PROOF OF CONCEPT: ORAL FLUIDS AND QUANTITATIVE ASSESSMENT FOR PORCINE CHRONIC RESPIRATORY DISEASE (PCRD) IN AUSTRALIAN FIELD CONDITIONS

A1-102

Final Report prepared for the Australasian Pork Research Institute Limited (APRIL)

By

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Executive Summary

Porcine chronic respiratory complex (PCRD) is a major issue causing high levels of morbidity and mortality on Australian pig farms, and internationally. It is a multi-faceted disease complex that causes respiratory distress in pigs, with many organisms involved in its pathogenesis, primarily antigens such as *Actinobacillus pleuropneumoniae* (APP), *Mycoplasma hyopneumoniae, Pasteurella multocida* (PM), Porcine Circovirus 2 (PCV2), *Streptocoocus suis*, and *Haemophilus parasuis*. Oral fluids (OF) are used increasingly for health diagnostics in humans and agricultural industries. The current gold standard for detection of PCRD and its known pathogens (e.g. *M. hyopneumoniae*) in pigs is through a real-time polymerase chain reaction (qPCR) assay performed on tracheo-bronchial mucus, collection of which is impractical on a farm level as it requires restraint of pigs, high labour requirements, and presents possible welfare concerns. Therefore, on farm collection and analysis of OF has become a topic of interest in surveillance of PCRD at a farm level. The current project aimed to further develop the use of OF technology in pigs in a commercial setting, to determine correlations between antigen levels in OF determined via qPCR, antibody concentrations in serum of pigs, and on-farm diagnosis of clinical and sub-clinical signs of infection (coughing, post-mortem pulmonary lesions, respiratory distress, etc.). Determining these correlations would therefore help us to develop a system for using OF as a mechanism for early detection of PCRD in commercial pigs.

This project consisted of a single experiment, conducted at Rivalea Australia's largest piggery site in Corowa, NSW. In the weaner phase, OF samples were collected from 5 pens of approximately 45 male pigs per pen, and blood samples were taken from n = 30 focal pigs for serology. Oral fluids samples were collected as per the methods of Dron et al. (2012) using a rope hung in the pen for 30 min. Cough scores were recorded twice-weekly, and blood and OF samples were taken at 5 and 9 weeks of age, as well as eye temperature (taken by thermal camera) of the focal pigs. Eighty of these pigs from the weaner phase were transferred to an individual grower-finisher facility at the Research and Innovation unit. In this facility, individual blood and saliva samples were taken and eye temperature was recorded at 12, 15, 18, and 21 weeks of age, and cough scores were recorded twice weekly. All pigs that died over the weaner and grower-finisher periods had a post-mortem examination carried out and samples of lung tissue preserved in formalin. Pigs from the grower-finisher facility were transferred to an on-site abattoir at approximately 21 weeks of age, where lungs were scored for pleurisy and pneumonia, and hot standard carcass weight (HSCW), backfat P2, and loin depth were measured. Serum samples were analysed for

antibodies against PCV2 (via antibody titration), APP (enzyme-linked immunosorbent assay; ELISA), and *M. hyopneumoniae* (ELISA). Oral fluids samples were analysed using qPCR for PCV2, APP, and *M. hyopneumoniae* in all samples, and additionally for *H. parasuis* and *M. hyorhinis* in the weaner samples only.

Most likely due to the nature of the design of the experiment and absence of major health challenges, no pigs presented conclusively with PCRD in the grower-finisher phase. Therefore, we were largely unable to determine a concentration of RNA from PCRD pathogens in OF that may be correlated with clinical or sub-clinical signs of infection. Furthermore, *M. hyopneumoniae* or its antibodies were not detected in OF or serum (respectively) for any pig at any timepoint. Antibodies against APP were detected in serum at most timepoints (although overall concentrations were low); however, APP was only detected in OF from a small number (n = 13) of pigs. Nonetheless, PCV2 antibodies were detected in serum, and DNA from PCV2 was detected in OF. There was a significant positive correlation between PCV2 antibody in serum and PCV2 DNA in OF at 18 weeks of age (r = 0.37; P = 0.046) and 21 weeks of age (r = 0.41; P = 0.028). Furthermore, the number of PCV2 DNA copies in OF at 15 weeks of age showed a positive linear correlation with PCV2 serology antibody levels (titre) at 18 weeks of age (r = 0.326); the correlation tended towards significance (P = 0.085). A dilution series was performed, and it was discovered that OF testing at a pen level may be successful in detecting PCV2 when as little as only one out of 100 pigs in a pen is infected. However, this was at an age where the PCV2 load in pigs was quite high, and at ages where viral load is lower (e.g. at 21 weeks of age), testing OF may be less sensitive.

In conclusion, these results show that measurement of PCV2 in OF in pigs may be used as an indicator for likelihood of infection and this knowledge will aid in the development of rapid on-farm diagnostic tests using OF. Further investigation is required in a more commercial setting, or using challenge models, with grower-finisher pigs housed in large groups, and in winter periods where PCRD may be more prevalent.

Table of Contents

1. Introduction

The non-specific diagnosis of Porcine Chronic Respiratory Disease (PCRD) in pigs often involves key pathogens such as *Actinobacillus pleuropneumoniae* (APP), *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, Porcine Circovirus 2 (PCV2), *Streptocoocus suis*, *Haemophilus parasuis,* and potentially *Bordetella bronchiseptica,* and *Mycoplasma hyorhinis*. Clinical signs of this respiratory disease complex often manifest themselves in non-specific coughing, fever, and increased mortalities resulting in poor feed conversion ratio (FCR) and poor average daily gain (ADG), as well as increased medication costs in growing pigs. Seasonal fluctuations of disease severity are commonly associated with housing in poorly ventilated sheds where shed closure during the colder months to improve thermal comfort decreases air quality dramatically.

The gold standard sample for ante-mortem assessment of *M. hyopneumoniae* prevalence in individual pigs consists of collection and submission of tracheo-bronchial mucus and a real time polymerase chain reaction (qPCR) assay (Vangroenweghe et al., 2015). Collection of tracheo-bronchial mucus from individual pigs is highly impractical and can only be performed on a small number of animals. Alternative ante-mortem diagnostic testing for *M. hyopneumoniae* include testing for antibodies which has also been identified as not very reliable to assess herd prevalence, changed management, or vaccination strategies. It is important to note that the seroconversion timing for *M. hyopneumoniae* is difficult to predict as vertical (dam to offspring) and horizontal (offspring to offspring). Transmission is slow and can take up to 14-21 days if horizontal, but it has been demonstrated that gilts can shed *M. hyopneumoiae* for as long as 240 days (Maes et al., 2018). Identification of persistently infected pigs or their prevalence after weaning would help veterinarians with management, vaccination, and improve medication strategies.

Oral fluids (OF) have been used for diagnosis of respiratory pathogens in humans since the early 1900s (Pollaci et al., 1909; cited by Prickett et al., 2008), and more recently in domestic animals (Costa et al., 2012; Fekadu et al., 1982; Giménez-Lirola et al., 2013; González et al., 2017; Neto et al., 2014; Prickett et al., 2010; Smith et al., 2004; Smith et al., 2005). Recently, OF have been used successfully for surveillance of agents of PCRD, or antibodies to them, in commercial pig

populations (Bjustrom-Kraft et al., 2018; Prickett et al., 2008; Prickett et al., 2010). A pooled sample of OF collected from cotton ropes within a pen may be an effective strategy to monitor antigen or antibody levels on a pen basis (Prickett et al., 2008). Real time PCR may be one way to quantify antigen levels in OF in order to detect PCRD early in commercial herds. Antigens such as porcine influenza virus (SIV), PCV2, porcine reproductive and respiratory syndrome virus (PRRSV) and APP (Allan et al., 2000; Heinen et al., 2001; Prickett et al., 2008; Shibata et al., 2003; Wills et al., 1997) have all been previously detected in OF, and monitoring these pathogens in commercial herds via the pooled rope collection method has shown success (Costa et al., 2012; Dufresne, 2011; Prickett et al., 2008).

The current project aimed to demonstrate a close correlation between the number of DNA copies of primary and secondary respiratory pathogens in OF with clinical symptoms, alternative diagnostic methods like histopathology, serology, and final evaluation of lung pathology at slaughter.

2. Methodology

All on-farm experimental methods were approved by the Rivalea Animal Ethics Committee (protocol number 18V055C) in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013). The experimental data collection ran from December 2019 until March 2020, in the Australian summer-autumn period.

2.1 On Farm Data Collection

2.1.1 Weaner Phase

Approximately *n* = 225 male weaner pigs (Primegro™ Genetics, Large White x Landrace F1 cross, Corowa NSW, Australia; weaned at approximately 26 days of age) were commercially housed in group pens (5 pens with ~45 pigs/pen) at Rivalea's Module 5 site (Corowa, New South Wales). At 5 and 9 weeks of age, OF samples were collected from each pen by allowing pigs to chew on cotton ropes in the pen (2 ropes per pen, ~20-25 pigs/rope). At the time of OF sampling, a pen cough score was recorded (by counting the number of coughs heard from that pen in 30 sec) and an individual blood sample was obtained from a cohort of pigs (*n* = 30, 5 from each pen). Blood samples were collected in a vacuum tube containing a clot activator (Vacutainer®; BD, Franklin Lakes NJ, USA) and left to clot for >2 hours. Blood samples were then centrifuged at 2,000-3,000 x *g* for 5 mins to extract serum, and frozen at -20°C until further analysis. Eye temperature was recorded for each pig just prior to blood sampling after the pig was secured with a snare, using a hand-held thermal camera (FLIR Ex Series E6; FLIR® Systems Inc., USA). Cough scores were collected twice weekly (on Tuesday and Friday), measured as number of coughs per pen in 30 sec.

2.1.2 Grower-Finisher Phase

A total of *n* = 80 castrated male pigs (Primegro™ Genetics, Corowa NSW, Australia) from the weaner group were individually housed in pens in the individual grower-finisher research facility (Grower Discovery Centre) at Rivalea's Corowa site, under otherwise commercial conditions. At 12, 15, 18, and 21 weeks of age, an individual OF sample was collected from each pig, where possible, using the methods of Dron

et al. (2012). At 12, 15, 18, and 21 weeks of age, eye temperature was recorded just prior to blood sampling, and an individual serum sample was obtained (from all 80 pigs, but only 30 serum samples were sent for analysis due to budget constraints) for serology analysis. Samples were collected, processed, and stored as described above. An individual cough score (coughs counted over 30 sec) was recorded for each pig twice weekly (on Tuesday and Friday).

All pig mortalities during the weaner and grower-finisher phases were recorded and post-mortem necropsy was carried out on each pig that died during the experiment. A sample of lung and bronchial lymph node tissue was collected from each (n = 30) and preserved in formalin for histopathological examination.

2.1.3 Oral Fluids Collection

Oral fluids were collected according to the methods of Dron et al. (2012). Briefly, cotton ropes (approx. 30 cm in length) were hung on the walls inside each pen (Figure 1), so that they fell approximately at shoulder height of the animals in the pen. Ropes were placed on the gate of each pen such that pigs from neighbouring pens could not access the rope. Pigs were allowed to manipulate and chew on the rope for 30 min before the rope was removed and OF were extracted. Oral fluids were extracted by squeezing the wet end of the rope and wringing the fluid into a snap-lock bag. Oral fluids were then poured into separate sterile specimen containers and frozen at -20°C until being sent for analysis. For the weaner phase of the experiment, the 2 samples per pen were pooled for analysis.

Figure 1: A cotton rope hung on a wall inside a commercial pen for collection of oral fluids (OF).

2.1.4 Abattoir Data and Sample Collection

Prior to sale, pigs were each given an individual tattoo (3-digit number) to allow for identification at the abattoir. At the abattoir (on-site; Rivalea Australia, Corowa NSW, Australia), samples of lung and lymph node tissue were collected and stored at -20°C before being sent off for analysis.

At slaughter, all lungs were scored for severity of pneumonia using the system developed by Goodwin et al. (1969), with scores out of 55, 0 being no signs of pneumonia, and 55 being all lung lobes completely affected. Pleurisy was also scored for each pig from 0 to 3; 0 = no pleurisy, 1 = mild pleurisy, 2 = medium pleurisy, and 3 = extreme pleurisy. Pneumonia and pleurisy were scored by the same technician for each pig. Tracheal swabs (*n* = 20 random carcasses) were collected at slaughter from each pig using a sterile cotton swab (Eurotubo®; Deltalab, Barcelona, Spain) which was stored on ice until further analysis. Samples of lung and bronchial lymph node from all carcasses were collected and stored in formalin for histopathological examination. All oral, tissue, and serum samples were sent to ACE Laboratories (Bendigo, Vic) for further analysis.

2.2 Laboratory Analysis

2.2.1 Histopathology

Lung and bronchial lymph node samples that were collected from pigs at the abattoir from a subsample of pigs (*n* = 30) were sent for macroscopic and microscopic histopathological diagnostics. Formalin fixed samples were processed and stained with H & E (Hematoxylin and Eosin) Stains and examined by the veterinary pathologist.

2.2.2 Analysis of Oral Fluids

Each OF sample collected in the weaner phase of the experiment was tested for pathogens by qPCR for *M. hyopneumoniae,* APP, PCV2, *M. hyorhinis,* and *H. parasuis*. Oral fluid samples from the grower-finisher phase and tracheal swabs from the abattoir were then tested individually by qPCR for *M. hyopneumoniae,* PCV2, and APP.

2.2.2.1 DNA Extraction

The DNA was extracted from OF samples (either from pooled pens or individual animals) using the MagMax™ CORE Nucleic Acid Purification Kit (Applied Biosystems™; Thermo-Fisher Scientific; Waltham MA, USA) following 'Workflow B' of the manufacturers protocol, while DNA was extracted from tracheal swabs following 'Workflow A'. Extracted DNA was stored at -20°C until required for PCR.

2.2.2.2 PCR Amplification

All DNA amplifications for qPCR were performed on the ABI 7500 fast real time PCR machine (Thermo-Fisher Scientific, Waltham MA, USA). Commercial kits or in-house ACE prescribed methods were used for amplification, as follows:

- *M. hyopneumoniae* – VetMax™ *M. hyopneumoniae* PCR kit (Thermo-Fisher Scientific, Waltham MA, USA). Note that DNA was diluted 1:5 for this method;
- PCV2 – PrimerDesign PCV2 Advanced Detection Kit (Integrated Sciences, Chatswood NSW, Australia);
- APP – ACE in-house protocol (all serovars detected);
- *H. parasuis* – ACE in-house protocol (all serovars detected);

- *M. hyorhinis* – ACE in-house protocol (SYBR detection).

All PCR methods were verified to detect the correct strains and not cross react with other microorganisms. Verification was performed by bioinformatics (ACE in-house methods only – primer sequences are not provided by kit manufacturers), as well as testing with known purified cultures and/or known positive controls. More information on the in-house PCR methods used by ACE can be supplied upon request.

All qPCR assays were performed with a standard curve for quantitation of the target with the exception of *M. hyorhinis*. Quantified positive controls for the standard curve were either created by the kit manufacturer or by dilutions of a known positive pure culture. However, this was not possible for *M. hyorhinis* as a pure culture was not achievable. Internal controls were used in order to detect PCR inhibition.

2.2.2.3 Sensitivity Testing – Dilution Series

In order to determine what proportion of a herd needs to be positive to be detectable by OF sampling, a dilution series of a PCV2 positive OF sample, diluted in PCV2 negative OF was performed (collected from unrelated animals known to be negative for exposure to PCV2). The positive OF was diluted in the negative OF at four dilutions: 1:5, 1:10, 1:50, and 1:100. The pooled OF samples were then extracted and amplified as per the protocols described above for all dilutions. All quantities for the qPCR results were recorded as copies per µL of OF.

2.2.3 Serology

The testing for APP, *M. hyopneumoniae* and PCV2 were conducted using commercially available ELISA kits. All kits followed the same basic principles. These assays are designed to measure the relative amount of antibody in porcine serum. Antigen is coated on to 96 well plates. Upon incubation of the test sample in the coated well, antibody specific for the bacteria/virus forms a complex with the coated antigens. After washing away the unbound materials from the wells, a conjugate is added which binds with any attached porcine antibody in the wells. Unbound conjugate is washed away and enzyme substrate is added. Subsequent colour development is directly related to the amount of antibody present in the test sample. For all of the assays the presence or absence of antibody is determined by

relating the colour density of the unknown to the Positive control mean. The Positive control is standardised and represents significant antibody levels in porcine serum. The relative amount of antibody in the sample is determined by calculating the sample to positive (S/P) ratio and reported as a S/P%, S/P or end point titre which is also calculated from the S/P.

A commercial indirect enzyme-linked immunosorbent assay (ELISA) kit (IDScreen® APP Screening Indirect; IDvet, Grabels, France) was used to quantify *A. pleuropneumoniae* (serovars 1 to 12) specific antibodies in each serum sample according to the manufacturer's instructions. *A. pleuropneumoniae* serovar 15 is the serovar found to cause the majority of respiratory disease at Rivalea's Corowa site, which serologically cross reacts with serovars 3, 6, and 8 (Gottschalk et al., 2010).

From the APP ELISA results, the sample to positive ratio (S/P%) was obtained for each serum sample, calculated as:

$$\text{SP Ratio} = \frac{(Sample\ optical\ density\ (OD) - negative\ control\ OD)}{(Positive\ control\ OD - negative\ control\ OD)} \times 100$$

For the APP assay, any sample obtaining an S/P ratio between 30-40% was considered 'suspect' and any sample obtaining an S/P ratio above 40% was considered 'positive'. These values were 40%-50% as 'suspect' and above 50% as 'positive' for the APP ELISA.

The IDEXX *M. hyopneumoniae* antibody test kit (IDEXX Laboratories Pty Ltd, Mount Waverley Vic, Australia) was used to quantify the *M. hyopneumoniae* antibodies in each serum sample. The relative amount of antibody in the sample is determined by calculating the sample to positive (S/P) ratio as:

$$\text{SP Ratio} = \frac{(Sample\ optical\ density\ (OD) - negative\ control\ OD)}{(Positive\ control\ OD - negative\ control\ OD)}$$

Negative samples had a S/P ratio of <0.30, the suspect range is 0.30 - =/<40 and samples having a ratio above 0.40 were considered positive.

Porcine circovirus 2 (PCV2) was analysed for anti-PCV2 antibodies using the Biochek Porcine Circovirus type 2 Antibody test kit (Biochek BV, The Netherlands). The relative amount of antibody in the sample is determined by calculating the sample to positive (S/P) ratio as:

$$\text{SP Ratio} = \frac{(Sample\ optical\ density\ (OD) - negative\ control\ OD)}{(Positive\ control\ OD - negative\ control\ OD)}$$

The end point titre was calculated using the following equation:

$$\text{Log}_{10}\ \text{Titre} = 1.1 * \text{Log}_{10}(\text{S/P}) + 3.361$$

A titre of > 1070 was considered 'positive'.

2.3 Animal Management and Farm Health Status

The commercial herd used in this study is endemic for respiratory pathogens *M. hyopneumoniae*, PCV2, and APP (mainly serovar 15). Pigs were vaccinated against *M. hyopneumoniae* (Ingelvac MycoFLEX®) and PCV2 (Ingelvac CircoFLEX®; both Boehringer Ingelheim, Macquarie Park NSW, Australia) at approximately 3 weeks of age, just prior to weaning. Pigs were vaccinated against APP at 9, 12, and 16 weeks of age (autogenous killed APP vaccine; DEDJTR, Victoria, Australia).

The weaner shed used in the current study was located at Rivalea Australia's Module 5 facility. Pens were heated with heat lamps hanging from above the pen, and temperature in the room was controlled by automatic blinds set to the optimum temperature for the age of the pigs. Each pen had several drinker nipples for *ad libitum* water access, and pigs had *ad libitum* access to commercial weaner diets through group feeders in the centre of the pen. Pens floors were slatted with a solid area at the front centre of the pen, and effluent was removed from the shed through flusher pits below pens. All pigs were housed in the one weaner room, with all pigs in the same room all weaned in the same week. In this system, each weaner room represented one age (weaning week) of pigs, and pigs remained in this room until they were moved into the grower-finisher facility.

Pigs were transferred to the individual grower-finisher facility by truck at approximately 9 weeks of age. This facility was located at a separate farming unit (Research & Innovation unit) on the same site and included 112 pens, of which 80 pens side-by-side were used for experimental pigs, and others were kept empty. Pigs had access to a drinker nipple and *ad libitum* access to commercial grower and finisher diets through a single feeder located in each pen. Pens were slatted at the back with a solid area at the front near the feeder, and nose to nose contact with pigs in neighbouring pens was allowed through barred walls between pens. Temperature of the shed was controlled through automated vents at the top of the side walls of the shed and misting fans. At entry to the shed, pigs were individually injected with tulathromycin (Draxxin®; Zoetis, Rhodes NSW, Australia) for treatment of respiratory pathogens, as per commercial procedures for this facility. Lincomycin (Lincomix® 800 soluble powder; Zoetis, Rhodes NSW, Australia) was administered through the waterlines when the pigs were 14 and 15 weeks of age, as per normal commercial procedures for control of *M. hyopneumoniae*. Pigs were immunologically castrated with Improvac® (Zoetis, Rhodes NSW, Australia). At sale, pigs were sent to an on-site abattoir at approximately 22 weeks of age, 1 week after the final measurements and OF samples were taken and marketed as per commercial procedures.

2.4 Statistical Analysis

From the cough scores obtained, a coughing index was calculated for the pigs that moved into the grower-finisher facility, which was calculated as the average percentage of pigs coughing per 30 sec of observation, based on a variation of the calculation used by Nathues et al. (2012). Cough scores are reported as arithmetic means from the raw data. Eye temperature data were analysed over several timepoints as a linear mixed model using the MIXED procedure of SPSS. Eye temperatures in the weaner facility and the grower-finisher facility were analysed as separate cohorts of data, as different pigs were sampled in each period. In the weaner period, as different pigs were measured at 5 and 9 weeks of age, a simple model was fitted with timepoint as a fixed effect (Eye temperature = timepoint[fixed] ± error). Pen was tested as a random effect (fitting an auto-regressive [AR1] heterogenous covariance structure) but did not have a significant effect ($P \geq 0.05$) and was therefore left out of the final model. In the grower-finisher period, timepoint was fitted as the repeated measure and pig as the subject

assuming an AR1 heterogenous covariance structure (Eye temperature = timepoint[repeated] + pig[subject] ± error).

Correlation analysis was performed using the CORRELATIONS procedure of SPSS (IBM SPSS Statistics, version 25; IBM, Armonk NY) to obtain Pearson's correlation coefficient and determine the significance of the linear correlation. For non-linear correlations between serology and OF results (where applicable), R^2 value was calculated using Microsoft Excel. All other results are represented as descriptive statistics calculated from the raw data. Results from pigs that presented with any clinical or sub-clinical signs of PCRD (namely, pleurisy at the abattoir) were also compared to the average result for the other 'healthy' pigs as an additional analysis. Results are reported as mean ± standard error of the mean (SE), and $P < 0.05$ was considered significant.

3. Outcomes

3.1 On-Farm Data Collection

3.1.1 Mortality and Post-Mortem Results

In the weaner phase, one pig died of unknown causes, which could not be further explained during the post-mortem analysis (no gross respiratory lesions were observed). Two pigs died in the grower-finisher phase, one at 11 weeks of age, and the other at 20 weeks of age. Post-mortem analysis revealed that the pig that died at 11 weeks of age had an empty stomach at the time of death and scours were observed in the individual pen. There were no post-mortem lung lesions observed in this pig. Similarly, for the pig that died at 20 weeks of age, the post-mortem analysis revealed that this pig may have died from a twisted gut and was not showing any signs of respiratory infection.

No significant macroscopic changes in lung tissue were observed from pigs that died throughout the course of the on-farm portion of the experiment. However, in the weaner phase, several microscopic changes were observed in the one pig that died during this period. Bronchi and bronchioles contained high numbers of degenerate white blood cells (presumed neutrophils), which extended into the adjacent alveoli and throughout extensive areas of the lung. These were admixed with moderate amounts of eosinophilic proteinaceous material. In many areas the tissue was markedly congested and haemorrhagic. Interlobular septae and the pleura also showed extensive signs of inflammation. These microscopic signs were diagnosed as severe, acute, neutrophilic, haemorrhagic pleuropneumonia. Such inflammation would be consistent with a bacterial infection (e.g. from APP; Dron et al., 2012; Marsteller et al., 1999). The lung sections from the other two pigs that died in the grower-finisher phase did not exhibit any significant diagnostic histological changes.

These low morbidity and mortality rates were unexpected, as PCRD and its associated antigens are endemic to the herd used in the current study (e.g. APP, *M. hyopneumoniae*, and PCV2). However, given that pigs were housed from 10 weeks of age in an individual facility and medicated when required through the water lines as per commercial production herd health program, it is unsurprising that most pigs remained clinically healthy in the current study. High stocking density has been well reported as an important risk factor for transmission of respiratory pathogens (Dron

et al., 2012; Gardner et al., 2002; Maes et al., 2008; Pointon et al., 1985), hence this may have been a factor in the relatively low prevalence of PCRD pathogens seen in the current study. This individual housing likely would not have allowed for frequent nose to nose contact, which would reduce the likelihood of transmission between animals.

Unfortunately, pigs were not weighed in the current study. This may have been important information as infection with *A. pleuropneumoniae* (Hoflack et al., 2001; Holmgren et al., 1999; Straw et al., 1990), PCV2 (Kekarainen et al., 2015), and *M. hyopneumoniae* (Holst et al., 2015; Maes et al., 2018) have all been associated with a reduction in weight gain of growing pigs; however, we did not find any signs of 'wasting' in any pigs, and carcass weights were within normal limits. Similarly, feed intake data would have been another useful measure that could indicate PCRD (Sassu et al., 2018) but was not measured in this study. Daily welfare checks were carried out and no pigs were identified as not eating, which is another indicator that PCRD was not prevalent in the experimental population.

3.1.2 Cough Scores and Eye Temperatures

Average eye temperatures at each timepoint from the raw data are shown in Figure 2. From the results of the linear mixed models for eye temperature, there was a significant effect of timepoint in the weaner phase ($P = 0.004$), with pigs averaging an eye temperature of 34.5 ± 0.21°C at 5 weeks of age, and 33.7 ± 0.21°C at 9 weeks of age. In the grower-finisher phase, timepoint was also significant ($P < 0.001$), with average eye temperature 36.1 ± 0.09°C, 35.6 ± 0.12°C, 36.2 ± 0.16°C, and 35.3 ± 0.13°C, at 12, 15, 18, and 21 weeks of age, respectively.

No pigs showed signs of fever (40.5-41.0°C) at any timepoint during this study in terms of eye temperature, which is a predominant sign of APP and other respiratory infections (Sassu et al., 2018). Pleuropneumonia in pigs can be characterised by a fever followed by a subsequent drop in body temperature (Sassu et al., 2018), which was not observed in any pigs in the current study.

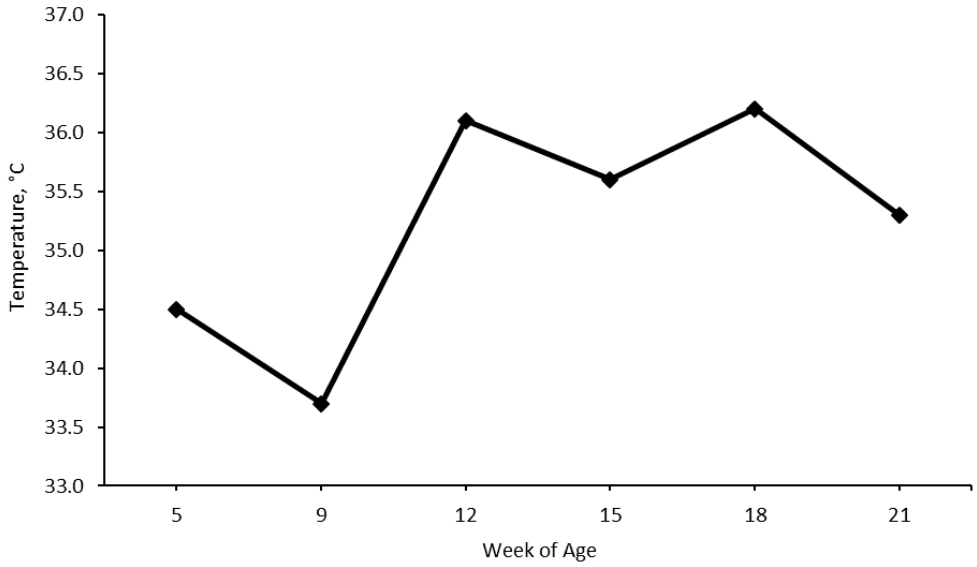

Figure 2: Arithmetic means of eye temperatures (˚C) in pigs from 5 to 21 weeks of age. Temperatures were taken from a subset of pigs in the weaner phase (*n* = 30; *n* = 5 pigs per pen) and all pigs in the grower-finisher phase (*n* = 80).

Coughing is a clinical sign of infection from *A. pleuropneumoniae* (Straw et al., 1990) and *M. hyopneumoniae* (Garcia-Morante et al., 2016; Sibila et al., 2009), among other respiratory pathogens. Results from the cough scoring data are shown in Figures 3 and 4 (presented as means from the raw data). Cough scores seemed to be highest around the time that pigs entered the new facility, which could have been due to stirring of dust as pigs entered a clean environment. At most timepoints, only 1 or 2 pigs had a cough score of > 0 in the grower-finisher facility, and the impact of this result on the overall proportion of pigs coughing can be seen in Figure 4, being 0 at most timepoints and not exceeding 3% of pigs. Signs of PCRD can often be exacerbated by poor air quality from build-up of particulate matter (such as dust) or ammonia (Michiels et al., 2015). Given that the air quality in the grower-finisher facility was ideal due to its ventilation system, coupled with the low stocking density and the fact that the PCRD pathogens measured were largely not detected, the low cough scores that were observed in the current study were unsurprising.

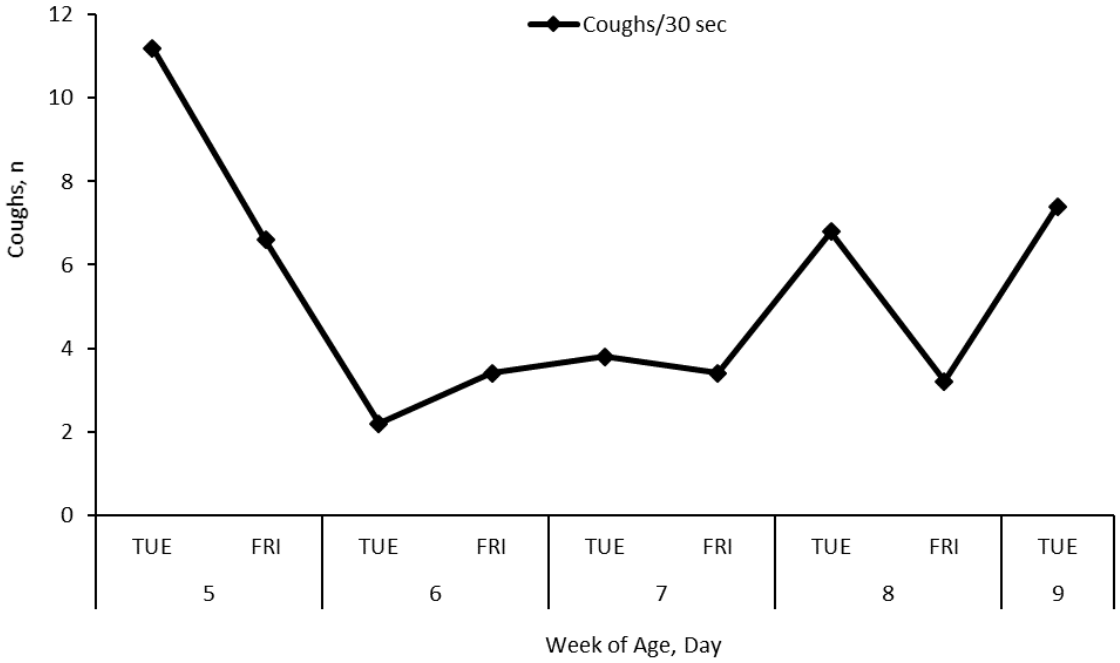

Figure 3: Arithmetic means of weekly cough scores (number of coughs per 30 sec) in the weaner phase.

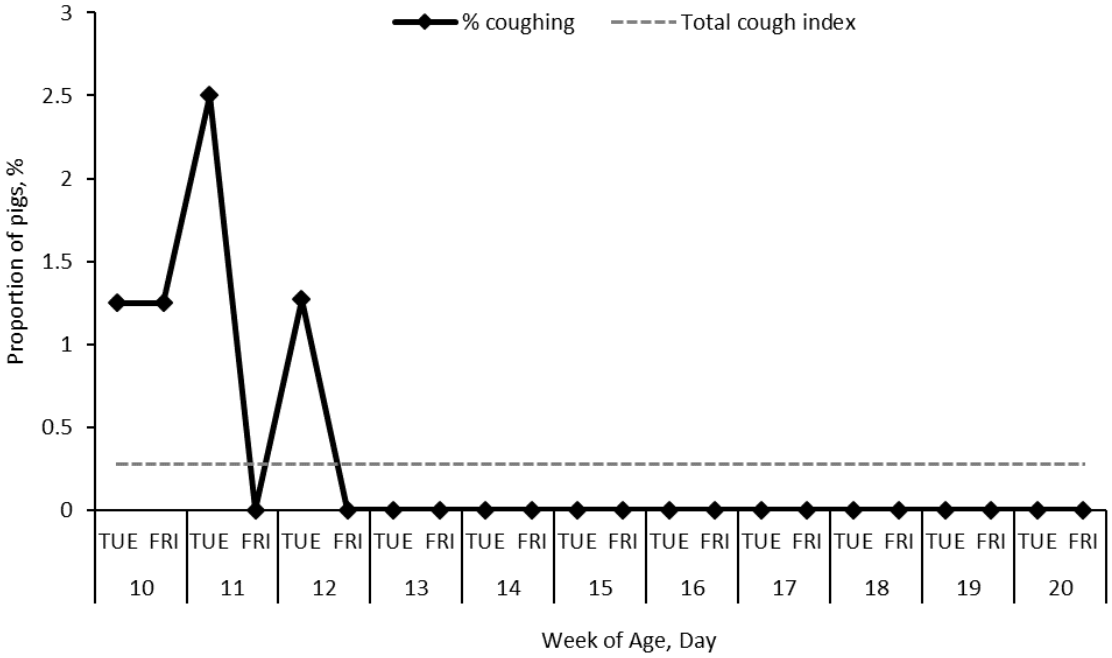

Figure 4: Weekly cough scores presented as the proportion of pigs coughing per 30 sec in the grower-finisher phase (Nathues et al., 2012). Coughs from individual pigs were counted at different times and the proportion of pigs coughing in 30 sec of all experimental pigs (*n* = 80) was calculated.

Given that the overall presence of PCRD in this particular herd is high it was surprising that little to no pigs in this experiment showed signs (clinical or subclinical) of PCRD. However, morbidity and mortality from PCRD in this herd usually occurs in the grower-finisher phase. Given that pigs were housed in individual housing, with ideal (controlled) environmental conditions and sufficient vaccination and medical treatments, this may have minimised the spread of antigens such as APP, and therefore may be why morbidity and mortality rates due to PCRD and associated pathogens was so low in the current experiment.

3.2 Abattoir Data Collection and Histopathology

The mean hot standard carcass weight (HSCW) of all pigs was 82.4 ± 0.90 kg (*n* = 72; mean ± SE), carcass P2 backfat thickness was 12.9 ± 0.26 mm, and loin depth at the P2 site was 54.4 ± 0.83 mm. Lungs from 6 pigs were unable to be observed at the abattoir. Pleurisy was observed in a total of 25 pigs, either mild (*n* = 2), medium (*n* = 5), or extensive (*n* = 18). Pigs that presented with extensive pleurisy had a lung score of 0 as the lung surface was torn away when stuck to the rib cage. These lung scores were not included in the averages calculated. The average lung score was 4.1 ± 0.64 (*n* = 53; lungs from one pig were missed at the abattoir), with the maximum lung score of all pigs being 20 (*n* = 1).

From the lung and lymph node samples collected at the abattoir, there was only one pig showing histological signs of respiratory distress. There was evidence to suggest an acute, mild to moderate neutrophilic bronchopneumonia (consistent with a bacterial infection) in the lungs of this pig, and pleurisy was observed at the abattoir. On microscopic examination, there were occasional, scattered, small focal areas observed where bronchioles and alveoli contain low to moderate numbers of neutrophils. However, the lymph node of this pig was within normal size limits. Other tissues (from the rest of the pigs) were all within normal limits, and there were no other significant diagnostic changes in any of the tissues examined.

Carcass weights were within normal limits for all pigs, perhaps an indication of good respiratory health in these animals, and may suggest that there was no reduction in weight gain or potentially feed intake due to respiratory infections, as is usually seen in outbreaks of PCRD (Kekarainen et al., 2015; Maes et al., 2018; Straw et al.,

1990). Infection with respiratory pathogens such as APP or *M. hyopneumoniae* result in destruction of the lung tissue and hence pneumonia, with leaking of pulmonary capillaries causing pleurisy (Maes et al., 2018; Marsteller et al., 1999). Combined infections with other respiratory pathogens in PCRD result in even more severe lesions (Amass et al., 1994; Marois et al., 2009; Opriessnig et al., 2004). The lack of gross and histopathological lung lesions may explain why little coughing was observed in the current experiment, as coughing generally occurs as a direct consequence of lung necrosis in PCRD (Garcia-Morante et al., 2016; Maes et al., 2018). Collectively, results taken at the abattoir further suggest that these pigs were only minimally impacted by PCRD.

3.3 Serology

3.3.1 Weaner Phase

No pigs were seropositive for *M. hyopneumoniae* at 5 or 9 weeks of age, with the exception of one suspect and one positive pig at 5 weeks of age. It is most likely that these pigs were exhibiting maternal immunity, and/or immunity in response to vaccination at this age (Maes et al., 2018). The average S/P ratio for pigs that were blood sampled in this phase was 10 ± 2.6 at 5 weeks and 2 ± 0.8 at 9 weeks of age. For APP, 25/30 pigs (83%) were either positive or suspect for APP antibodies at 5 weeks of age, and 13/30 pigs (43%) were positive or suspect at 9 weeks of age. The average S/P ratios for APP serology were 119 ± 11.3% and 39 ± 7.7% at 5 and 9 weeks of age, respectively. At 5 weeks of age, the APP-specific antibody concentrations in pig sera are most likely of maternal origin (Marsteller et al., 1999). However, by 9 weeks of age, maternal antibodies would be waning, and shedding of APP usually occurs around 11 weeks of age (Dron et al., 2012; Marsteller et al., 1999; Sassu et al., 2018).

At 5 weeks of age, 28/30 (93%) pigs returned a positive antibody titre for PCV2, and this was the same at 9 weeks of age. The average antibody titre was 2823 ± 284.5 and 4026 ± 449.4 at 5 and 9 weeks of age, respectively. Porcine circovirus 2 is ubiquitous in nature, and therefore the infection of most pigs in the current study is unsurprising (Kekarainen et al., 2015; Segalés et al., 2013). These circulating PCV2 antibodies may also likely be a result of vaccination of these pigs just prior to weaning, and from maternally derived immunity acquired from colostrum

(Kekarainen et al., 2015; McKeown et al., 2005; Ostanello et al., 2005). The vaccine used in the current study to protect against PCV2 (Ingelvac CircoFLEX®) has been shown to prevent viraemia, which is not always the case with other PCV2 vaccines (Figueras-Gourgues et al., 2019). Regardless, it is likely that without the added challenge of *A. pleuropneumoniae* or *M. hyopneumoniae* co-infection, these pigs were able to fight PCV2 infection and not develop clinical disease (Kekarainen et al., 2015).

3.3.2 Grower-Finisher Phase

Serology results for *M. hyopneumoniae*, APP, and PCV2 antibody levels in the grower-finisher period are shown in Figure 5 (along with weaner results). Unfortunately, serology was not able to be conducted on blood samples collected at 12 weeks of age. None of the pigs for which immune serology was carried out (*n* = 30) were positive for *M. hyopneumoniae* at any age in the grower-finisher phase. One pig delivered a suspect result for *M. hyopneumoniae*, with an S/P ratio of 0.39 at 21 weeks of age (pen 16). The incidence of APP seroconversion (proportion of pigs considered APP positive) was 13.3% (*n* = 4/30), 20.0% (*n* = 6/30), and 23.3% (*n* = 7/30) at 15, 18, and 21 weeks of age, respectively. There were a further *n* = 3 samples considered 'suspect' for APP under the cut off values for the ELISA, which were treated as positive for the statistical analysis. All pigs were positive for PCV2 in this phase.

Pigs will not seroconvert for *M. hyopneumoniae* infection until about 6 weeks post infection, as first infection usually occurs in the farrowing house, and generally as a result of shedding of the bacteria from primiparous sows and their progeny (Maes et al., 2018). The young age of the primiparous sows results in shedding through farrowing and lactation of their first litter, leaving their progeny susceptible to infection, especially since it is believed that colostrum-derived immunity may be lacking in these progeny (Rooke et al., 2002). Therefore, progeny from multiparous sows may be exposed to *M. hyopneumoniae* from their gilt progeny counterparts at weaning, before they have had a chance to develop appropriate protective immunity (Maes et al., 2018). Unfortunately, in the current study, it was not recorded which pigs were progeny of primiparous sows and which were the progeny of multiparous sows. Previous studies have shown that at least 17% of the breeding herd at Rivalea may consist of GP (Craig et al., 2017), so it may be reasonable to

assume that this was the case with the pigs chosen for the current study. Initial group colonisation with *M. hyopneumoniae* (e.g. in the farrowing house from progeny born to primiparous sows, or at weaning when mixing occurs) has been shown to determine the patterns of infection and disease in later stages of growth (Fano et al., 2007). It is difficult to predict the incubation period of *M. hyopneumoniae*, but seroconversion is thought to occur 7 to 42 days post-infection (DPI; Leon et al., 2001; Maes et al., 2018). Therefore, we can conclude that these pigs may not have been exposed to significant levels of *M. hyopneumoniae* in this instance. This would have been assisted by vaccination and treatment of this pathogen (along with other respiratory pathogens) in these pigs as per common commercial procedures.

Antibodies for APP appearing in the grower-finisher facility indicate immunity in response to infection, as maternal immunity is most likely to have completely waned at this age (Dron et al., 2012; Sassu et al., 2018). Serum antibody tests for APP alone are often questioned for their ability to detect subclinical infections (Dron et al., 2012; Montaraz et al., 1996), mostly due to the high variation between virulence factors of each APP serotype, and the stages of immune competence in the pig (i.e. to distinguish between maternal immunity vs. acquired immunity, for example).

Since PCV2 is ubiquitous in nature, and maternally derived antibodies would have waned by 15 weeks of age (Kekarainen et al., 2015), it is likely that the high titre values from this age onwards indicate a persistent PCV2 infection in these pigs and are likely as a result of infection rather than maternally- or vaccine-derived immunity.

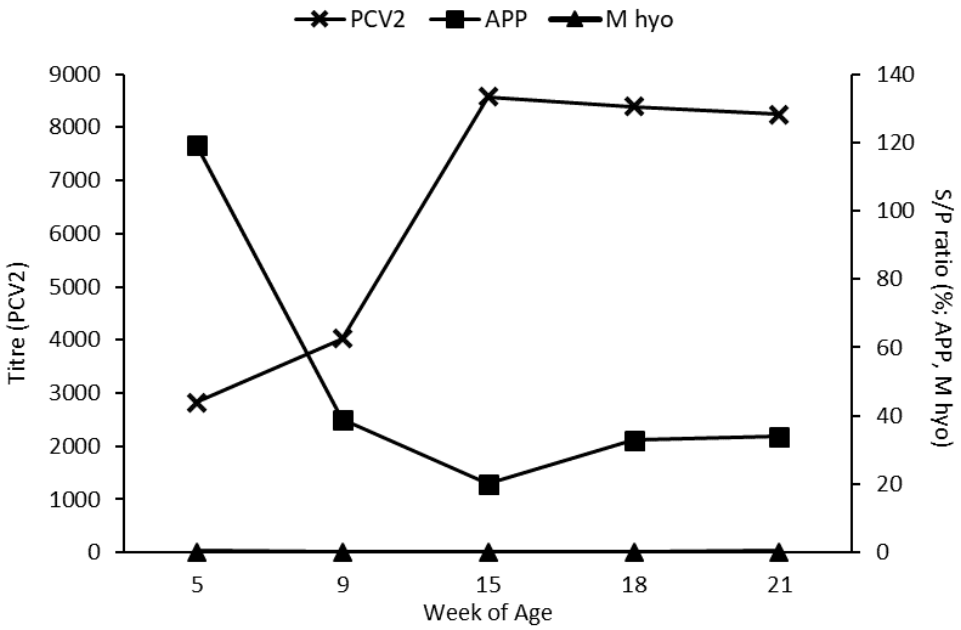

Figure 5: Arithmetic means for serology results for Porcine circovirus type 2 (PCV2; antibody titre), *Actinobacillus pleuropneumoniae* (APP; antibody enzyme-linked immunosorbent assay [ELISA] sample to positive ratio [S/P ratio]), and *Mycoplasma hyopneumoniae* (M hyo; antibody ELISA S/P ratio).

3.4 Oral Fluid PCR Results

3.4.1 Weaner Phase

In the weaner period, high quantity PCR results were observed for PCV2, *H. parasuis* and *M. hyorhinis*, while APP and *M. hyopneumoniae* were barely detected (*n* = 2 samples from 5 weeks of age were below the usual PCR cut off point for APP, but these values were left in for the analysis). Arithmetic means for the qPCR results (number of DNA copies per µL) for APP and PCV2 in OF at each experimental timepoint (weaner and grower-finisher phase) are shown in Figure 6.

All OF samples from the weaner phase were positive for *M. hyorhinis*, with an average C_t value of 27.3 ± 0.26 at 5 weeks of age, and 26.7 ± 1.00 at 9 weeks of age. *Haemophilus parasuis* was also detected in OF samples in the weaner phase, averaging 6799 ± 1941 copies/µL at 5 weeks of age, and 613 ± 202 copies/µL at 9 weeks of age. This was expected as *H. parasuis* is a normal coloniser of the upper respiratory tract in pigs (Costa et al., 2012).

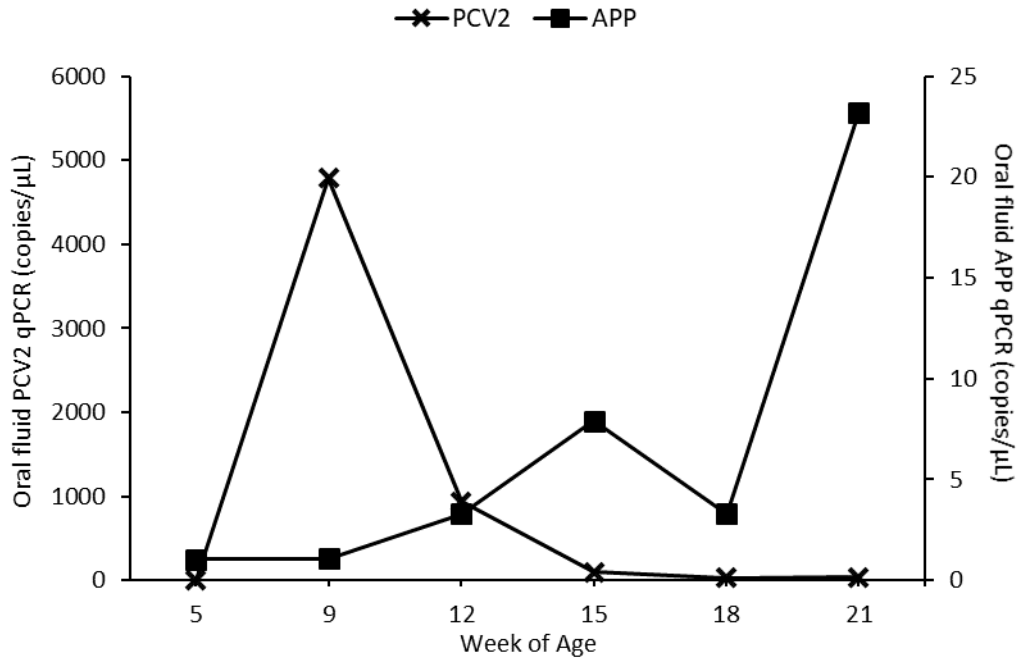

Figure 6: Arithmetic means for oral fluids (OF) PCR results for porcine circovirus type 2 (PCV2), and *Actinobacillus pleuropneumoniae* (APP), expressed as copies per µL of OF. *Mycoplasma hyopneumoniae* (*M hyo*) was not detected in any OF samples.

It seems from this data that PCV2 may present in OF earlier than APP, and hence OF may be a better tool for early surveillance of PCV2, rather than APP. It is unclear why this may be, but storage of samples between collection and analysis may have played a role, as currently these PCR tests are unable to be performed in real-time on farm. The impacts of storage of OF on PCR results needs to be further investigated. It was identified by Dron et al. (2012) that APP in saliva degraded at the same rate when OF were frozen or refrigerated, and it was assumed that the same is true for other respiratory pathogens. In support of this, other respiratory pathogens, such as *Betaarterivirus suid* 1 (Porcine Reproductive and Respiratory Syndrome Virus, PRRSV), have been shown to be resistant to prompt freezing and degradation of viral RNA is low using this method (Prickett et al., 2010; Ramirez et al., 2012).

3.4.2 Grower-Finisher Phase

The qPCR results for OF samples collected in the grower-finisher phase are shown in Figure 6. It was interesting to note that no OF samples tested positive for *M.*

hyopneumoniae across all age groups. Prior to commencement of the project, *M. hyopneumoniae* had been successfully detected in OF by the protocols used in this project (T. Limm; pers. comm.), so the absence of this pathogen is actual rather than related to the method. In previous studies, sensitivity of OF diagnostics on pooled samples has been shown to be quite high for some pathogens, e.g. 69-99% sensitivity was found for detection of Swine Influenza Virus A (Romagosa et al., 2012), and low for others such as *M. hyopneumoniae* (Pieters et al., 2017), depending upon prevalence of the pathogen of interest, and OF results can often be inconsistent (Hernandez-Garcia et al., 2017).

In the grower-finisher phase, APP was detected in approximately 12.5% of individuals across all timepoints (Table 1). However, the quantity detected varied greatly, while some quantities were quite strong, many values were in the suspect range. It is important to note that a number of samples (*n* = 18/299) gave a result that was below the usual cut off point for the qPCR for APP; however, these values were left in for the calculation of arithmetic means. A number of previous studies have investigated the ability to detect APP specific antibodies in pig OF, as serum and OF tend to have a similar antibody profile, albeit at lower concentrations in OF (Cameron et al., 2005; Prickett et al., 2008). Dron et al. (2012) suggested that subclinical APP infection may not be able to be detected through PCR of OF samples when APP is endemic in the herd – as is the case with the herd used in the current study. However, OF may be a useful tool for surveillance of APP in non-endemic herds, and allow for farmers to take early action to prevent widespread dissemination of APP bacteria on their farms (Dron et al., 2012; Johnson et al., 2011; Loftager et al., 1993). Detection of APP in OF may be hindered by the fact that the bacteria is more often found in the deep crypts of tonsillar cavities or in nasal cavities, and therefore may not be represented efficiently in the OF itself (Bossé et al., 2002; Chiers et al., 2001; Costa et al., 2012).

Porcine circovirus type 2 (PCV2) was detected in all OF across all ages at some level. From the qPCR results, a number of OF samples were considered 'suspect' for PCV2, including *n* = 23 samples at 15 weeks of age, *n* = 49 samples at 18 weeks of age, and *n* = 36 samples at 21 weeks of age. For the statistical analysis, these were considered a positive sample. It was noted that the highest quantity values were observed from OF at 9 to 12 weeks of age and were lower either side of this time, with significant variation over all timepoints. The study of Hernandez-Garcia et al. (2017), the

number of OF samples negative for PCV2 increased with age, similar to what was seen in the current study.

Table 1: Results of porcine circovirus type 2 (PCV2) and *Actinobacillus pleuropneumoniae* (APP) antibody serology tests (titre and S/P ratio, respectively) and real-time polymerase chain reactions (qPCR) from 12 to 21 weeks of age.

	% positive samples			
	Serology		Oral fluid	
Week of Age	PCV2	APP	PCV2	APP
12	-	-	98.7% (76/77)	13.0% (10/77)
15	100% (30/30)	16.7% (5/30)*	100% (68/68)*	9.9% (7/71)
18	100% (30/30)	26.7% (8/30)*	97.3% (73/75)*	10.5% (8/76)
21	100% (30/30)	23.3% (7/30)	100% (74/74)*	14.7% (11/75)
22 (mucosal swabs)	-	-	100% (19/19)**	0% (0/20)

* Includes samples considered as 'suspect' by the cut off limits of the assay.
** One sample result was inconclusive.

3.4.3 Tracheal Mucus Swabs

Quantities (copies/µL) of each PCR target for the tracheal fluid swabs that were submitted for analysis (n = 20) were 0.2 ± 0.07 copies/µL for *M. hyopneumoniae*, and 31.4 ± 12.53 copies/µL for PCV2 (presented as arithmetic mean ± SE), and APP was not detected in any of the swabs. However, note that this is not a true quantitation, as the amount of sample that is absorbed onto a swab is unknown and uncontrolled. The volume of sample going into the extraction is completely unknown, thus the number of copies/µL of the original sample cannot be determined, and hence these data were not used in the formal statistical analysis. It must also be noted that results for all tracheal fluid samples in which *M. hyopneumoniae* was detected (30%, n = 6/20) were below the usual cut off for the qPCR assay. Prevalence of PCV2 and APP from swab results are shown in Table 1. These results were consistent with the OF results for PCV2; however, APP was not detected in any of swabs that were taken, which may be as a result of the localisation of APP bacteria deep in the tonsillar crypts (Chiers et al., 2001) where it may have not been accessed by swab on the surface of the tonsil.

3.4.4 Dilution Series

From the dilution series that was carried out, the positive sample used was a mid to high-range PCV2 positive sample and was easily detectable at a 1:100 dilution. Thus, in this instance, one affected pig within a pen of 100 pigs would be easily detectable. However, it must also be noted that if testing at 21 weeks where the quantity of PCV2 is much lower, detecting one affected pig in a pen of 100 pigs is less likely to be possible. For the dilution, PCV2 was the only pathogen able to be used as these samples were more conclusively positive. While 10 individuals were consistently seropositive for APP throughout the grower-finisher stage, the quantity of APP detected varied greatly. As with PCV2, detection of an APP affected individual within a pen is possible if the pathogen/shedding level is high, it may not be possible at time points when pathogen levels are low.

Therefore, OF testing at a pen level may be successful in detecting PCV2 when only one out of 100 pigs in that pen is infected. i.e. the test was quite sensitive and could be effective in detecting a respiratory pathogen when infection rates are quite low within the pen, as long as all pigs (or at least the infected ones) are chewing on the rope. This is in contrast to the results of Dron et al. (2012) and Prickett et al. (2008) who found that the best ratio was 20-25 pigs to one rope. These authors investigated the sensitivity of OF to detect APP, whereas PCV2 was the only pathogen used for sensitivity testing in the current study. If practical, it is recommended that one rope per 20-25 pigs is used in order to successfully detect APP, PCV2, and/or other respiratory pathogens in OF via this pooled sampling method to maximise detection sensitivity.

Laboratory tests such as PCR are preferred for detection of respiratory pathogens over other methods due to their high sensitivity and specificity (Dron et al., 2012), and tests like these are becoming quicker and cheaper, which makes them ideal for real time monitoring and surveillance of pathogens in commercial settings. Initially, OF were used as a way to detect respiratory pathogens in individual pigs. Later on, authors from the USA (Prickett et al., 2011; Prickett et al., 2008; Prickett et al., 2010) further developed OF testing to be able to use pooled OF samples to detect these pathogens, allowing for samples to be collected at a pen level (Dron et al., 2012). This technique is preferred over individual sampling as more pigs can be tested at once, and collection of OF is just a matter of setting a rope in a pen for

30 min which can be easily achieved on busy farms. Furthermore, the one OF sample can then be used to test for a number of different pathogens or specific antibodies (Dufresne, 2011).

3.5 Correlations Between Oral Fluids, Serology, and Clinical Diagnostics

Oral fluid contains serum transudate and therefore its composition resembles that of serum (Cameron et al., 2005; Dron et al., 2012). Previous studies have focused on detection of viral antigens in OF, but research is focusing more on detection of bacterial antigens as well, such as APP (Costa et al., 2012), *M. hyopneumoniae*, and *H. parasuis* (Bjustrom-Kraft et al., 2018), as were included in the focus of the current study. There have been few studies until recently that have investigated numerous pathogens contributing to PCRD on the same OF samples (Biernacka et al., 2016; Hernandez-Garcia et al., 2017; Prickett et al., 2008; Ramirez et al., 2012). The proportion of pigs that seroconverted for PCV2 and APP, and the prevalence of these pathogens in OF samples are presented in Table 1. *A. pleuropneumoniae* was detected in OF at all ages examined in the current study, albeit at low concentrations. This was unsurprising given that the main colonisation site for this pathogen is the tonsils (Chiers et al., 2001) and that APP is known to be endemic to this herd.

Correlations between serology results and OF results were carried out for PCV2 results, as this was the most prevalent pathogen in OF and the pathogen for which pigs showed the strongest immune response from the serology results. A matrix scatter plot of serum antibody levels and OF qPCR quantities of PCV2 for all 30 pigs sampled for serum and OF in the grower-finisher facility is shown in Figure 7.

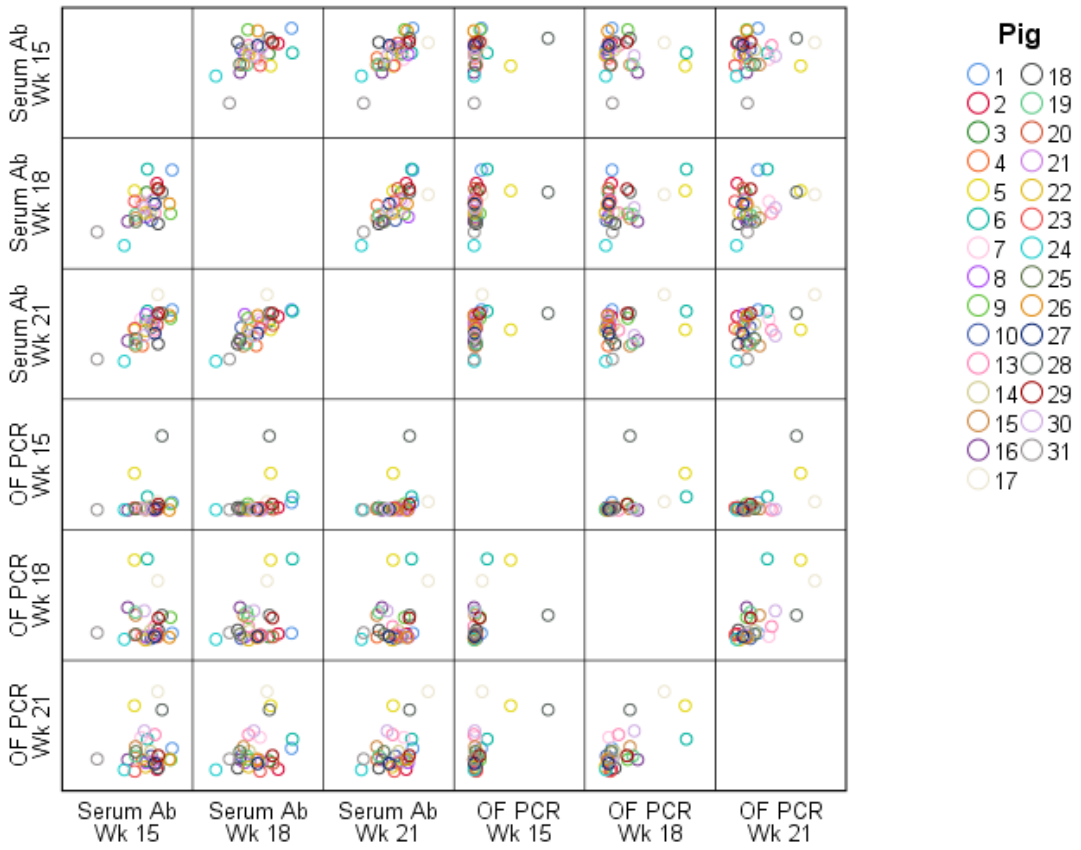

Figure 7: Scatter plot matrix of raw data for serology for porcine circovirus type 2 (PCV2; antibody titre) and oral fluids PCR (PCV2 viral DNA) results.

The highest correlation of these relationships modelled in Figure 7 was between PCV2 antibody titre and OF qPCR result when both were taken at 21 weeks of age (r = 0.408; P = 0.028; Table 2). However, for on-farm diagnostic purposes, sampling OF at 21 weeks of age does not demonstrate a pro-active approach, as most pigs will be marketed around this time and would have already been impacted by PCRD.

The correlation between serology and OF results at 18 weeks of age was also significant (r = 0.374; P = 0.046; Table 2), showing a moderate positive correlation between the two measures. Perhaps the most interesting of these correlations is the moderate positive correlation between PCR result from OF at 15 weeks of age and serology results at 18 weeks of age for PCV2 (r = 0.326; P = 0.085; Table 2). This represents an opportunity to screen for presence of PCV2 using the less invasive OF technique, which may be an indicator of the pigs' ability to develop immunity to PCV2 later in life. However, it would be of more interest to see if these positive correlations can be picked up even earlier (i.e. in the weaner phase). It was

unfortunate that the weaner results couldn't be matched to the same pigs in the grower-finisher facility in the current study, and future research should investigate this relationship.

Table 2: Correlations between serum antibody (titre) and oral fluid PCR DNA (copies/µL) for porcine circovirus type 2 (PCV2) tested at 15, 18, and 21 weeks of age.

		Titre (serology)		
	Age	**15 Weeks**	**18 Weeks**	**21 Weeks**
PCR DNA copies/µL (oral fluid)	**15 Weeks**	*r* = +0.167 *P* = 0.39	*r* = +0.326 *P* = 0.085	*r* = +0.274 *P* = 0.15
	18 Weeks	*r* = -0.046 *P* = 0.81	*r* = +0.374 *P* = 0.046	*r* = +0.261 *P* = 0.17
	21 Weeks	*r* = +0.111 *P* = 0.57	*r* = +0.324 *P* = 0.087	*r* = +0.408 *P* = 0.028

Investigating this relationship between serology and OF PCR results in PCV2 further, it was found that this relationship may be better explained by a quadratic equation (Figure 8). The quadratic equation gave a higher R^2 value than the linear equation in the comparison between serology and OF results at 15 (R^2 = 0.045 vs. R^2 = 0.027), 18 (R^2 = 0.158 vs. R^2 = 0.14), and 21 weeks of age (R^2 = 0.244 vs. R^2 = 0.172, respectively). Figure 9 further shows the correlations between OF result at 15 weeks of age, and the titre result at 18 weeks of age further. In Figures 8 and 9A it can be seen that one or two high outliers for the qPCR result may be skewing the data. In Figure 9B, these outliers have been removed for this comparison, and both the linear (R^2 = 0.386) and the quadratic equation (R^2 = 0.516) fit the data more appropriately in this model (vs. R^2 = 0.106 for both equations in the full data model). In all cases the quadratic model was the best fit for the data. These data seem to suggest that there is potential for early detection of PCV2 infection in grower-finisher pigs using OF diagnostics. Prickett et al. (2008) suggested that surveillance for PCV2 in OF should occur every 2 to 4 weeks in a commercial setting in order to be most effective. Hernandez-Garcia et al. (2017) also concluded that pooled OF samples could be an effective way to monitor PCV2 in commercial herds.

It was unfortunate that *M. hyopneumoniae* was unable to be detected in many of the samples in the current study, as the experimental farm has a high prevalence

of PCRD, thought to be significantly impacted by the endemic presence of *M. hyopneumoniae* in this herd. The experimental design may not have allowed for sufficient spread of *M. hyopneumoniae* within experimental pigs, and further investigation of the patterns of *M. hyopneumoniae* presentation in OF is required in a more commercial setting, given the notability of this pathogen. However, Hernandez-Garcia et al. (2017) detected *M. hyopneumoniae* in OF and found that detection patterns were irregular over time and the apparent low sensitivity of the OF method was largely inconclusive for on-farm surveillance of this pathogen.

Most likely, OF may be more useful for early detection of PCRD pathogens in commercial herds where the risk of infection is higher due to increased mixing, overcrowding, and handling by farm staff (Bossé et al., 2002; Rosendal et al., 1983). In the current study, pigs were kept in individual pens for the grower-finisher period and were subjected to minimal handling. Furthermore, pigs were kept in a well ventilated shed, the study was carried out over the summer and early autumn months, pigs were kept on a strict health regime and were able to be monitored individually for signs of respiratory stress or other animal welfare indicators. Therefore, the pigs in the current study may have had maximum immune efficiency helping them to fight off respiratory antigens (Bossé et al., 2002; Rosendal et al., 1983), which is unfortunately not often the case in commercial production. Future studies must focus on pigs housed in a more typical commercial environment under more stressful environmental conditions.

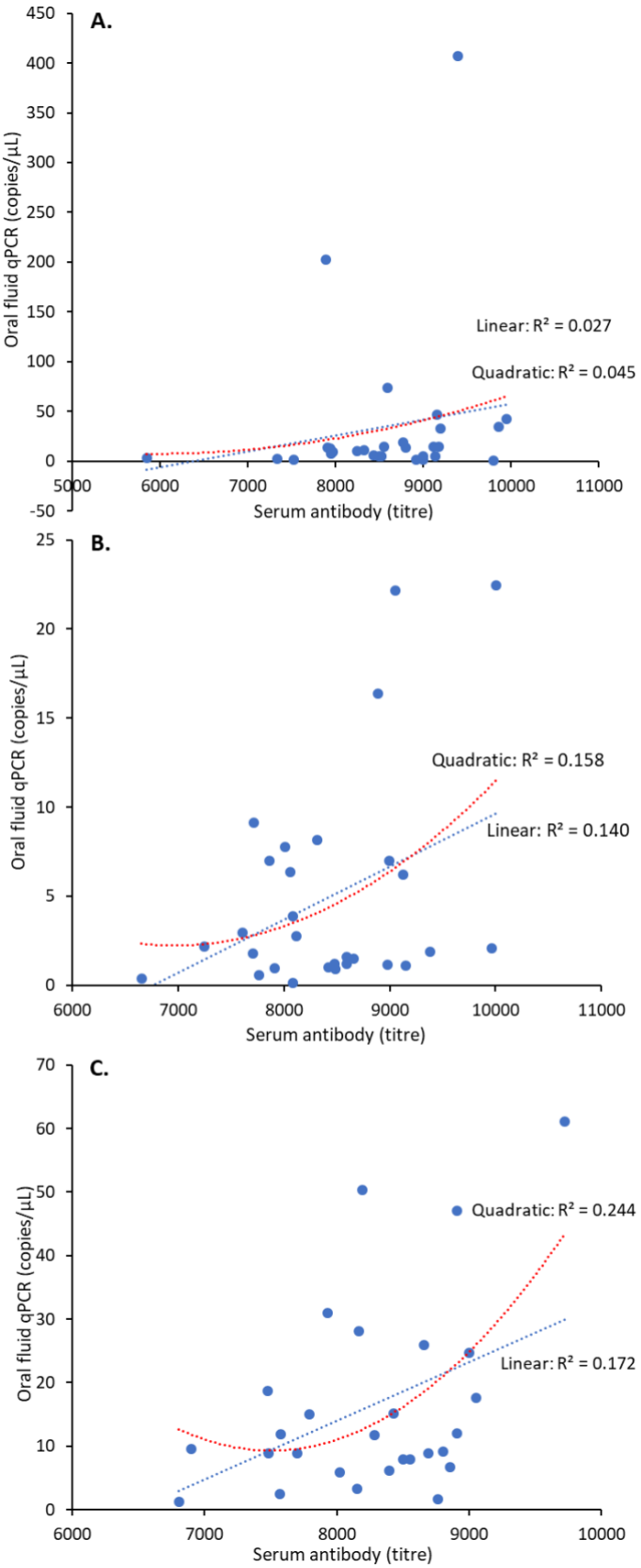

Figure 8: Correlations between individual serology (antibody titre) and oral fluids (antigenic DNA qPCR) results for PCV2 in the grower-finisher facility taken at 15 (A), 18 (B), and 21 (C) weeks of age.

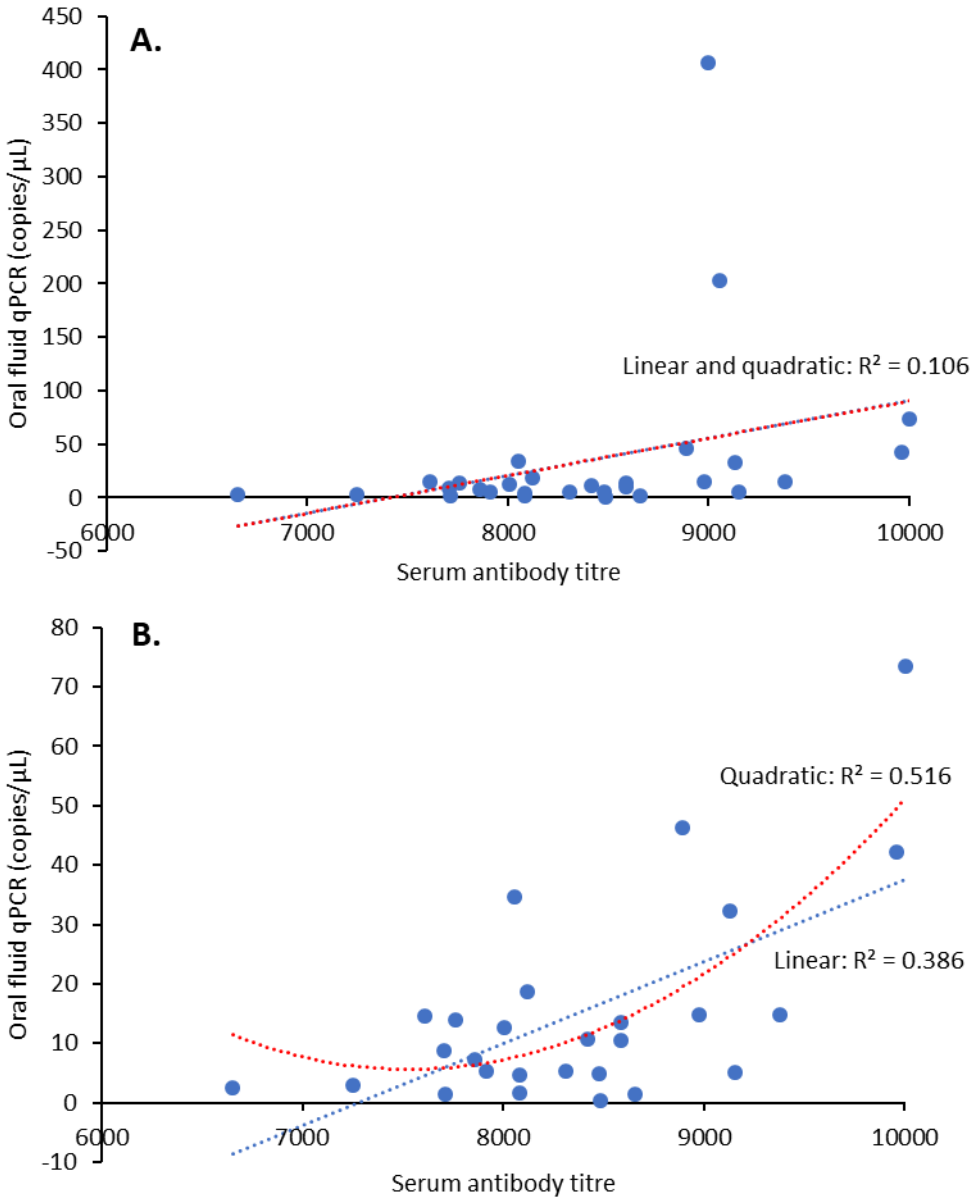

Figure 9: Correlations between individual oral fluids (antigenic DNA qPCR) taken at 15 weeks of age, and serology results (antibody titre) taken at 18 weeks of age for PCV2 in the grower-finisher facility: (A) with all data included; and, (B) with outliers for oral fluids results (> 100 DNA copies/µL; *n* = 2) excluded.

Of the pigs that were identified as suffering from some level of pleurisy at the abattoir (~35% of all trial animals), there was significant variation in eye temperature, serology, and OF results between these pigs (data not shown). Pigs that suffered from pleurisy showed a drop in serum PCV2 antibody titre from 15 to 18 weeks of age, whereas this level remained relatively constant for the remaining 'healthy' pigs (Figure 10A). However, the 'healthy' pigs still had higher

concentrations of PCV2 antigenic DNA in their OF compared to pigs suffering from pleurisy (Figure 10B). Pigs that suffered from pleurisy showed a spike in eye temperature from 12 to 15 weeks of age, whereas eye temperature remained relatively constant over the whole experimental period for 'healthy' pigs (Figure 10C). Given that individual responses in eye temperature, PCV2 OF, and serology results were quite varied for pigs that were either removed at the abattoir, or that were identified after slaughter as suffering from pleurisy, these warning signs may not be suitable indicators of PCRD when taken alone.

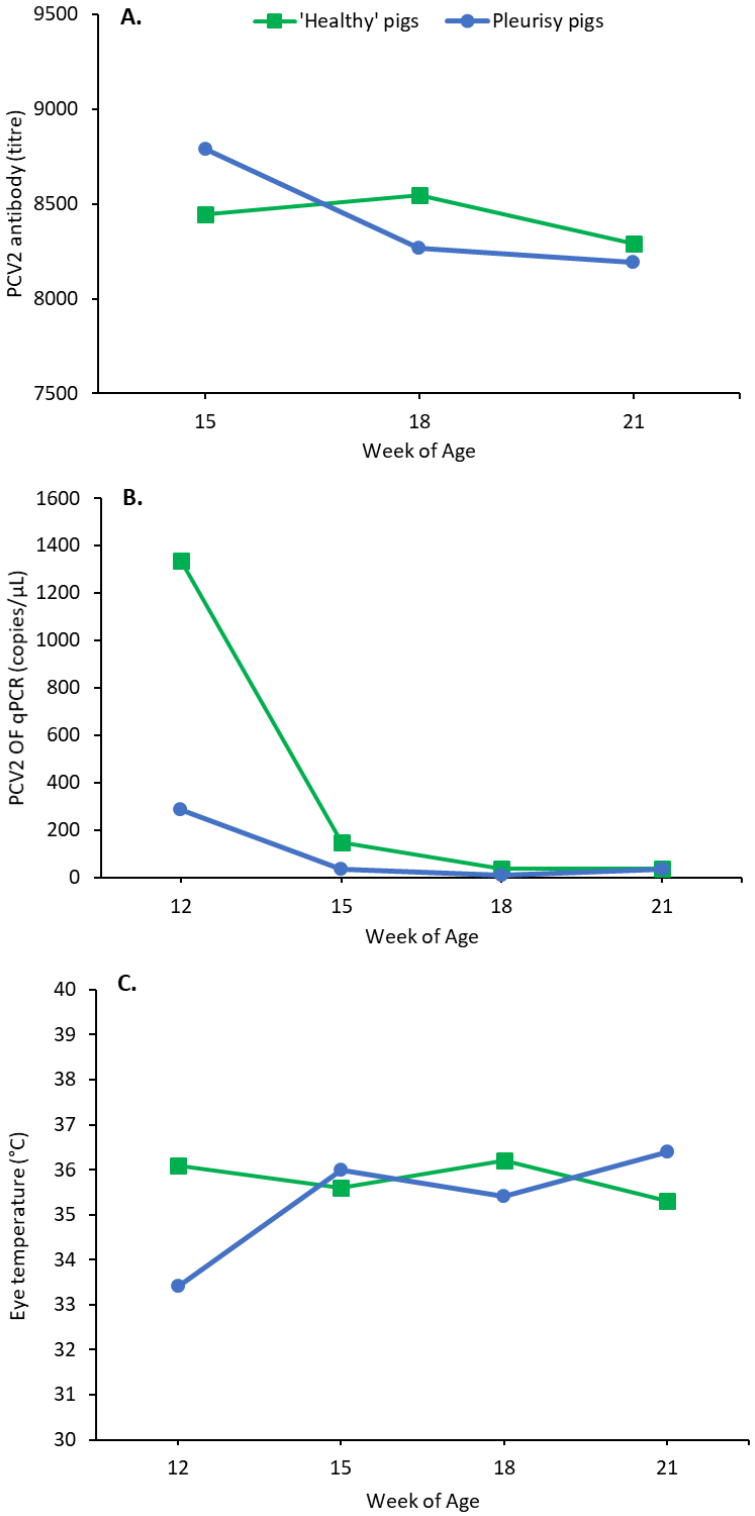

Figure 10: Average porcine circovirus type 2 (PCV2) antibody titre, qPCR result for PCV2 antigen in oral fluid, and eye temperatures of pigs in the grower-finisher facility for which pleurisy was identified at the abattoir compared to the average result for all other ('healthy') pigs, excluding pigs whose lungs were not able to be observed at slaughter (*n* = 47).

In a previous study, Dufresne (2011) found a high correlation between serology and OF PCR results for PRRSV and swine influenza virus (SIV), indicating a high sensitivity using this PCR detection method. Use of PCR for detection of APP antigen in OF may be more sensitive than that of detection of APP-specific antibody in serum (Costa et al., 2012), as the antigen can be detected earlier than seroconversion in the young pig, and hence this method may be more successful for on farm surveillance. However, Costa et al. (2012) found that this method had a lower sensitivity in comparison to PCR to detect APP antibodies in serum.

Similar to our results, Hernandez-Garcia et al. (2017) found that groups of pigs that had previously been shown to be positive for pathogens such as PCV2, *M. hyopneumoniae*, PRRSV, and SIV were then shown to be negative through PCR of OF, indicating a low sensitivity of this method. Our results, along with those of other previous studies (Finlaison et al., 2014; Hernandez-Garcia et al., 2017) suggest that pooled OF samples may actually be more sensitive than serology when blood samples are only taken from select individuals for that group. This is likely to be the case, as blood samples require significant amounts of labour for collection and require additional processing to extract serum – and hence less animals are likely to be tested from a large group. This poses an advantage of pooled OF testing over individual blood sampling.

4. Application of Research

The current project aimed to validate OF qPCR testing as a practical and economical diagnostic testing method for detecting PCRD at a commercial level. Uptake of this testing method by pig veterinarians will assist in making proactive economical health and management decisions improving herd productivity and improving the effectiveness of antibiotic medication programs. It also demonstrates that the pig industry is looking into alternative methods of health diagnosis which are non-invasive and welfare friendly. There is potential for future collaboration on further development of diagnostic tests using OF in pigs. Specifically, researchers in the USA are currently requesting for research proposals looking at sensitivity of PCR techniques for testing for presence of African Swine Fever Virus (ASF) and/or PRRSV in OF (National Hog Farmer, 2020), which may be used as part of an ASF or PRRSV surveillance program.

From our results it seems that pooled OF samples may be an effective way to monitor PCV2 infection patterns in commercial pig farms, consistent with the results of Finlaison et al. (2014) and Hernandez-Garcia et al. (2017). Our methods for collection of pooled OF samples (1 rope per 20-25 pigs, exposure for 30 min, etc.) were based on the results of previous studies (Costa et al., 2012; Dron et al., 2012; Prickett et al., 2008) and allowed for minimal environmental contamination, while maximising the time for all pigs in the pen to come in contact with the rope.

Collection of pooled OF samples from pens using ropes is an efficient way to collect samples for diagnostic analysis while minimising the stress of handling for pigs, as opposed to collection of tonsillar swabs, for example (Costa et al., 2012). However, a fallback of this method is that pigs experiencing clinical signs of PCRD may be less likely to contact the rope if they are depressed, are experiencing appetite suppression, or if their physical fitness has been compromised, which may result in false negative results (Dron et al., 2012; Escobar et al., 2007; Hart, 1988). However, this may also be the case with other forms of surveillance (swabs, blood sampling etc.), as sick animals may be less likely to be tested, for fear of worsening their condition or putting unnecessary stress on the animal. Addition of ropes to the pen introduces a fomite that may also facilitate sharing of OF between pen-mates, and hence may increase the risk of spread of PCRD pathogens within the pen (Costa et al., 2012; Detmer et al., 2009; Dufresne, 2011), and the impact of this on a large

scale deserves further investigation. Care must be taken in interpreting the quantitative PCR results from pooled OF samples, as some pigs in the pen may contribute to the sample more than others, with a higher proportion of contact with the rope, or with higher levels of pathogen shedding compared to others within the pen that may or may not be interacting with the rope (Hernandez-Garcia et al., 2017).

The type of facility in which OF collections are being carried out may also impact the success of this method, for example, in ecoshelters where enrichment is provided, pigs may be less likely to interact with the ropes (reviewed by Dron et al., 2012; Petersen et al., 1995; Stolba et al., 1980). Another technique that has been suggested to improve the rope OF collection method is to add buffered salts to the rope in order to yield further sample, by encouraging production of saliva and hence increase the volume of sample collected (Opriessnig et al., 2006; Wills et al., 1997). This may facilitate a greater concentration of antigen or antibody to be extracted (Dron et al., 2012). Oral fluid results may be impacted by environmental contamination, and efforts to reduce this as much as possible must be made (Cameron et al., 2005). Only allowing pigs access to the rope for a maximum of 30 min is one way to achieve this.

If testing OF for antibody concentrations, it is important to know what S/P ratios and titre results can be considered as protective against these pathogens. This would prove to be valuable information in the development of on-farm OF testing protocols in order to increase knowledge about the health of our pigs to make effective management decisions. However, this is further complicated by the inability to distinguish between maternally-derived, vaccine-derived, and infection-derived immune responses.

The collection of OF for on-farm diagnostics deserves to be studied further, as this method is relatively cheap and easy to carry out in a commercial situation. There is a current lack of timely disease monitoring on pig farms in Australia and throughout the world. This is mostly as a result of the absence of cheap, accurate, and timely tests able to detect the range of pathogens causing disease on farms today. Oral fluid is a medium that is easy to collect (especially if this can be done via a group sample from a rope in a pen), has a low labour requirement, and can be tested efficiently through the use of PCR for early detection of PCRD.

5. Conclusion

In conclusion, these results show that measurement of PCV2 in OF in pigs may be a strong indicator for infection and this knowledge will aid in the development of rapid on-farm diagnostic tests using OF. Further study is required in a more typical commercial setting, with grower-finisher pigs housed in large groups, and in winter periods where PCRD may be more prevalent.

6. Limitations/Risks

It must be noted that in the present study, animals were housed in an individual grower-finisher facility from 10 weeks of age until slaughter, under experimental conditions. Therefore, one limitation of the current study is that the experimental pigs represent 'high health' pigs (albeit from a herd with the pathogens examined being endemic) that were not necessarily exposed to the high antigen load that a pig housed in commercial group housing situations may have. However, once these surveillance methods have been fully developed, this is a quick and easy way to test for agents of PCRD in growing pig populations.

Previous studies have used PCR for surveillance of OF to detect pathogens responsible for PCRD, but not as a quantitative measure. Quantitative real time PCR was used in the current study, but unfortunately concentrations of antigen in OF were not able to be correlated to clinical on farm signs of PCRD, most likely due to the nature of the experimental design. Furthermore, presence of molecules in OF that can inhibit the effectiveness of PCR present a difficulty for this method (Decorte et al., 2013; Hernandez-Garcia et al., 2017; Ochert et al., 1994), and OF may need further processing on farm or in the laboratory to increase the sensitivity of these methods.

Variations in colonisation dynamics between the range of PCRD pathogens that may exist on farm present some difficulties for use of OF for early diagnosis. For example, some pathogens are early colonisers and some are late colonisers, some have several serovars that exhibit a range of virulence factors (e.g. APP), and the kinetics of the PCRD complex is further complicated by several other host, management, and environmental factors (Costa et al., 2012; Dron et al., 2012; Prickett et al., 2008). Veterinarians must take the full range of these factors into account for any on-farm PCRD surveillance programs.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

- Collection of pooled OF samples via the methods of Prickett et al. (2008), Ramirez et al. (2012), and others can be used as an easy on-farm method of surveillance of PCV2 infection patterns in Australian herds.
- Further research is required in a more typical commercial setting to correlate antigen and/or antibody concentrations in OF with concentrations in serum and other clinical and sub-clinical manifestations of PCRD, in order to fully validate this method for early detection of PCRD.

8. References

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