then showing the diminishing pattern seen for maternally-derived antibody, however before samples became negative the S:P ratio spiked towards the end of the experimental period. This was anticipated to represent animals shedding maternal antibody in OF initially, but then encountering a PRRSv challenge later during the sampling period and therefore producing an antibody response which could be seen in the OF. In these cases corresponding sera were in agreement with the OFs and suggested a new PRRSv infection.

The findings reported in Chapter 6 illustrate the versatility of pen-based porcine OF when used longitudinally as a means to investigate disease dynamics on farms. The variety of outcomes yielded by the 8 units studied serves to illustrate the diversity of potential disease situations across all farms, the understanding and recognition of which may be facilitated by using longitudinal OF monitoring, as may the monitoring of success of subsequent intervention strategies.

7.6 Final conclusions

The research findings presented in this thesis contribute to a body of evidence showing the great potential and applicability of OF diagnostics for pig health and welfare. Investigation into collection methodologies demonstrated how the method may be successfully adapted, via providing multiple ropes for pigs to chew, for the diverse range of housing systems currently used within the UK pig industry.

A method to store OF at ambient temperature for molecular testing and diagnosis of PRRSv by RT-PCR was successfully achieved. This should facilitate adoption of the OF diagnostic approach by producers, whilst also offering superiorr sample archiving possibilities without compromising accurate and reliable results.

Large scale and longitudinal validation studies have shown that OF test performance is comparable with that of the current Gold Standard using blood serum for the detection of anti-PRRSv antibody. However, care must be taken when interpreting data as it is likely that maternally-derived, as well as infection-associated antibodies, can be detected within OF.

Appendix A: The basic principles of three commonly used ELISA test types

Blocking ELISA

The blocking, also known as direct ELISA method uses an enzyme-linked antibody (hereafter referred to as antibody conjugate) that will bind directly to the specific antigen either coated directly on the ELISA test plate, or bound to a monoclonal “capture” antibody bound to the plate. The antibody conjugate is added after the sample, so any target antibody present within the sample should bind before the antibody conjugate is added. Thus for this method, lower end readout values convey stronger sample positive results.

Indirect ELISA

Indirect ELISA tests adopt specific antigen coated test plates, however the antibody conjugate for this method is a secondary antibody. Once the sample is added, and any target antibody present bound to the antigen on the plate, the antibody conjugate will then be added and label up bound target antibody from the sample. The result from an indirect ELISA readout is therefore directly proportional to the amount of antibody present, as opposed to the previously explained blocking method. Most commercial indirect ELISA results are then expressed as sample-to-positive (S:P) ratios, which describe the proportion of assay reactivity within the sample test well relative to the internal kit positive control wells. A higher S:P ratio is indicative of a higher antibody titre in the test sample.

Capture ELISA

The third type of ELISA relevant to the current review is the “sandwich” or capture ELISA. This method uses a monoclonal capture antibody that is coated to the test plate, and will specifically bind the target antibody in the sample. A recombinant antigen is then added to bind to the target antibody. The antibody conjugate will then bind the recombinant antigen and complete the “antibody sandwich”. The use of the monoclonal capture antibody to bind the target from the sample makes the sandwich ELISA a slightly more specific option than the

blocking and indirect tests. However the majority of currently available commercial diagnostic kits adopt either the blocking or indirect ELISA methods.

Appendix B: Protocol for the analysis of porcine oral fluid samples for anti-PRRSv antibody using the IDEXX PRRS X3 antibody test kit for oral fluids (IDEXX Laboratories, Westbrook, ME)

Preparation of reagents

Wash solution

Determine the amount of Wash Solution needed for washing the microtitre plates. Dilute the Wash Concentrate (10X) 1:10 with distilled/deionised water (1 part concentrate with 9 parts water, e.g., 30ml Wash Concentrate (10X) + 270ml distilled water).

Preparation of samples

Dilute samples to be tested 1:2 with the Sample Diluent (e.g., by diluting 100µl of sample with 100µl of Sample Diluent). Do not dilute controls.

Be sure to change tips for each sample and record the position of each sample on the plate using a worksheet. Samples should be mixed prior to dispensing into the PRRSv coated wells.

Test Procedure

All reagents must be allowed to come to 18-26°C before use then mixed by inverting and swirling. Use a separate pipette tip for each sample.

1. Obtain antigen-coated plate(s) and record the sample position on a worksheet.
2. Dispense 100µl of undiluted Negative Control into two wells of the assay plate.
3. Dispense 100µl of undiluted Positive Control into two wells of the assay plate.
4. Dispense 100µl of diluted samples into appropriate wells.
5. Incubate for 30 minutes (±2 min.) at 18-26°C.
6. Aspirate liquid contents of all wells into an appropriate waste reservoir.

7. Wash each well with approximately 300µl of Wash Solution three to five times. Aspirate liquid contents of all wells after each wash. Avoid plate drying between plate washings and prior to the addition of conjugate. Following the final wash fluid aspiration, gently, but firmly tap residual wash fluid from each plate onto absorbent material.
8. Dispense 100µl of Conjugate into each well.
9. Incubate for 30 minutes (±2 min.) at 18-26°C.
10. Repeat steps 6 and 7.
11. Dispense 100µl of TMB Substrate solution into each test plate well.
12. Incubate for 15 minutes (±1 min.) at 18-26°C.
13. Dispense 100µl of Stop Solution into each well of the test plate to stop the reaction.
14. Measure and record the optical density (OD) of the plate at A(450) and at reference A(650), for samples and controls. Calculate the difference between the OD values A(450) – A(650) and use these to calculate the sample to positive (S:P) ratios for the OF samples.

Calculation of results

Calculation of Negative Control Mean, NCx

$$NCx = \frac{NC1\ (A450\text{-}A650) + NC2\ (A450\text{-}A650)}{2}$$

Calculation of Positive Control Mean, PCx

$$PCx = \frac{PC1\ (A450\text{-}A650) + PC2\ (A450\text{-}A650)}{2}$$

Calculation of test sample to positive (S:P) ratio

$$S:P = \frac{Sample\ (A450\text{-}A650) - NCx}{PCx - NCx}$$

Appendix C: Housing type, stage of sampling[a], group size and blood sample representation for each farm visit included in the study in Chapter 5

Phase	Visit number	Housing	Stage sampled	Group size	Number of blood samples collected	% Representation
I	1	Straw	Nursery	175	8	5
I	2	Slatted	Finishing	31	5	16
I	3	Slatted	X-section	50	6	12
I	4	Straw	Nursery	300	6	0.2
I	5	Straw	Finishing	50	6	12
I	6	Straw	X-section	100	6	6
I	7	Straw	Nursery	36	6	8
I	8	Slatted	Finisher	30	6	20
I	9	Straw	Nursery	300	6	0.2
I	10	Straw	Finisher	175	6	3
I	11	Straw	Nursery	200	6	3
I	12	Straw	Finisher	250	6	2
I	13	Straw	Nursery	250	6	2
I	14	Straw	Finisher	125	6	5
I	15	Straw	X-section	200	6	3
I	16	Straw	Nursery	250	6	2
I	17	Straw	Finisher	160	6	4
I	18	Slatted	X-section	50	6	12
II	19	Slatted	X-section	25	7	28
II	20	Straw	Nursery	100	14	14
II	21	Straw	Nursery	250	14	6
II	22	Slatted	X-section	30	7	23
II	23	Slatted	X-section	30	7	23
II	24	Outdoor	X-section	100	10	10
II	25	Slatted	X-section	30	14	47

II	26	Outdoor	Grower	60	14	23
II	27	Straw	Grower	250	14	6
II	28	Straw	Grower	150	14	9
II	29	Straw	Nursery	150	14	9
II	30	Slatted	X-section	30	7	23
II	31	Straw	Nursery	150	14	9
II	32	Straw	Nursery	100	14	14
II	33	Slatted	Nursery	30	10	33

[a] Farrow to finish units were sampled in cross section, where samples were collected from nursery, growing and finishing stages. For these cases, stage sampled is shown as “X-section” in the table.

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