Evaluation of oral fluid samples for herd health monitoring of pathogens and the immune response in pigs

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Executive Summary

The use of porcine oral fluid as a sample matrix for disease surveillance strategies in the piggery is an expanding field and the current focus of much research. While traditional diagnostic strategies involve the sampling of individual animals within a population, oral fluid sampling targets groups of animals within a population by sampling a group or pen of animals by means of a cotton rope placed in the pen. By sampling groups of animals oral fluid testing has the potential to facilitate surveillance and detection of disease in a population though either detection of the pathogen or antibodies directed against the pathogen.

Oral fluid is a challenging sample to work with in diagnostic tests. Sample processing was evaluated for real-time PCR and an optimal extraction method identified. In addition, a small study was undertaken to evaluate the effect of inhibition of processed samples on the PCR reaction. The reduced sensitivity of detection of virus in oral fluid compared with a viral transport media indicated that viral nucleic acid degradation, virus binding to particulate matter in the sample or reduced extraction efficiency of nucleic acid from the sample are the most likely causes.

By real-time PCR PCV2 and *Lawsonia intracellularis* could be detected in oral fluids and quantitated, and Bungowannah virus could be detected in oral fluids after 'spiking'. A commercially available PCV2 ELISA for detection of antibodies in serum was optimised for detection of PCV2 antibodies in oral fluid.

Significant efforts were made to detect antibodies to *Lawsonia intracellularis* in oral fluids both by modifying and developing new ELISA techniques and by purifying and concentrating total IgG in oral fluids. While both strategies increased the sensitivity of detection for *L. intracellularis* antibody in serum, the same increased sensitivity was insufficient to detect antibodies in oral fluids where the starting concentration of antibodies was significantly lower (less than 1% of serum antibody titres). Detection of *L. intracellularis* antibodies in oral fluids at this point is possible in heavily infected animals, but difficult and unreliable in the majority of animals. The development of a more sensitive ELISA using increased concentration of a highly immunogenic *L. intracellularis* recombinant protein could be possible in the future; however it is likely that the cost of this may outweigh advantages in increased sensitivity.

The effects of storage temperature on nucleic acid detection by real-time PCR and antibody detection by ELISA were evaluated. The study demonstrated that optimal storage/transport temperatures are critical for test accuracy. Samples should be placed at 4°C as soon as possible after collection and frozen if not submitted to the laboratory on the day of collection. Samples should be well packed so they remained chilled while in transit to the laboratory. Under these conditions there is minimal impact on the detection of nucleic acid or antibodies for the tests evaluated.

A total of 12 submissions from 10 piggeries containing 869 sera and 152 porcine oral fluids (from 89 pens) were received for the study. Each sample was tested for PCV2 by real-time PCR and PCV2 antibodies by ELISA. All oral fluid samples were also tested for *L. intracellularis* by real-time PCR, but only serum samples were tested for *L.intracellularis* antibodies. The results for porcine oral fluid and the related sera were compared. Oral fluid sampling for PCV2 was found to be more sensitive than blood collection from a subsample of animals for detection of PCV2 infection within a group. Simple correlation coefficients showed significant correlations between the serum variables evaluated (geometric mean, arithmetic mean or maximum viral load) and the mean viral load in oral fluid. The sensitivity of detecting PCV2 antibodies in oral fluids compared to blood samples from a subsample of the group was 83% from this study. A significant correlation was identified between the mean or median result of the serum samples and the mean result of the oral fluid samples. In addition, there was good agreement of positive and negative result interpretation for the mean results of serum and oral fluids collected from an individual pen. A significant correlation was also observed between the mean *L.intracellularis* antibody concentration in serum and the mean load of *L.intracellularis* in oral fluids.

This proof of concept project indicates that oral fluid testing should be a cost-effective means of herd health monitoring that has the potential to be used to detect a wide range of both viral and bacterial pathogens and associated antibody responses. The techniques and understanding developed as part of this project could lead to significant innovations in Australia in herd health monitoring, animal welfare and nutrition, which are all significant milestones for the Pork CRC.

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Abbreviations

APP	*Actinobacillus pleuropneumoniae*
BHV-1	Bovine herpesvirus 1
Ct	Curve threshold
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
IAV	Influenza A virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L. intracellularis	*Lawsonia intracellularis*
PBGS	Phosphate buffered gelatine saline
PCR	Polymerase chain reaction
PCV2	Porcine circovirus 2
PRRSV	Porcine reproductive and respiratory syndrome virus
qPCR	quantitative real-time PCR
RNA	Ribonucleic acid
S/N ratio	The optical density of sample/mean optical density of the negative control

1. Introduction

The use of oral fluid as a sample matrix for diagnostic testing as part of disease surveillance strategies for pigs is a rapidly expanding area particularly in the USA where it is widely used for porcine reproductive and respiratory syndrome virus (PRRSV) surveillance (both by real-time PCR and serology), and also detection of influenza A virus (IAV), porcine epidemic diarrhoea virus and transmissible gastroenteritis virus (K. Harmon, pers. Comm.). At Iowa State University the number of samples received for testing has expanded from 11,000 in 2010 to greater than 50,000 in 2012 (J. Zimmerman, APV Conference, 2012).

Oral fluid consists of a combination of excretions of the salivary glands, serum transudate that originates from the circulatory system, nasal and bronchial secretions, bacteria, viruses, fungi, epithelial cells, inflammatory cells and food debris (Kaufman and Lamster, 2002). The use of oral fluids for detection of locally and systemically produced antibody dates back to the 1960's in human medicine although most tests have been developed from the mid-1990's onwards (Prickett and Zimmerman, 2010). In addition from the mid-1990s as polymerase chain reaction (PCR) technology has become more readily available, oral fluid samples have also been used for pathogen detection (mostly viruses).

In porcine medicine, field and experimental studies conducted in other countries have demonstrated that pathogens and antibodies to a number of infectious agents may be detected in oral fluid samples. Infectious agents to which antibodies have been detected in oral fluid include: African swine fever virus (ASFV) (Mur et al., 2013), IAV (Panyasing et al., 2012), PRRSV (Prickett et al., 2008), and PCV2 (Christiansen Leth et al., 2011; Prickett et al., 2011). Pathogen detection studies utilising PCR have been able to detect the presence of a range of viruses including PRRSV (Prickett et al., 2008), PCV2 (Prickett et al., 2008; Christiansen Leth et al., 2011; Prickett et al., 2011), IAV (Detmer et al., 2011; Romagosa et al., 2012; Goodell et al., 2013), foot and mouth disease virus (Vosloo et al., 2012), torque teno viruses 1 and 2 (Ramirez et al., 2012) and bacteria (APP, *Haemophilus parasuis* and *Streptococcus suis*) (Costa et al., 2012) in oral fluid samples.

Recent work published from the USA indicates that testing of oral fluids may be a useful approach for herd health monitoring. While traditional diagnostic strategies involve the sampling of individual animals within a population, oral fluid sampling targets groups of animals within a population by sampling a group or pen of animals by means of a cotton rope placed in the pen for a period of 20-30 minutes. By sampling groups of animals oral fluid testing has the potential to facilitate surveillance and detection of disease in a population. Prickett and Zimmerman (2010) suggest that "Oral fluid testing offers an opportunity to easily collect herd-level disease data on a periodic basis. Integration of longitudinal disease data with herd records (interventions, morbidity, mortality and production parameters) would provide for: (1) appropriately timed and targeted interventions, (2) 'real time' evaluations of interventions and (3) improved, herd specific estimates of the impact of specific pathogens on pig health and productivity. This proactive approach to disease monitoring translates diagnostic costs into improved growth performance rather than a historical vignette of past disease events".

Testing of oral fluids is dependent on the availability of diagnostic assays that have high analytical sensitivity. Both ELISA technology and real-time PCR meet this requirement as well as being very rapid and cost effective. Emerging technologies such as those based on microspheres (e.g. MAGPIX®) may add a further dimension in the future by having high sensitivity, a requirement for a small sample volume and an opportunity to multiplex (i.e. the capacity to test for multiple agents concurrently in the same test well).

Three agents have been selected for investigation in this project: PCV2, Bungowannah virus and *Lawsonia intracellularis*. PCV2 is recognised to cause significant production losses in many pig producing countries. Porcine circovirus associated diseases include post-weaning multisystemic wasting syndrome, respiratory disease, enteritis, reproductive failure and porcine dermatitis and nephropathy syndrome. The amount of PCV2 DNA present in serum and tissues as detected by quantitative real-time PCR (qPCR) has been

shown to be predictive of clinical outcome. Bungowannah virus is a significant reproductive pathogen. While currently only present on a single piggery in Australia its introduction into a naïve population would result in significant economic losses due to a marked increase in the numbers of stillborn and weak born piglets, and neonatal deaths on first introduction to a piggery. *L. intracellularis* is an endemic pathogen present in the majority of Australian herds causing reduced weight gain, decreased feed conversion efficiency, increased P2 backfat, increased variation in pig weights and reduced profits (by up to AUD$13 per pig, Holyoake et al., 2010). Ileitis can be controlled with vaccination and antibiotic medication, but sub-clinical disease and the associated production losses often goes undetected, unless pigs are monitored for *L. intracellularis* infection.

These agents have been selected to cover several strategies that oral-fluid testing needs to address:

1) PCV2 DNA detection – as an example of monitoring pathogen levels and correlation with disease.

2) Bungowannah virus RNA detection – representing a method of surveillance for introduction of new diseases.

3) PCV2 antibody and *L. intracellularis* antibody and pathogen detection – a tool to evaluate strategies to assist with optimal timing of vaccination including determining time of loss of maternal antibodies and monitoring for pathogen introduction by detection of pathogen or antibodies in oral-fluid samples.

This project aims to establish as a 'proof of concept' that oral fluid testing offers a cost effective means of herd health monitoring for a wide range of both viral and bacterial pathogens (both endemic and potentially exotic) in the Australian pig industry. Oral fluid sampling offers broader surveillance of the pig population compared with individual sampling. It also offers easier sample collection and decreased costs for diagnostic testing on a herd basis. Future technologies that are becoming available may also allow for detection of multiple pathogens or antibodies from this sample in a single test.

The aims of this project are five-fold.

1) Develop capability and knowledge for the collection of oral fluid samples and determine storage and transport conditions needed for optimal quantitative PCR and ELISA performance under Australian conditions;

2) Determine if the currently recommended methods for oral fluid processing sufficiently reduce inhibitors that affect the ability of qPCR to detect pathogens;

3) Demonstrate that PCV2 (DNA and antibodies), Bungowannah virus (RNA) and *Lawsonia intracellularis* (antibodies and pathogen) can be detected in oral fluid samples;

4) Determine if the presence or quantity of pathogen or antibody detected in oral fluids correlates with that detected in serum and that this technique therefore has the potential for herd health monitoring of a range of both endemic and exotic diseases in the Australian pig population;

5) Make diagnostic testing for PCV2 and *Lawsonia intracellularis* using qPCR and serology, by means of oral fluid sampling/collection, available for Australian pig veterinarians and producers.

2. Methodology

a) Collection of sera and oral fluid samples

The planned strategy was to initially sample 2 piggeries known to be infected with PCV2 and Lawsonia. Oral fluids would be collected from a cross-section of up to 5 age groups at each piggery with sampling of 4-5 pens of each age group and concurrent collection of blood from 10 pigs within each sampled pen. The target group/pen size for this initial study was 20-50 pigs. This sampling strategy proved to be unachievable. During our discussions with co-operators to organise sampling it was realised that since the submission of the project proposal a large proportion of the Australian pig population is now vaccinated against PCV2. In addition, while we wanted to sample 4-5 pens per age group, it was going to be difficult for our co-operators in most instances to collect from more than one pen at each age group. As the total number of samples received for this project was less than initially anticipated, to continue to meet the aims of this project we tested all of the sera individually rather than in pools (as was planned for a proportion of samples in the initial protocol). By testing all sera individually it was expected this would provide superior information on the range of results for serum within a sampled pen for each assay and how this compares with/affects the interpretation of the result we get from the oral fluid sample. We also hoped that by testing sera individually and making these results rapidly available to the submitter we would be able to encourage more submissions.

Collaborators were advised of the following protocol for sample collection:

- Selected farms must not be vaccinating against porcine circovirus 2 (PCV2);
- Pen size must be at least 15 pigs/pen, and ideally not more than 100 pigs/pen;
- Five age groups of pigs should be sampled (i.e. 6, 9, 12, 15 and 18 week old pigs, or thereabouts);
- From each selected pen clotted blood should to be collected from 10 animals; In addition, cotton rope(s) from the TEGO™ Oral Fluids Kit(s) (ITL Animal Healthcare) must to be hung in the pen for 20-30 minutes.
- For pen sizes up to 100 pigs include 1 rope per ~30 pigs;
- If there are >100 pigs in the pen hang 1 rope per 50 pigs;
- Blood and oral fluid samples must be identified by the age of the animals in the sample pen (or a pen ID);
- Oral fluids must be chilled (and preferably frozen) prior to submission;
- Details must be provided on the number of pigs in each of the pens sampled;
- If more than 1 rope is collected from a pen – the oral fluid from each rope should go into a separate collection tube.

b) Evaluation of extraction (processing) methodologies prior to real-time PCR testing of oral fluid samples

Two extraction methodologies were evaluated for the detection of RNA and DNA viruses in oral fluid samples. The two methods selected were the extraction kit currently utilised in the Virology Laboratory at EMAI for high-throughput testing of samples (MagMax™-96 Viral RNA Isolation Kit (Life Technologies™, Cat. No. AM1836); Kit A) and the extraction kit based on a published method that was found to have the best sensitivity and specificity in a study comparing extraction kits for detection of PRRSV (an RNA virus) in oral fluid samples (MagMax™ Pathogen RNA/DNA Kit - Oral Fluid Samples Protocol (Life Technologies™, Cat. No. 4462359; Kit B) (Chittick et al., 2011).

Bungowannah virus (an RNA virus) and bovine herpesvirus-1 (BHV-1; also referred to as infectious bovine rhinotracheitis virus, a DNA virus) were 'spiked' into separate aliquots of pooled porcine oral fluid and phosphate buffered gelatin saline (PBGS – a viral transport medium), and then diluted in a 10-fold dilution series. The oral fluid had been collected earlier on the day of use. (BHV-1 while not a pig virus is closely related to pseudorabies virus – both viruses belong to the varicellovirus genus, of the *Alphaherpesvirinae* subfamily and *Herpesviridae* family). Bungowannah and BHV-1 viruses were chosen as they would not be present in the oral fluid we collected for 'spiking' and we believed it was important to check for any difference in the inhibitory effect of oral fluid on a DNA versus an RNA virus. After 45 minutes at room temperature a 300 µL aliquot of porcine oral fluid for each dilution of virus and the uninoculated control was transferred to 450 µL of lysis/binding buffer of Kit B. All samples were held at -20°C prior to testing by real-time PCR. In addition, an aliquot of each sample of oral fluid collected before pooling was retained and a 300 µL aliquot was added to 450 µL of lysis/binding buffer of Kit B for PCV2 real-time PCR. Limited repeatability and reproducibility studies were undertaken with the residual 'spiked' oral fluid and a second preparation of 'spiked' oral fluid. Table 1 summarises the 5 different methods of sample extraction evaluated for the oral fluid samples.

The oral fluids samples were extracted using both Kit A and Kit B. The PBGS samples seeded with virus were extracted using Kit A only as a reference control. For all samples nucleic acid was eluted in a final volume of 50 µL. By using different extraction methodologies the aim was both to compare the two kits and also assess if differences in sample processing technique or sample volume had an effect on the sensitivity of the assay.

The samples were tested for Bungowannah virus, BHV-1 virus or PCV2 nucleic acid as described in 2.d).

Table 1: Summary of the extraction methodologies evaluated

Extraction methodology
A1 = MagMax 96 Viral isolation kit (50 µL unclarified sample)
A2 = MagMax 96 Viral isolation kit (50 µl clarified sample)
A3 = MagMax 96 Viral isolation kit (25 µL unclarified sample)
B1 = MagMax Pathogen RNA/DNA Kit (unclarified sample)
B2 = MagMax Pathogen RNA/DNA Kit (oral fluid added to lysis buffer 45 minutes after spiking)
B3 = MagMax Pathogen RNA/DNA Kit (clarified sample)

NT = not tested.

c) Processing of oral fluid samples

Based on the results of both section 2.b) and previously published methods (Kittawornrat et al., 2012; Panyasing et al., 2012; Romagosa et al., 2012), and to facilitate sample handling in the laboratory, the oral fluid samples were processed prior to testing by the following method:

i. Samples were centrifuged at 3,000g for 10 minutes at 4°C;

ii. The supernatant was harvested and stored at -20°C prior to testing and the pellet discarded.

d) Nucleic acid extraction and real-time PCR for Bungowannah virus, infectious bovine rhinotracheitis virus, Porcine circovirus 2 and Lawsonia intracellularis

For virus detection by real-time PCR, nucleic was extracted from porcine oral fluid (based on the results from 2.b)), PBGS or serum using the MagMax™-96 Viral RNA Isolation Kit (Ambion, Austin, Texas, Cat. No. AM1836). A Kingfisher® 96 magnetic particle handling system (Thermo Electron Corporation, Finland) was used for the extraction process and total nucleic acid was eluted in 50 µL of kit buffer. The same DNA extractions from field samples were used for *L.intracellularis* detection.

The Bungowannah virus (Finlaison et al., 2009), BHV-1 (Wang et al., 2007), PCV2 (Opriessnig et al., 2003) and *L.intracellularis* (Collins et al., 2007) real-time PCR assays were performed with 5 µL of extracted nucleic acid using the AgPath-ID™ One-Step RT-PCR Reagents (Life Technologies™, Cat. No. AM1005) in a 25 µL reaction volume. The reaction was undertaken in an Applied Biosystems 7500 Fast Real-Time System run in standard mode using the following cycling conditions: 48°C for 10 minutes, 95°C for 10 minutes, then 45 cycles of (95°C 15 seconds, 60°C 1 minute). The background fluorescent signal was adjusted automatically and the threshold set manually at 0.05. Results were expressed as the amplification cycle at which the threshold was crossed (Ct value), with a positive result having a Ct value <40.00.

e) Effect of temperature on detection of viral nucleic acid in oral fluid samples

Varying temperatures during the storage and transport of samples may affect the detection of either viruses or antibodies. To evaluate the effect of temperature over time on the detection of viral nucleic acid, BHV-1 and Bungowannah virus were spiked into both porcine oral fluid and PBGS. PBGS is a viral transport medium and was included in the experiment to provide a reference control.

Fresh oral fluid was collected on day 0 of the experiment. BHV-1 and Bungowannah were diluted to provide three concentrations in a 10-fold dilution series (Dilutions 1 to 3; highest to lowest concentrations of virus respectively) in PBGS and added in parallel to both porcine oral fluid and PBGS. Each dilution of spiked oral fluid or PBGS was placed at either 4°C, 25°C or 37°C. At day 0, 3, 7, 10 and 13 duplicate 200 µL (porcine oral fluid) or 150 µL (PBGS) aliquots were taken from each sample and stored at -20°C prior to testing. PCV2 was present in the available porcine oral fluid at high concentrations so a spiking experiment was not undertaken. The effect of temperature over time for PCV2 was therefore monitored at a single concentration only.

In the initial experiment, the amount of BHV-1 spiked into the samples was at the limit of sensitivity for the real-time PCR assay at the middle dilution (Dilution 2) so the experiment was repeated using a BHV-1 stock with a higher concentration.

Prior to extraction for PCR the samples were thawed and then centrifuged at 3000g for 10 minutes at 4°C. Nucleic acid extraction and real-time PCR were performed as described in 2.d).

The mean of the Ct value for each dilution of virus in porcine oral fluid for the two experiments was evaluated. For BHV-1 there was only a single replicate for the highest concentration of virus (Dilution 1). For the purposes of presenting the results where no viral nucleic acid was detected by PCR a Ct of 40 was used.

f) Detection of PCV2 immune responses in oral fluids

The Synbiotics SERELISA® PCV2 Ab Mono Blocking test kit (Synbiotics, Lyon France) was utilised for the detection of PCV2 antibodies in sera and porcine oral fluids. This kit uses a blocking ELISA technique and has been validated for the detection of PCV2 antibodies in porcine serum or faeces. The kit can be used qualitatively for the detection of PCV2

antibodies in serum (1/100 dilution only) or faeces, or quantitatively (1/100, 1/1,000 and 1/10,000 dilutions) to measure an antibody titre in serum.

Prickett et al. (2011), Kittawornrat et al. (2012) and Panyasing et al. (2012) describe serum antibody assays that have been successfully adapted to detect the lower concentration of antibodies in porcine oral fluid compared with serum. This has been achieved by using a lower sample dilution compared to serum, larger sample volumes in the assay, longer incubation periods for the samples and/or modifying the conjugate concentration.

For oral fluid samples the modified sample dilutions and incubation periods evaluated for this study were:

i. Undiluted, 1/2, 1/5, and 1/10 at 37°C for 1 hour;

ii. Undiluted, 1/2, 1/5 and 1/10 at 4°C for 16 hours (overnight);

iii. 1/5 and 1/10 at 25°C for 16 hours (overnight);

Aside from the modifications outlined above the test was performed on oral fluid samples as described by the kit insert for the "Qualitative method on serum". Oral fluid samples were tested in duplicate. Serum samples were tested in single wells using the "Qualitative method on serum". The result for a sample is determined from its S/N ratio (optical density of sample/mean optical density of the negative control). An S/N ratio ≤0.40 is considered positive for the presence of PCV2 antibodies; an S/N ratio >0.40 is considered negative for the presence of antibodies.

As PCV2 is known to be widespread in the pig population in NSW and Australia it was not readily possible to source PCV2 antibody free serum to spike for evaluation of the ELISA. For this reason oral fluid samples from the field were utilised for optimisation of the assay.

Initial experiments indicated that the optimal dilution and incubation temperature/time for oral fluids in this test was likely to be undiluted, 1/2 or 1/5 at 4°C for 16 hours. All oral fluids (n=152) collected for the project were tested at each of these dilutions with an overnight incubation at 4°C.

g) Effect of temperature on detection of PCV2 antibodies in oral fluid samples

The effect of temperature over time on the detection of PCV2 antibodies in processed porcine oral fluid (as per 2.c)) was evaluated in 2 parts:

i. The effect of storage at 4°C was evaluated for 6 samples collected from the field and processed as for 2.c). The oral fluid samples selected had moderate levels of PCV2 antibodies (S/N ratio ~0.2). An aliquot of each sample was harvested at day 0, 7, 14 and 28 and stored at -20°C prior to testing.

ii. The effect of storage at 4°C, 25°C or 37°C was evaluated for 8 samples collected from the field and processed as for 2.c). Oral fluid samples with a range of S/N ratios were selected for the experiment (strong positive to weak positive and one negative sample). A 300 µL aliquot of each sample was harvested at day 0, 3, 7, 10 and 14 and stored at -20°C prior to testing.

h) Evaluation of the inhibitory effect of porcine oral fluid samples on detection of DNA and RNA viruses by real-time PCR

Oral fluid samples are known to be challenging samples to process and achieve optimal sensitivity by PCR (Chittick et al., 2011). To further evaluate if the reduction in sensitivity between 'spiked' porcine oral fluid and PBGS samples observed in 2.b) and 2.e) was occurring during the PCR step an exogenous control (XIPC RNA or DNA) and primer probe set (Schroeder et al., 2012) was added to the qPCR mix and the qPCR repeated on the previously extracted nucleic acid (selected samples from 2.b) and 2.e). The 32 samples

selected had previously tested positive for Bungowannah virus or BHV-1 nucleic acid in PBGS and in oral fluids by some of the extraction methodologies utilised.

i) Detection of Lawsonia immune responses in oral fluids

Oral fluid samples were collected from herds where previous blood sampling demonstrated antibodies (IgG) to *Lawsonia intracellularis* in the serum of grower and finisher pigs. *L. intracellularis*- specific antibodies in sera were confirmed with both the indirect fluorescent antibody test (IFAT) used for research at EMAI and with the commercial ileitis ELISA (Bioscreen, Germany). Faecal samples collected from the same grower and finisher pigs were also tested for *L. intracellularis* numbers using the quantitative PCR to confirm infection. Using this data we were able to increase the probability of selecting groups of pigs with *L. intracellularis* antibodies (IgG) in oral fluid samples.

As the concentration of IgG in oral fluid samples was expected to be much lower than sera (< 1%), oral fluids were only diluted from 1/2 to 1/10 and incubated with *L. intracellularis* antigen for 16 hours at 4˚C, rather than 1 hour at 37˚C. Antibody detection was evaluated with 3 different *L. intracellularis* immunoassays: the *L. intracellularis* IFAT, the commercial ileitis ELISA (the Bioscreen Ileitis blocking ELISA) and a modified direct *L. intracellularis* ELISA (whole bacteria extracted from affected intestinal tissue).

j) Evaluation of field samples (PCV2 and L.intracellularis real-time PCR and serology).

All sera and oral fluid samples were processed and tested for the presence of PCV2 and *L.intracellularis* DNA by real-time PCR as described in 2.c) and 2.d). All sera were tested for the presence of PCV2 antibodies using the Synbiotics SERELISA® PCV2 Ab Mono Blocking test kit as per the kit qualitative method. For detection of PCV2 antibodies in oral fluids the samples were tested using an optimised method (1/2 sample dilution and with sample incubation at 4˚C for 16 hours/overnight).

Simple correlation coefficients were calculated between the variables from serum and oral fluid for the PCV2 qPCR and PCV2 ELISA.

3. Outcomes

a) Collection of sera and oral fluid samples

Sera and oral fluids were collected from a total of ten piggeries (12 submissions) where pigs had not been previously vaccinated against PCV2 (Table 2). A total of 869 sera and 152 porcine oral fluids were received. Two or three oral fluid samples were collected from 60 pens of pigs which allowed an evaluation of the variation of the results obtained by oral fluid sampling within a group of pigs.

Table 2: Summary of the details of each submission.

Submission No.	No. of pens sampled	Age of pigs sampled (wks)	No. sera/pen	No. oral fluid/pen	Total pigs/pen
1	16	19	9-10	2	50
2	20	19	10	2	50
3	5	6, 9, 12, 15, 18	10	1	15-30
4	2	13, 17	10	1	30
5	16	19	10	2	50
6	5	7.5, 11.5, 15.5, 19.5, 23.5	10	1-2	15
7	3	9.5, 14.5, 19.5	10	2 (pooled)	200
8	4	8, 12.5, 16.5, 20.5	10	2	200
9	3	8, 10, 12	5	1	10-15
10	3	7, 10, 13	12	3	150
11	5	6, 9, 12, 16, 18	10-11	1	31-35
12	7	6, 9, 12, 14, 16, 18, 20	10	1	24-26
Total	89		869	152	

b) Evaluation of extraction (processing) methodologies prior to real-time PCR testing of oral fluid samples

Fresh oral fluid was collected on the day of 'spiking' with virus. It was more difficult than expected to collect a large volume of oral fluid for this experiment. While the plan was to collect approximately 80-100 mL we were only able to collect approximately 40 mL of porcine oral fluid on each of the 2 collection days (Experiment 1 and 2). This limited the number of replicates that could be prepared. In this experiment the virus was 'spiked' directly into the oral fluid and the dilution series made with oral fluid. In retrospect this may have affected the dilution series as this sample type can be quite viscous and may have resulted in some pipetting errors. This may explain the difference in Ct values observed between Experiment 1 and Experiment 2 for Bungowannah virus for Extraction A1. This technique was refined for 2.e).

Table 3 summarises the results of each of the different processing/extraction techniques for each of the viruses evaluated. Kit A generally out-performed Kit B, despite the sample extraction volume being 4.8 times greater (240 µL) for Kit B compared with the 50 µL extracted with Kit A. In a real-time PCR assay that is running at 100% efficiency it would be expected that 4.8 times more sample template would be reflected by a Ct value approximately 2.3 cycles lower for Kit B versus Kit A (a lower Ct value in the same assay means that a greater quantity of nucleic acid is present in the sample).

While in some instances there was a slight improvement in sensitivity using Kit B compared with Kit A, it was often difficult to repeat the original result. In comparison, results for Kit A were generally reproducible except at the higher dilutions that were near the limit of sensitivity of the PCR assay (Ct values of approximately 35 or higher).

For Bungowannah virus, there was little difference in the real-time PCR result for extraction methods A1 and B1 (Table 3); there was a slight improvement in sensitivity of extraction B2 over extraction A1 in Experiment 1. In Experiment 2, clarification of the sample prior to sampling for extraction (method A2) resulted in a marginal improvement in sensitivity for detection of Bungowannah virus (approximately 2-fold or 1 Ct value), while extraction of a 25 µL volume (Extraction A3) rather than 50 µL resulted in an approximate 2-fold decrease (1 Ct value decrease in sensitivity). Bungowannah RNA was not detected by extraction methods B1 or B3 in Experiment 2.

For BHV-1, the results were similar to Bungowannah virus although extraction methods B1 and B2 both gave lower Ct values for the oral fluid samples compared to extraction method A1 in Experiment 1. In Experiment 2, extraction method A1 gave lower Ct values than extraction method A2 although A2 was able to detect BHV-1 nucleic acid over 3 dilutions.

For PCV2, extractions based on Kit A detected more positive samples than methods using Kit B although when viral nucleic acid was detected by Kit B methods, the sensitivity was slightly higher (1-2 Ct values lower), which is consistent with the larger volume of sample. Clarification of the oral fluid sample by centrifugation prior to extraction resulted in improved sensitivity. Extraction of 25 µL versus 50 µL of clarified oral fluid resulted in a similar decrease in sensitivity to that observed for Bungowannah virus.

Processing/extraction method A2 was selected for testing oral fluids for the remainder of this project. This method is consistent with the existing PCR and extraction methods in use in the laboratory at EMAI. The loss in sensitivity was minimal when compared with Kit B when extractions were successful. The failure to be able to reproduce results using Kit B was a concern. Extraction method B2 was not evaluated further as this would require initial sample processing in the field rather than at the laboratory. The results for Experiment 2 also indicated generally improved sensitivity for detection of pathogen nucleic acid if the sample was clarified by centrifugation prior to extraction (Extraction method A2). Subsequent discussions with the manufacturer suggest that storage conditions of one of the components of Kit B may have affected its efficiency. Should future work show that Kit B is a superior extraction method for processing of oral fluids, then it would be important that samples are processed as a reasonable size batch to make this a cost and time effective extraction technique for the laboratory.

Since the commencement of this project a study of the use of an exogenous (internal control) nucleic acid to monitor the efficiency of sample extraction and PCR amplification commenced in the laboratory. Unfortunately there was not sufficient time to repeat all of these experiments with an internal control included but some preliminary work was undertaken (see 2.h) and 3.f)).

Overseas research still suggests that the 'perfect' method for processing of oral fluid samples is still evolving and the results of other studies should be monitored. A recent publication by Olsen et al. (2013a) considered the effects of the collection substrate and sample processing on pig oral fluid testing results. They found that for PCR-based assays oral fluid should be collected onto cotton-based materials but didn't evaluate the effect of sample processing. For serological testing, collection onto cotton-based material was found to have little impact on detection of IgG by ELISA and minimal post-collection processing is optimal.

Table 3: Summary of results (Ct values) comparing processing/extraction methods of porcine oral fluids for real-time PCR

Pathogen	Dilution	Experiment 1						Experiment 2					
		PBGS		Extraction A1		Extraction B1		Extraction B2	Extraction A1	Extraction A2	Extraction A3	Extraction B1	Extraction B3
Bungowannah virus	10^{-1}	19.50[a]	(3/3)[b]	23.42	(2/2)	23.48	(2/2)	22.39	26.63	25.45	26.49	-	-
	10^{-2}	22.63	(3/3)	26.97	(2/2)	26.89	(1/2)	25.67	31.3	30.39	30.67	-	-
	10^{-3}	25.51	(3/3)	29.39	(2/2)	30.63	(1/2)	28.59	34.44	33.62	34.55	-	-
	10^{-4}	28.86	(4/4)	32.69	(2/2)	32.79	(1/2)	31.85	-	34.97	-	-	-
	10^{-5}	32.22	(4/4)	35.76	(1/2)	36.18	(1/2)	34.4	-	-	-	-	-
	10^{-6}	34.71	(3/4)	-	(0/2)	36.17	(1/2)	-	-	-	-	-	-
	10^{-7}	-	(0/3)	-	(0/2)	-	(0/2)	36.94	-	-	-	-	-
	NC	-	(0/3)	-	(0/2)	-	(0/2)	-	-	-	-	-	-
BHV-1	10^{-1}	27.00	(3/3)	27.0	(2/2)	25.33	(1/2)	24.67	26.48	29.69	-	-	-
	10^{-2}	30.22	(4/4)	30.4	(2/2)	28.74	(1/2)	28.3	29.42	32.7	-	-	-
	10^{-3}	33.57	(4/4)	32.9	(2/2)	38.84	(1/2)	31.01	-	36.8	-	-	-
	10^{-4}	35.62	(2/4)	37.4	(1/2)	-	(0/2)	34.92	-	-	-	-	-
	10^{-5}	37.94	(1/3)	-	(0/2)	-	(0/2)	-	-	-	-	-	-
	10^{-6}	-	(0/3)	-	(0/2)	-	(0/2)	-	-	-	-	-	-
	10^{-7}	-	(0/3)	-	(0/2)	-	(0/2)	-	-	-	-	-	-
	NC	-	(0/3)	-	(0/2)	-	(0/2)	-	-	-	-	-	NT
PCV2 (Field samples)				34.05		32.94		33.25	33.63	NT	NT	32.05	NT
				35.09		33.01		33.00	-	35.89	36.67	-	33.03
				23.52		24.07		-	25.31	22.07	22.97	-	-
				19.44		-		-	23.82	22.30	24.07	-	-
				20.55		-		-	-	25.97	27.13	-	-
									-	-	-	-	-

[a] Mean Ct value of positive samples; [b] number of positive samples/total samples where more than one replicate was tested. Extraction methods: A = MagMax 96 Viral isolation kit (50 µL unclarified sample); A1 = MagMax 96 Viral isolation kit (50 µl clarified sample); A2 = MagMax 96 Viral isolation kit (25 µL unclarified sample); B1 = MagMax Pathogen RNA/DNA Kit (unclarified sample); B2 = MagMax Pathogen RNA/DNA Kit (oral fluid added to lysis buffer 45 minutes after 'spiking'); B3 = MagMax Pathogen RNA/DNA Kit (clarified sample). NC = negative control; NT = not tested.

c) Effect of temperature on detection of viral nucleic acid in oral fluid samples

The results of this study are illustrated in Figures 1 to 3. An increase in the Ct value of approximately 3.2 reflects a 10-fold decrease in the amount of viral nucleic acid detected.

PBGS is a viral transport medium that is used for preservation of viral infectivity and viral nucleic acid. Before evaluating the effect of temperature on the detection of viral nucleic acid in porcine oral fluid it is worth considering the effects of temperature on the control samples. BHV-1 DNA was extremely stable in PBGS regardless of the storage temperature (Figures 2a to 2c) with little change in the Ct value detected over the 13 day period (and in some cases the Ct value was actually lower at the end than the start of the study – the reason for this is unclear). In contrast, Bungowannah virus (an RNA virus) was less stable in PBGS (Figures 1a to 1c). At 4°C a less than 1 Ct value increase was observed over the 13 days; at 25°C increases of 1.5 and 2.5 Ct values were observed for dilutions 1 and 3; for dilution 2 a mean 9 Ct value increase was observed after the 13 days at 25°C – why this occurred is not clear but the effect was observed in both experiments; at 37°C the effect was greater with a mean increase of the CT value of between 3.9 and 6.0 observed for each of the virus dilutions. This indicates that the nucleic acid of Bungowannah virus (an RNA virus) is inherently less stable than the nucleic acid of a herpesvirus (a DNA virus). This difference may occur primarily because DNA is inherently more stable than RNA. However, as viral nucleic acid in an intact virus particle is protected by a protein nucleocapsid, the resilience to degradation of this protein may also be a factor.

In porcine oral fluid, detection of BHV-1 nucleic was stable over the 13 days of the study after storage at 4°C. In contrast, the amount of nucleic acid detected decreased over time following storage at 25°C or 37°C. BHV-1 DNA could be detected for the 13 day study for Dilutions 1 and 2 at 25°C with the Ct values increasing by 4.4 and 5.7 during the study; Dilution 3 could no longer be detected on day 13. At 37°C, BHV-1 DNA could no longer be detected at day 3 for Dilution 3 and from day 7 for the higher concentrations (Dilution 1 and 2).

Bungowannah RNA was less stable in porcine oral fluid compared with BHV-1 DNA. At 4°C only a mild effect was seen with the Ct values increasing between 1 and 1.9 over the 13 days. At 25°C the Ct values for Dilution 1 and 2 increased by 9.5 and 8.9 respectively. Bungowannah RNA was only intermittently detected after day 3 in Dilution 3. At 37°C Bungowannah RNA was not detected from day 3 for Dilution 3, day 10 for Dilution 2 and the increase in Ct value was ≥10.4 for Dilution 1 over the 13 day period.

PCV2 is known to be very stable in the environment and the effect of temperature on detection of PCV2 in oral fluid was generally much less than that observed for BHV-1 and Bungowannah virus. The exception was a 4°C where a 1.5 Ct increase (a <1 $\log_{10}$ decrease in quantity detected) was observed over the 13 days study (Figure 3).

In addition to the effect of temperature, the inhibitory effect of porcine oral fluid on the detection of viral nucleic acid by real-time PCR compared with PBGS is also demonstrated by the results. At Day 0, before the effect of temperature on the samples, this is demonstrated by the difference between the Ct values for the same dilution of BHV-1 and Bungowannah virus in oral fluid compared with these viruses in PBGS. The Ct values for BHV-1 virus spiked into oral fluid samples ranged between 3.0 and 4.4 cycles higher compared with the PBGS samples. For Bungowannah virus the Ct values were between 5.4 and 5.9 cycles higher for porcine oral fluid compared with PBGS. (See also Table 4b).

These results indicate optimal transport temperature for samples is 4°C and at this temperature there is minimal decline in the concentration of viral DNA/RNA in the sample. This is important as it suggests that as long as samples are well packed extended transport over 2-3 days should not impact on the result (positive/negative) or the quantity of virus detected (at least for these 2 agents – note that while BHV-1 is not present in pigs it belongs to the same subfamily of herpesviruses as pseudorabies virus). If samples cannot be submitted immediately they should be held frozen at -20°C. During hot weather freezing of samples prior to shipping to the laboratory should also be considered due the

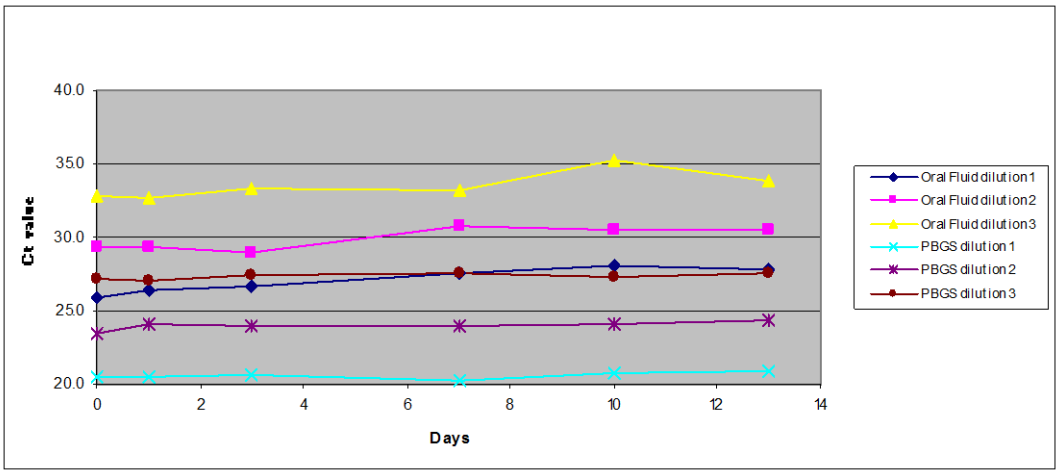

Figure 1a: Effect of storage at 4°C over 13 days on the detection of Bungowannah virus in PBGS or porcine oral fluid by PCR.

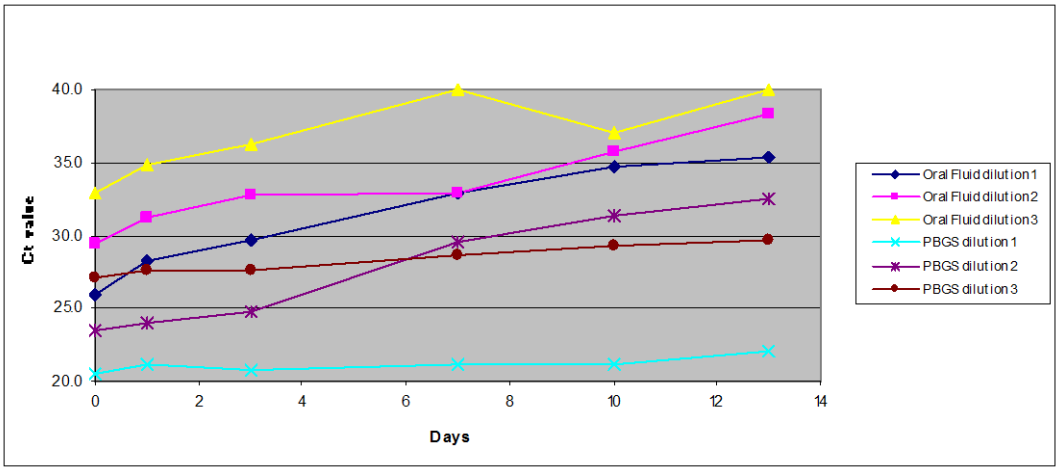

Figure 1b: Effect of storage at 25°C over 13 days on the detection of Bungowannah virus in PBGS or porcine oral fluid by PCR.

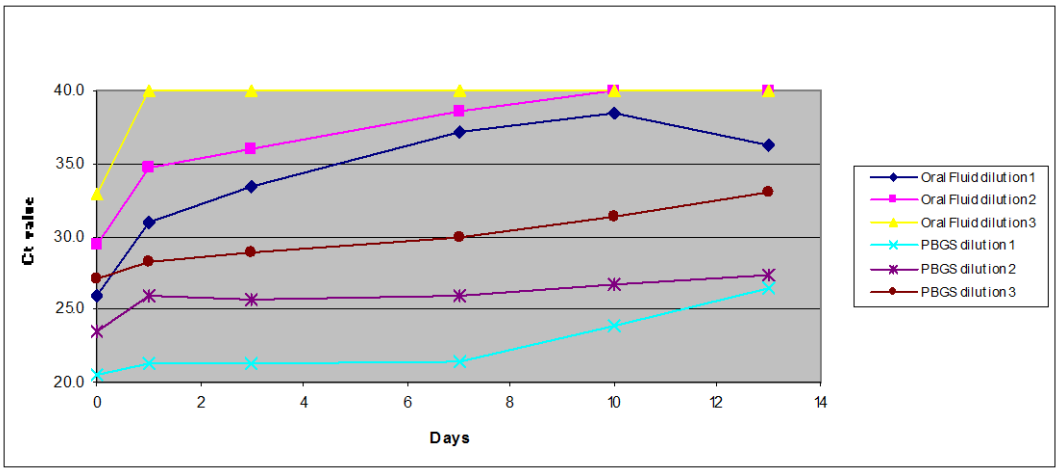

Figure 1c: Effect of storage at 37°C over 13 days on the detection of Bungowannah virus in PBGS or porcine oral fluid by PCR.

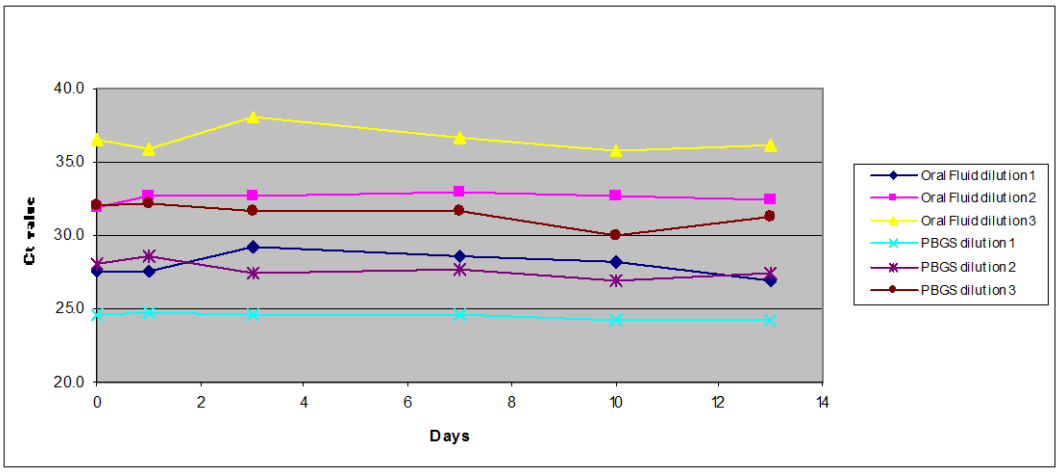

Figure 2a: Effect of storage at 4°C over 13 days on the detection of BHV-1 in PBGS or porcine oral fluid by PCR.

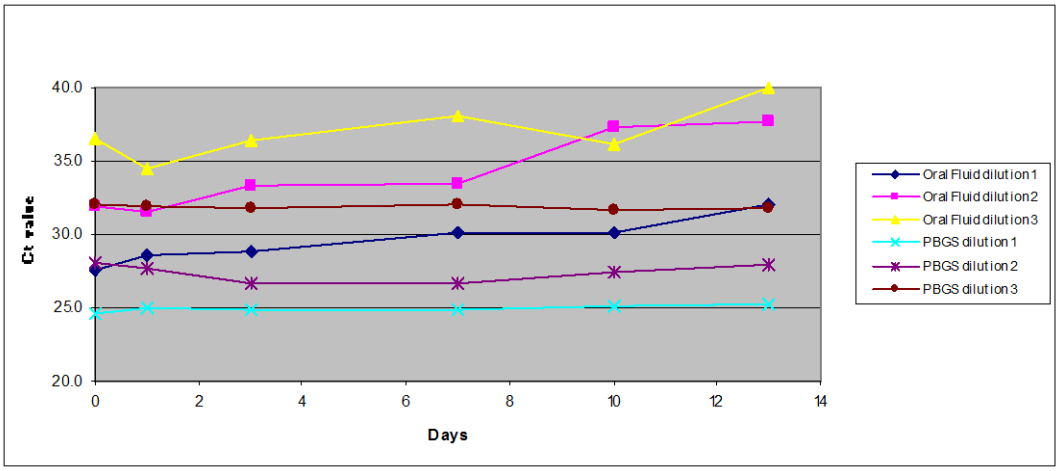

Figure 2b: Effect of storage at 25°C over 13 days on the detection of BHV-1 in PBGS or porcine oral fluid by PCR.

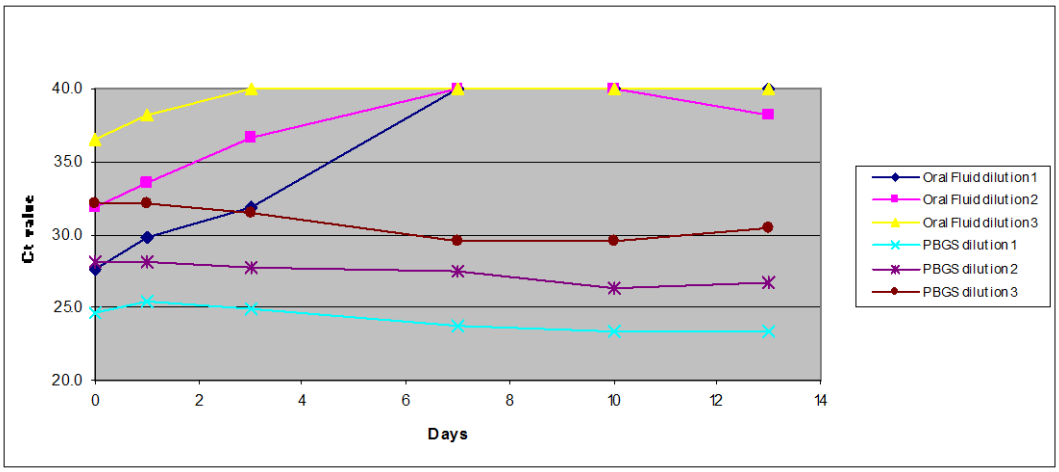

Figure 2c: Effect of storage at 37°C over 13 days on the detection of BHV-1 in PBGS or porcine oral fluid by PCR.

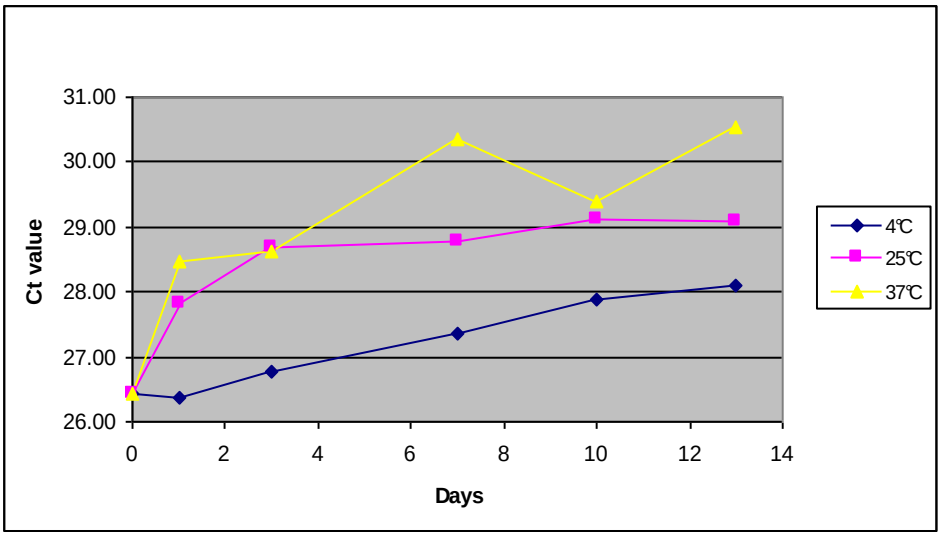

Figure 3: Effect of storage at 4°C, 25°C or 37°C on the detection of PCV2 in oral fluid over a 13 day period.

more rapid deterioration of the viral nucleic acid in oral fluids at higher temperatures. Our results are consistent with those of Prickett el al. (2010) who conducted a detailed study evaluating the stability of PRRSV in porcine oral fluid. They found that prompt freezing or refrigeration at 4°C maintained the integrity of PRRSV RNA.

d) Detection of PCV2 immune responses in oral fluids

Preliminary experiments indicated that the optimal sample incubation temperature and time for oral fluid samples in the Synbiotics® SERELISA® PCV2 Ab Mono Blocking kit was likely to be undiluted, 1/2 or 1/5 at 4°C for 16 hours (overnight). Under these conditions the in-kit controls had optical densities that met test validation criteria. To further clarify the optimal dilution for oral fluid 6 samples were selected for further evaluation based on moderately high antibody levels in 10/50 animals in the pen sampled. All of these samples gave positive results with similar reactivity regardless of sample dilution in the test. The remaining 146 oral fluid samples collected for this project were tested at each of the 3 dilutions with a sample incubation time of 16 hours at 4°C.

When all 152 samples were evaluated there was generally little difference observed between the S/N ratios of samples tested undiluted or at 1/2. At a 1/5 dilution the S/N ratio of some samples started to increase and in some instances this caused the result interpretation to change from positive to negative. There was a higher percentage of samples with poor duplicates for samples tested undiluted and for this reason we would recommend that oral fluids are tested at a 1/2 dilution in the Synbiotics® SERELISA® PCV2 Ab Mono Blocking kit. The results of the 1/2 dilution were used for comparison and analysis of results and evaluation of the effect of temperature on detection of pCV2 antibodies.

e) Effect of temperature on detection of PCV2 antibodies in oral fluid samples

For the six oral fluid samples selected with moderate positive reactivity in the PCV2 antibody ELISA there was no effect on the ELISA result during the 28 day period the samples were held at 4°C. The results are illustrated in Figure 4a. All 6 samples remained positive for PCV2 antibody for the duration of the study with minimal change in the reactivity.

In the second experiment oral fluid samples with a wider range of reactivity in the PCV2 antibody ELISA were evaluated. Increasing storage temperature was shown to have an

effect on the detection of PCV2 antibodies in oral fluid over time. The findings of the initial study were reproduced in this second study. While all samples remained positive when stored at 4°C an increase in the S/N ratio was observed in 4 samples (Figure 4b). When stored at 25°C, after 7 days five initially positive samples gave intermittent negative results and hovered around the positive/negative test threshold for the remainder of the study (Figure 4c). Storage at 37°C had a marked effect on the detection of PCV2 antibodies. From day 3, PCV2 antibody could no longer be detected in 3 samples and at day 7 a further two samples gave negative results (Figure 4d). Interestingly, two of the samples remained positive throughout the 14 days stored at 37°C although an increase in the S/N ratio was noted. Although it is not clear why these two samples were affected less by storage at 37°C we hypothesise this was related to the higher level of antibody present in these samples as reflected by the lower starting S/N ratios of these two samples on Day 0.

These results are comparable with those reported by Prickett et al. (2010) for detection of PRRSV antibodies. The effect of temperature on the detection of antibodies in oral fluid indicates that the optimal storage requirements suggested for real-time PCR should be followed. If samples cannot be submitted immediately they should be held frozen at -20°C. Samples should be well packed with chilling blocks to maintain a temperature near 4°C while being shipped to the laboratory.

f) Evaluation of the inhibitory effect of porcine oral fluid samples on detection of DNA and RNA viruses by real-time PCR

In section 3.c) the higher Ct values for viruses 'spiked' into porcine oral fluids versus PBGS were noted. An exogenous (internal control) nucleic acid can be added to samples to monitor both nucleic acid purification and the efficiency of the PCR reaction. By adding the internal control (XIPC DNA or RNA) to the PCR reaction mix we were able to assess if there were any substances that had an inhibitory effect on the PCR.

The results of this study indicate that following purification of porcine oral fluid with Kit A there appears to be few inhibitory substances present that impact on the PCR (or reverse transcription of the RNA). This is reflected by the less than 1 Ct value difference in the mean of the XIPC RNA (25.83) or XIPC DNA (24.97) control and the XIPC values for the extracted samples tested (Table 4a and 4b). While the number of samples evaluated for Kit B was low there is some indication that without clarification of the porcine oral fluid prior to nucleic acid extraction/purification some inhibitory substances are not removed by the purification step. In this project this resulted in losses ranging from >2- to 100-fold in sensitivity based on the Ct value of the internal control (Table 4a – extraction method B1). For extraction method B1 (Table 4a) it is interesting to note that for the 2 samples where <5 µL of sample was available for testing, the Ct value of the XIPC RNA is lower than other samples extracted with this method. The lower Ct values indicate less inhibition of the PCR – this is possibly due to a lower amount of inhibitory substances due to the reduced volume of sample tested. It is worth noting that at the start of this experiment the porcine oral fluid collected was pooled and mixed prior to the 'spiking' experiment so the variations in Ct values observed for the XIPC controls reflect differences in a single sample and not inherent differences between the samples themselves.

The results indicate that with the preferred processing and extraction method there is little inhibitory effect on the PCR after extraction and the losses in sensitivity that occur with the oral fluid matrix occur either prior to or during the extraction step, perhaps due to viral degradation or binding of virus to substances in the oral fluid.

It is not clear if RNA viruses (such as Bungowannah virus) are more severely affected by the inhibitory effects of oral fluid in the PCR test than DNA viruses. The results presented in Table 4a suggest the RNA virus studied is more severely affected while those in 4b suggest the difference in inhibitory effect is negligible. The difference observed between the two experiments may relate to how the virus was 'spiked' in and serially diluted in these samples and be independent of nucleic acid type.

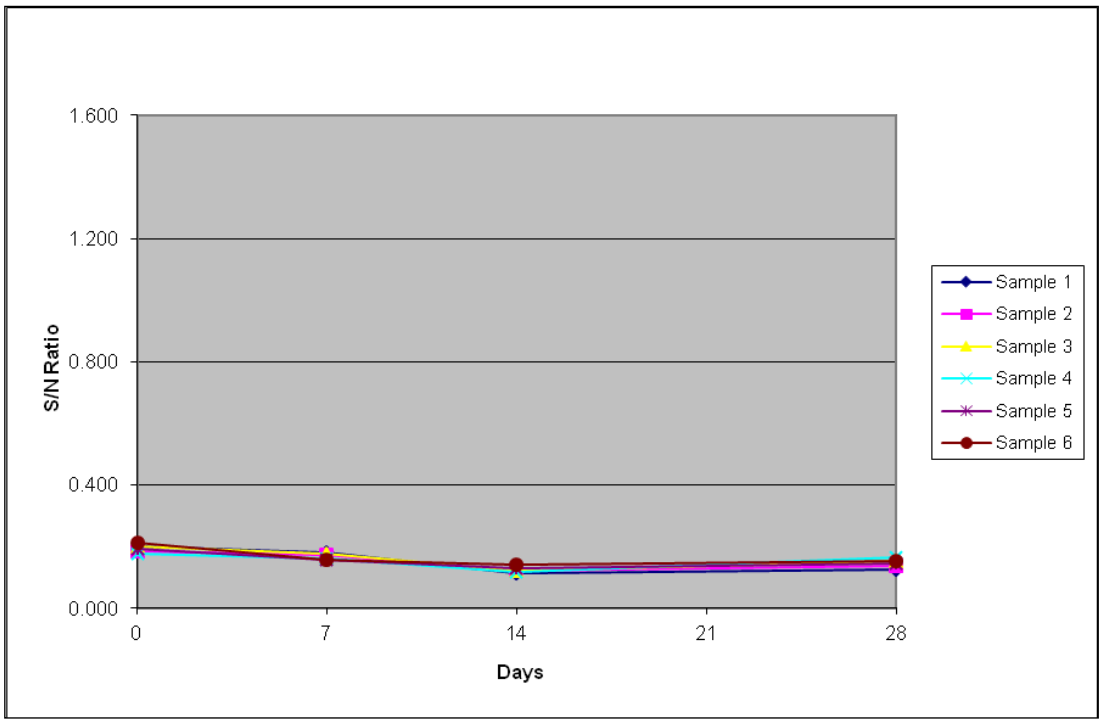

Figure 4a: Effect of storage of processed oral fluid at 4°C over 28 days in the PCV2 antibody ELISA. A sample with an S/N ratio ≤0.4 is considered positive for the presence of PCV2 antibody.

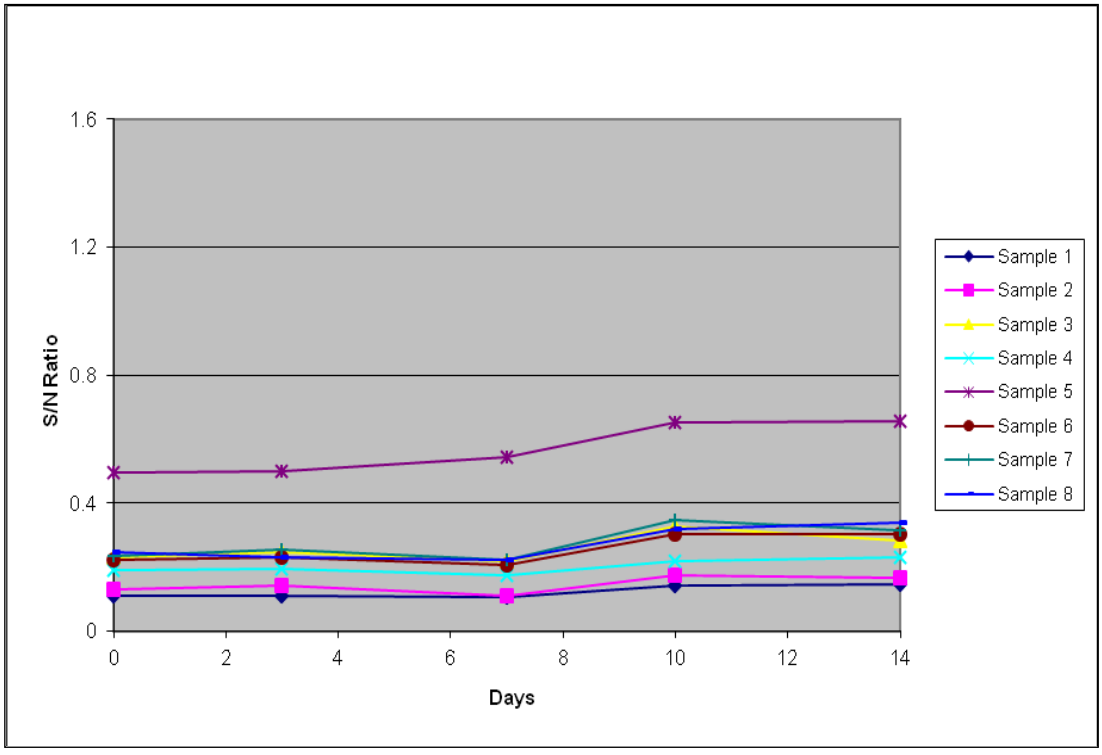

Figure 4b: Effect of storage of processed oral fluid at 4°C over 14 days in the PCV2 antibody ELISA. A sample with an S/N ratio ≤0.4 is considered positive for the presence of PCV2 antibody.

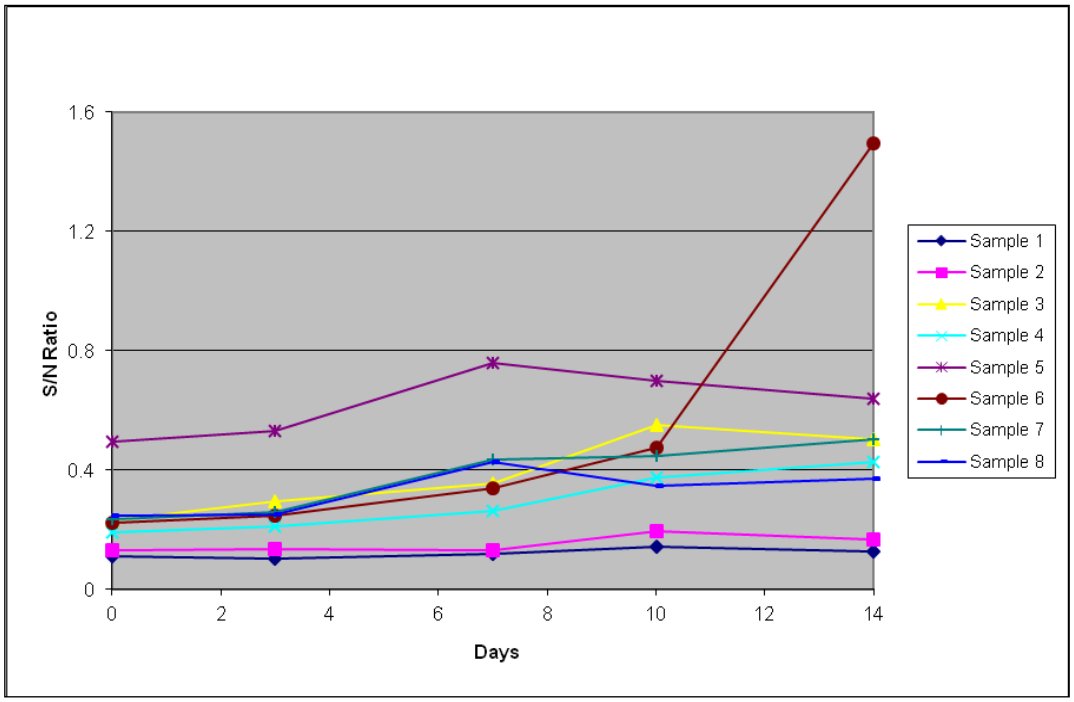

Figure 4c: Effect of storage of processed oral fluid at 25˚C over 14 days in the PCV2 antibody ELISA. A sample with an S/N ratio ≤0.4 is considered positive for the presence of PCV2 antibody.

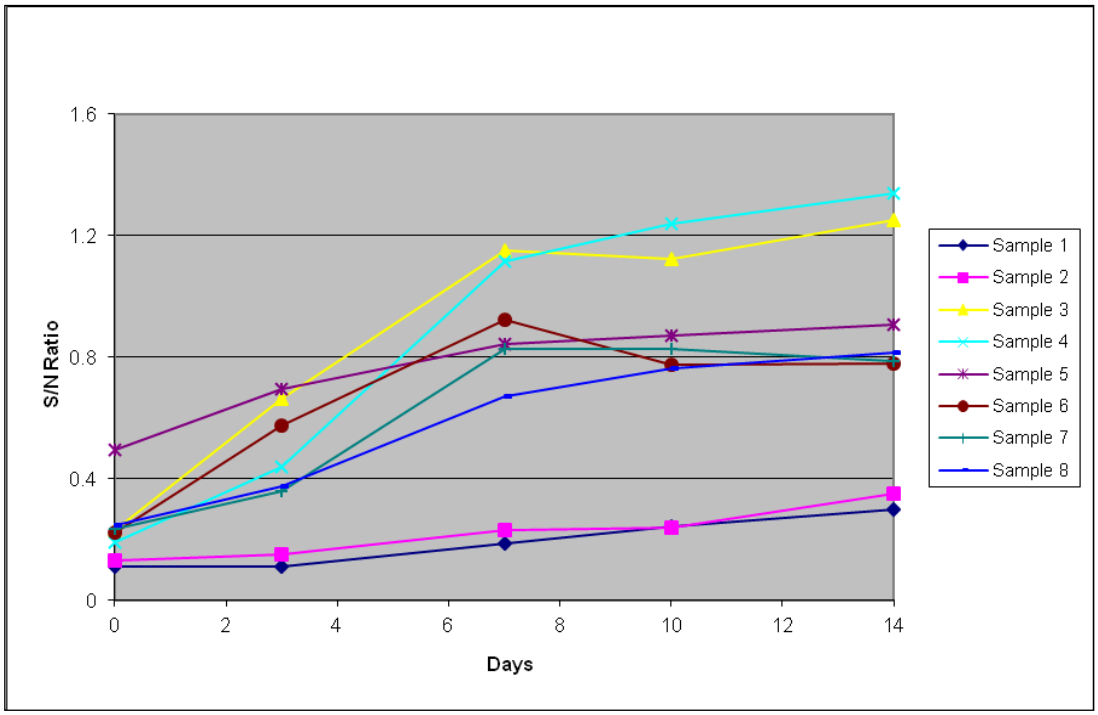

Figure 4d: Effect of storage of processed oral fluid at 37˚C over 14 days in the PCV2 antibody ELISA. A sample with an S/N ratio ≤0.4 is considered positive for the presence of PCV2 antibody.

Table 4a: Summary of real-time PCR results (Ct values) for nucleic acid purified from PBGS and porcine oral fluid samples 'spiked' with Bungowannah virus or BHV-1 and processed with different extraction methods.

Pathogen	Dilution	PBGS	XIPC[a]	Extraction A1	XIPC	Extraction A2	XIPC	Extraction B1	XIPC	Extraction B3	XIPC
Bungowannah	10^{-1}	17.81	24.99	26.58	26.40	23.71	26.12	26.47[b]	25.68	21.27	25.65
Virus	10^{-2}	20.82	25.76	28.93	26.50	27.67	26.34	27.79[b]	25.66	26.61	26.12
	10^{-3}	24.07	26.15	30.84	26.54	30.24	26.34	31.07	27.27	29.21	26.11
	10^{-4}	27.64	26.23	-	26.90	31.04	26.32	-	28.34	31.34	26.26
BHV-1	10^{-1}	25.91	25.23	25.76	25.32	28.63	25.39	26.43	28.38	28.06	25.19
	10^{-2}	29.08	25.40	28.75	25.28	31.77	25.32	28.43	28.02	30.33	25.21
	10^{-3}	31.65	25.50	31.6	25.18	38.2	25.24	-	30.15	33.76	25.30
	10^{-4}	34.8	25.52	-	25.22	-	25.31	-	28.8	-	25.53

[a] XIPC RNA or DNA was added to the PCR reaction mix; [b] Less than 5 µL of RNA tested. Mean Ct for XIPC RNA only control = 25.83; Mean Ct for XIPC DNA only control = 24.97.

Table 4b: Summary of real-time PCR results (Ct values) for nucleic acid purified from PBGS and porcine oral fluid samples 'spiked' with Bungowannah virus and BHV-1. Samples were clarified before extraction with Kit A.

		Experiment 1		Experiment 2	
Sample	**Dilution**	**Bungowannah virus**	**XIPC RNA**	**Bungowannah virus**	**XIPC RNA**
Oral fluid	$10^{-1.3}$	25.19	26.08	24.98	26.24
Oral fluid	$10^{-2.3}$	28.53	26.20	28.73	26.32
Oral fluid	$10^{-3.3}$	30.25	26.13	30.47	26.19
PBGS	$10^{-1.3}$	20.28	25.55	20.86	25.65
PBGS	$10^{-2.3}$	23.46	25.80	24.37	26.03
PBGS	$10^{-3.3}$	26.87	26.15	26.68	26.14
Sample	**Dilution**	**BHV-1**	**XIPC RNA**	**BHV-1**	**XIPC RNA**
Oral fluid	$10^{-1.3}$	32.19	25.18	27.47	25.48
Oral fluid	$10^{-2.3}$	37.05	25.25	33.05	24.97
Oral fluid	$10^{-3.3}$	-	25.22	38.02	25.29
PBGS	$10^{-1.3}$	28.49	25.44	24.28	25.34
PBGS	$10^{-2.3}$	30.45	25.70	27.63	25.38
PBGS	$10^{-3.3}$	34.05	25.45	30.37	25.37

[a] XIPC RNA or DNA was added to the PCR reaction mix. Mean Ct for XIPC RNA only control = 25.83; Mean Ct for XIPC DNA only control = 24.97.

Overcoming the inhibitory effect of oral fluid samples is one of the keys to optimising the sensitivity of real-time PCR assays for this sample matrix. Practically, the inhibitory effect means that when low levels of virus are present the viral nucleic acid may not be detected until it reaches a certain quantity which may be 2 $\log_{10}$ higher than when tested in an optimal sample matrix. This is of greatest relevance if oral fluids samples are to be used for surveillance for emergency animal diseases or early detection of a new pathogen on a high disease status farm.

g) Detection of Lawsonia immune responses in oral fluids

i. Evaluation of immunoassays to detect *L. intracellularis*- specific IgG in oral fluids

The *L. intracellularis* IFAT was developed in the Microbiological Diseases and Diagnostics Research (MDDR) laboratory at EMAI so it was easy to make changes to the assay to increase sensitivity. The IFAT uses whole *L. intracellularis* as antigen, which bind to *L. intracellularis*-specific antibodies in serum or oral fluids. The *L. intracellularis* antigen-antibody complex is detected with a polyclonal anti-pig IgG conjugated to the fluorophore FITC. Fluorescing *L. intracellularis* are visualised with a microscope under UV illumination, ensuring the specificity of the assay. When initial tests with oral fluids were unsuccessful, the concentration of *L. intracellularis* antigen was increased 2 and 4 fold, and the concentration of the fluorescent detection antibody was also increased 2- and 4-fold, but it was not possible to detect *L. intracellularis*-specific IgG in any of the oral fluid samples. Background fluorescence became too great once concentrations of antigen, oral fluids or the fluorescent detection antibody were increased further.

L. intracellularis-specific antibodies were not detected in oral fluids using the commercial blocking ELISA either (Bioscreen Enterisol Ileitis ELISA), and we were unable to modify reagent concentrations to optimise the assay sensitivity. This blocking ELISA was expected be more specific than the IFAT as *L. intracellularis*-specific antibodies in samples are bound to the antigen on the ELISA plate and detected with a monoclonal antibody specific for *L. intracellularis*. However, the commercial blocking ELISA was not more sensitive that the IFAT even when the incubation time for binding of antibodies to *L. intracellularis* antigen was increased from one to 16 hours.

In order to increase the sensitivity of *L. intracellularis* antibody detection, two different modified *L. intracellularis* ELISAs were developed at EMAI. The first ELISA used whole *L. intracellularis* bacteria bound to ELISA plates to detect *L. intracellularis*-specific IgG in pig oral fluids with a polyclonal anti-pig IgG secondary antibody. While a two-fold increase in optical density could be detected in oral fluids from positive grower and finisher pigs relative to negative pigs, the sensitivity of the test required further improvement or modification. Increasing the concentration of antigen or secondary detection antibodies led to higher background readings and so ultimately did not improve the sensitivity of the test.

The second ELISA evaluated a purified *L. intracellularis* component as the antigen. This outer membrane molecule (a lipopolysaccharide or LPS) reacted with sera from *L. intracellularis* infected pigs, but not with uninfected pigs in a Western Blot assay, suggesting that it is specific for *L. intracellularis*. Using the LPS as antigen in an ELISA, *L. intracellularis* IgG was detected in the serum of infected pigs, but it has not been possible to detect antibodies in oral fluids. While large amounts of the *L. intracellularis* LPS antigen were purified by the MDDR group at EMAI, it was not possible to increase binding of the sugar-lipid antigen, even with increased concentrations of LPS. This is possibly because of the high purity of the LPS extract. Most ELISA plates are designed to bind proteins not sugars, and although a wide range of ELISA plates were tried, it was not possible to increase LPS binding to a level that allowed detection of *L. intracellularis* antibodies in oral fluids.

At the current time there are no other *L. intracellularis* immunoassays available. A purified recombinant *L. intracellularis* protein may be required to increase the sensitivity and specificity of an ELISA for oral fluids (Zimmerman, personal comm). It is possible that

even with higher concentrations of purified *L. intracellularis* antigen it will not be possible to detect *L. intracellularis* antibodies in oral fluids due to their very low concentration.

ii. Concentration of antibodies in oral fluid samples:

The low concentration of antibodies in oral fluids (< 1% of antibodies found in sera) meant that it was not possible to detect *L. intracellularis*-specific antibodies in oral fluids from known *L. intracellularis* positive herds using the commercial blocking ELISA, the IFAT, the LPS ELISA or the modified whole cell ELISA. Consequently research was focussed on concentrating and purifying total IgG from oral fluids to increase the sensitivity of the above assays.

Initially size exclusion chromatography was used to remove all proteins less than 75kDa from the oral fluids, allowing larger proteins such as IgG (150kDa) to be concentrated in the sample. However, size exclusion alone was not enough to concentrate the antibodies to a sufficiently high level for detection with our current ELISAs.

In the next stage, affinity chromatography was used to capture total IgG from the oral fluids prior to concentration with size exclusion chromatography. Pig IgG binds with strong affinity to Protein A, so the IgG in oral fluids were purified on a Protein A glass matrix column. Contaminating proteins in the sample including albumin and plasminogen passed through the pores in the column and were discarded in the waste. The bound IgG was eluted and then concentrated on a size exclusion column and dialysed to remove salts.

Purification of IgG with the above method significantly reduced the total concentration of proteins in the oral fluids, largely by removing the significant concentration of contaminating proteins (55% albumin). However, significant amounts of pig IgG were also lost with this purification process; from 7 µg/mL to 0.5 µg/mL of pig IgG, calculated with the pig IgG ELISA developed in Project 2A-103. At this low concentration, it was not possible to detect *L. intracellularis*-specific IgG using the currently available immunoassays.

To overcome the loss of IgG from oral fluids by affinity chromatography, the capture of pig IgG from much larger volumes of oral fluids on magnetic beads coated with Protein A was investigated. Pig IgG from 20mL of oral fluids was captured by the magnetic beads and eluted in 0.4 mL volume (50 x concentration). However, it was still not possible to detect *L. intracellularis* specific IgG in this concentrated oral fluid using any of the serological assays available. This may have been due to the limited binding capacity of the Protein A coated magnetic beads (only 8mg/mL beads, restricted by the diameter and surface area of the beads). While significant attempts were made to optimise the capture of IgG on beads, it was never at sufficient levels to be detected in current *L. intracellularis*-specific immunoassays (ELISA and IFAT).

All of the Protein A methods required altering the IgG protein from its native state in order to disassociate it from Protein A. The final method investigated involved concentration of IgG in its native state in oral fluids by filtration under pressure in a stirred cell apparatus. It was hoped that this method would avoid losses associated with affinity columns and Protein A coated beads. The high recovery Amicon XM50 membrane allowed small molecules (<50,000 dalton) to pass through, thus concentrating larger molecules including IgG. Under pressure (35psi), 30 mL of oral fluid was concentrated down to 2 mL (15x). However, it was still not possible to detect *L. intracellularis*– specific IgG in these concentrated oral fluid samples.

While all of the techniques tested have concentrated total antibodies in pig oral fluids, not all of these would be feasible for diagnostic assays due to cost as well as the time required to purify the IgG. Even with a cost-effective IgG purification method, it is possible that the concentration of *L. intracellularis*-specific IgG in oral fluids will always be too low to detect with the current immunoassays. Previous attempts to detect *L. intracellularis*-specific IgG in oral fluids have had limited success (Pedersen; Zimmerman, *personal communication*). In a very small study, increasing antibody titres were observed in the sera of vaccinated gilts at 28 and 49 days post vaccination, but corresponding increases in antibody titres in oral fluids were less obvious (Johnson et al., 2011). Pigs affected with

ileitis routinely have titres of about 240 (i.e. can detect IgG in sera diluted 1/240). It is expected that titres in oral fluids will be less than 1% of this. Detection of viral antibodies such as PCV2 and PRRS in oral fluids has been more successful because pigs commonly raise titres of 10,000 to 20,000 to PCV2 in serum (Prickett et al., 2008).

h) Evaluation of field samples (PCV2 real-time PCR and serology and L.intracellularis real time PCR).

i. PCV2 real-time PCR:

Based on testing of serum by qPCR PCV2, viraemia was detected in at least one pig for 78/89 pens. In contrast PCV2 DNA was detected in oral fluid samples from 88/89 pens and in 151/152 individual oral fluid samples. The single negative oral fluid sample was from a pen where the serum from all pigs sampled was also negative. The quantity of PCV2 DNA detected in all oral fluid samples ranged from <2.3 $\log_{10}$ copies of PCV2 DNA/mL (i.e. no PCV2 DNA detected) to 8.19 $\log_{10}$ copies of PCV2 DNA/mL. The quantity of PCV2 DNA detected in oral fluid samples where all the sera tested from that pen gave negative results ranged from 2.40 to 5.78 $\log_{10}$ copies of PCV2 DNA/mL. In pens where two or three ropes were placed for oral fluid collection the range of quantities detected within a pen was within one order of magnitude (i.e. <10-fold difference).

Simple correlation coefficients were calculated for the variables listed in Table 5 and plotted in Figures 5a to 5c. Significant correlations were detected for each of the compared variables with the geometric mean of the quantity of PCV2 in serum for the animals sampled correlating best with the quantity of PCV2 DNA detected in oral fluid. The graphs illustrate the greater sensitivity of oral fluid compared with serum from a subsample of the group. This is illustrated by the points on the graph that indicate positive results in the oral fluid sample despite negative results in serum (≤2.3 $\log_{10}$ copies/mL) or a low mean quantity of PCV2 DNA detected in serum compared with oral fluid. In addition, for 93% of pens sampled the mean quantity of PCV2 DNA detected in oral fluid was greater than the geometric mean of the quantity detected in serum (P<0.001). While not statistically significant, for 46% of pens sampled the mean quantity of PCV2 DNA detected in oral fluid was greater than the maximum viral load in serum for the pigs sampled (P=0.458). These findings suggest that either PCV2 DNA is detected in higher quantities in oral fluid compared to serum or that the quantity of PCV2 DNA detected in oral fluid better reflects the viral load of the pigs shedding the highest quantity of virus within the group.

Table 5: Summary of simple correlation coefficients calculated between variables from serum and oral fluid in the PCV2 qPCR.

	Variable		Result
Test	**X**	**Y**	**R value**
PCV2 qPCR	Geometric mean (serum)	Oral fluid (OF) mean	0.438***
	Arithmetic mean (serum)	Oral fluid (OF) mean	0.431***
	Maximum viral load (serum)	Oral fluid (OF) mean	0.415***

*** Denotes significant at p<0.001.

These findings indicate that there is strong potential for oral fluid sampling to be a more sensitive means of surveillance than blood sampling a small sub-group of individual pigs within a population. This is highlighted by PCV2 being detected in more pens by oral fluid sampling than blood collection. This finding is unlikely to be due to false positive results as probe based real-time PCR assays have very high specificity. The difference is therefore

either due to the greater sensitivity of oral fluid sampling as a greater number of pigs within the pen are sampled or environmental contamination due to the exploratory nature of the pig. When monitoring for an emergency animal disease this second factor is not relevant and the first highlights the potential advantage of this sample type for disease surveillance. When monitoring an endemic disease the detection of the presence of an agent (+/- the quantity) is likely to be used in conjunction with the disease exhibited and results observed across multiple age groups (i.e. the result trend) to make management decisions.

The sensitivity of oral fluid sampling is affected by multiple factors such as behavioural aspects of pig-rope interaction, including the proportion of pigs within a group that interact with the rope and the number of ropes per pen, the quantity of a pathogen present in oral fluids and optimisation of the detection assay. Therefore sensitivity of this approach is likely to vary between pathogens. PCV2 is known to be endemic on many piggeries in Australia with a high proportion of viraemic pigs in a pen at peak infection. This study shows that in this type of situation oral fluid surveillance is a highly sensitive approach for disease monitoring. Studies such as those described by Olsen et. al. (2013b) which examine the probability of detecting an agent based on within-pen prevalence will add further insight into the usefulness of disease surveillance by oral fluid sampling.

ii. PCV2 serology:

PCV2 antibodies were detected in the serum of at least one pig from 87/89 pens. All three oral fluid samples collected from the two pens with negative serology gave negative results for the presence of antibody based on the cut-off ranges used for the test. Oral fluids negative for the presence of PCV2 antibody were also detected in an additional 20 pens (n=25 oral fluids) giving a sensitivity of 83% for the detection of antibody in oral fluid when detected in at least one pig by testing of serum. There are a number of factors that may have contributed to this reduced sensitivity including the lower quantities of IgG antibody present in oral fluid compared to serum and sub-optimisation of the cut-off values for the testing of oral fluid samples in the ELISA test. Interestingly when the mean S/N ratio of the sera from the corresponding pen for the oral fluid samples that gave a negative result is evaluated, the mean S/N ratio is >0.4 (negative result) for 14/20 pens indicating a correlation between low serum antibody levels and a negative test result for an oral fluid sample . In contrast, for oral fluids samples giving positive results only 2 pens were found to have mean serum S/N ratios >0.4. In the absence of oral fluids 'spiked' with PCV2 antibody or a large number of oral fluid samples of known antibody status it was not possible to accurately optimise the cut-off values for oral fluid any further for the Synbiotics SERELISA® PCV2 Ab Mono Blocking test kit.

Table 6 and Figure 6 summarise and illustrate the correlation between the mean S/N ratio for serum and oral fluid samples in the Synbiotics SERELISA® PCV2 Ab Mono Blocking test kit. A significant correlation was identified between the mean or median S/N ratio in serum and the mean S/N of the oral fluid samples. In addition, the kappa value of 0.742 indicates good agreement of positive and negative result interpretation for the mean S/N ratios of serum and oral fluids collected from an individual pen.

Of the 60 pens where two or three oral fluid samples were collected the same result was reported for each oral fluid sample in 59/60 pens. For the one pen with two negative and one positive result the S/N ratios for the samples were close to the cut-off value (0.43, 0.37, 0.43). The variation in S/N ratios varied from 0.02-0.34 within a pen for those with oral fluids giving negative results (n=5) and from 0.00-0.13 (mean 0.03; median 0.02) within a pen for those with oral fluids giving positive results (n=55).

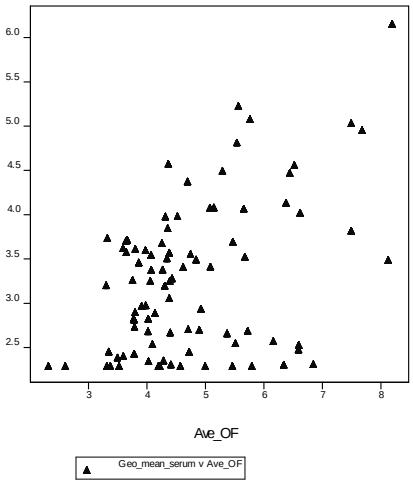

Figure 5a: Plot comparing the geometric mean of PCV2 in serum of sampled animals compared with oral fluids for animals in the same pen as measured by qPCR (viral load measured as log10 copies/mL of serum or oral fluid).

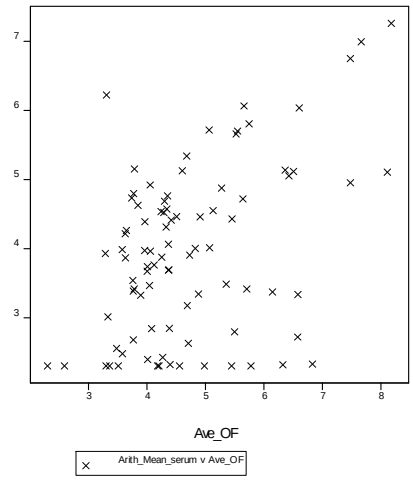

Figure 5b: Plot comparing the arithmetic mean of PCV2 in serum of sampled animals compared with oral fluids for animals in the same pen as measured by qPCR (viral load measured as log10 copies/mL of serum or oral fluid).

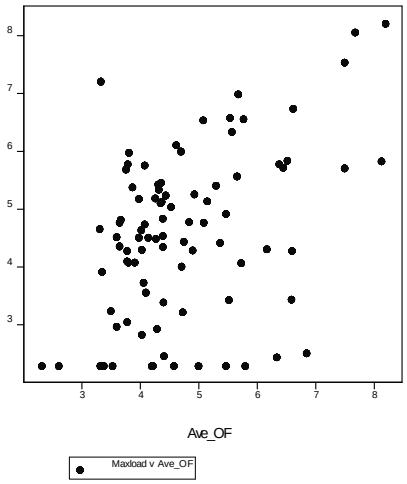

Figure 5c: Plot comparing the maximum viral load of PCV2 in serum of sampled animals with oral fluids for animals in the same pen as measured by qPCR (viral load measured as log10 copies/mL of serum or oral fluid).

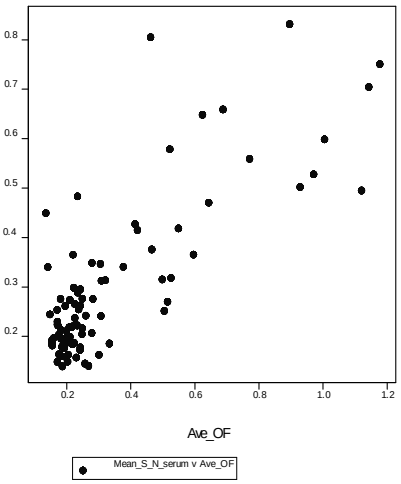

Figure 6: Plot comparing the mean S/N ratio for serum of the sampled animals compared with oral fluids for animals in the same pen as measured by the PCV2 ELISA.

Table 6: Summary of simple correlation coefficients calculated between variables for serum and oral fluid in the Synbiotics SERELISA® PCV2 Ab Mono Blocking test kit.

Test	Variable		Results	
	X	Y	R value	Kappa
ELISA	Serum Mean S/N	Oral fluid (OF) mean S/N	0.798***	0.742***
	Serum Median S/N	Oral fluid (OF) mean S/N	0.778***	

*** Denotes significant at p<0.001.

iii. *Lawsonia intracellularis* real-time PCR:

L.intracellularis DNA was detected in 97 of the 152 oral fluid samples (63.8%), with *L.intracellularis* numbers ranging from 2.6 x 10^2 to 3.2 x 10^7. *L.intracellularis* was not detected in oral fluids collected from any age group in 5 of the 12 herds. In the other 7 herds, *L.intracellularis* DNA was detected in pigs from 12.5 weeks of age and up to 19 weeks of age, with *L.intracellularis* most commonly found in the oral fluids of older pigs.

Matching sera from these same pens were tested for antibodies to *L.intracellularis*, to determine which samples and which assay identified *L.intracellularis* infection earlier. Herds with only a single age group were omitted from this analysis (herds 1, 2 and 5). In herds 6, 7 and 8, qPCR detection of *L.intracellularis* DNA in oral fluids occurred at the same time as *L.intracellularis* antibody detection in sera. However, in herds 3, 10, 11 and 12 qPCR detection of *L.intracellularis* was either delayed or not detected relative to the presence of specific antibodies in sera.

A significant correlation was demonstrated between the number of *L.intracellularis* detected in oral fluids and the concentration of *L.intracellularis* antibodies in sera (R = 0.45, p = 0.019). However, only a moderate (not significant) correlation was found between the proportion of seropositive pigs and the proportion of qPCR positive pigs (R = 0.377, p = 0.053). The results suggest that qPCR detection of *L.intracellularis* in oral fluids is not more sensitive than serology. However, the qPCR may prove useful in monitoring *L.intracellularis* infection when antibody titres are high and the prevalence of infection is high (60%).

4. Application of Research

The research undertaken during this project has established a capability and understanding of the issues related to collection and testing of the oral fluid sample matrix. It has also encouraged some of the pig veterinarians and pharmaceutical companies servicing the pig industry to develop skills in the collection of oral fluids, as opposed to their normal practice of blood collection. The adoption of oral fluid sampling could be easily introduced at the farm level as collection kits are commercially available. Before oral fluids can be used for disease surveillance (including emergency animal disease surveillance) by pathogen or antibody detection the diagnostic tests need to be validated. This project evaluated adaptation of several tests for the oral fluid matrix, the best practice for storage and transport of samples, and investigated some if the issues that can affect pathogen detection with this sample type. In this section we comment on both commercial application of oral fluid sampling for disease diagnosis and surveillance, and the laboratory aspects that need to be considered for the use of this sample matrix to be successfully applied.

A testing capability for the detection of PCV2 and *L.intracellularis* DNA and PCV2 antibodies in oral fluids has been established at the Microbiology and Virology Laboratories at EMAI. The project has demonstrated that the quantity of PCV2 detected in an oral fluid sample significantly correlates with the geometric, arithmetic and maximum viral load of PCV2 detected within the sera of a sampled sub-group of animals in the same pen. The results also indicate that the viral load detected in oral fluid is biased towards the maximum viral load of an individual animal in a pen rather than the geometric mean as 93% of pens sampled had a higher quantity of PCV2 detected in oral fluid than the geometric mean in serum. In addition, the sensitivity of detection of PCV2 within a pen was higher for oral fluids compared with serum. This is probably related to the high proportion of pigs that are likely to be infected within a group and also the potentially very high levels of virus which may be excreted by an infected pig. Oral fluid samples could be used to replace blood sampling of a sub-population of animals within a group to monitor PCV2 viral loads across age groups on a piggery as long as the limitations of this sample type is considered when interpreting the results.

During this project it was also possible to successfully detect PCV2 antibodies in oral fluid samples. As a sample matrix the main issue with oral fluid samples for serology by ELISA is the lower levels of antibody present compared with serum. This requires adaptation of assays designed for use on serum and it maybe necessary to establish new cut-off values. While testing for PCV2 antibodies is likely to have less field application compared with detection and measurement of viral load of PCV2 DNA it could be used to provide additional information about timing of seroconversion in a population in relation to time of infection or vaccination. A significant correlation was also identified between the results from oral fluid samples in the PCV2 ELISA and the mean result for serum collected from a sub-group of animals. In addition, there was a good correlation between the result interpretations of each sample type.

While the quantity of *L.intracellularis* detected in oral fluids correlated significantly with the mean concentration of antibodies, serology was still the better assay to determine the timing and prevalence of *L.intracellularis* infection. Unfortunately we were unable to develop a sufficiently sensitive ELISA for the detection of *L.intracellularis* antibodies in oral fluids, despite significant modifications of the assays and concentration of IgG in the oral fluid samples. The failure to successfully adapt a serum based antibody assay for *L.intracellularis* is presumably due to the relatively low levels of antibody produced in response to infection. Successful development of an antibody assay for this agent is likely to depend on a system which uses high quantities of purified recombinant antigen without causing unsuitable levels of background staining. As *L. intracellularis* is endemic in the Australian pig population, an ability to monitor time of infection by detection of antibodies in oral fluid to determine optimal time for vaccination or medication remains a desirable goal.

The results of the studies evaluating the effect of storage temperature on detection of viral nucleic acid by real-time PCR and PCV2 antibodies by ELISA are consistent with other reported studies despite different viruses being evaluated (Prickett et al., 2010). Oral fluid samples should be chilled to 4°C to as soon as practical after collection and frozen if not submitted to the laboratory on the day of collection. The results indicate that the optimal transport temperature is ≤4°C and at this temperature there is minimal decrease in the amount of viral DNA/RNA detected and little effect on ELISA results conducted for antibody detection over 7-14 days as long as this temperature is maintained (at least for the three viruses studied). Given the generally warm to hot climate conditions in Australia unless samples are to be dispatched to the laboratory soon after collection they should be frozen prior to shipping. Samples should be well packed with chilling blocks so they remain cold while in transit to the laboratory.

While oral fluid samples are an owner/veterinarian and welfare friendly approach to sample collection, there are some difficulties in testing this sample type. For testing by PCR the oral fluid sample has an 'inhibitory' effect on the detection of viral nucleic acid. This inhibition could potentially occur at a number of time points after collection including: 1) degradation of viral nucleic acid prior to extraction or 'binding' of virus to particulate matter that is lost when the sample is clarified; 2) reduced efficiency of extraction of nucleic acid from the sample, i.e. current extraction techniques may not be able to efficiently liberate viral nucleic acid from all the virions in the sample and/or once the nucleic acid is liberated it is bound by small particles in the oral fluid or there is some degradation by nucleases before they are completely inactivated by the extraction buffer; 3) an inhibitory effect on the PCR step resulting in reduced amplification of viral template. The inclusion of an exogenous nucleic acid to the sample at extraction is recommended so any loss in sensitivity of the assay subsequent to clarification of the sample can be monitored. In some cases a simple 1/2 to 1/4 dilution of the sample (Harmon, pers. comm.) may be sufficient to resolve the inhibitory affect and should have minimal impact on the ability to detect the agent by PCR unless it is present at very low levels, although this will require further evaluation with both field and 'spiked' samples. The inhibitory effect of oral fluids on the detection of viral nucleic acid will be a critical factor to overcome/manage should this sampling approach be used for emergency animal disease surveillance.

Assay validation with 'spiked' samples and later samples collected from experimentally or naturally animals of known infection status will be required for validation of real-time PCR assays or serological assays for each new pathogen evaluated. Samples collected from animals of know infection status is the next step in validating the real-time PCR for detection of Bungowannah virus in oral fluids from naturally infected animals. For real-time PCR these samples are necessary to demonstrate optimal sensitivity of the assay and also confirm that the pathogen is present in oral fluids at sufficient levels for detection compared to current sample types. The epidemiology of infection for a pathogen should also be considered. In particular, expected duration of shedding and amount of virus excreted in by the oral route (transient versus persistent and chronic infections) and how these may impact on pathogen detection. As observed in this project, it maybe difficult to use oral fluids to detect antibodies to pathogens that only induce a weak antibody response unless the assay is very sensitive.

The potential benefits to the cost of production have not been measured by the project but increased knowledge of disease transmission on a property may provide better targeting of treatments (vaccination/antimicrobials) in response to timing of disease transmission rather than as a response to the clinical signs of disease. Oral fluid sampling should be far more cost effective than bleeding animals and by sampling across age groups the submitter obtains a 'snap shot' of what is happening within a population in regard to a particular pathogen whether by pathogen detection or serological monitoring. The advantage of the oral fluid sample is that it is simple to collect and collection requires minimal training. Persons collecting samples should be advised of optimal sample collection time (morning) and processing to avoid cross contamination and for optimal sample integrity. Oral fluid sampling does not require anyone skilled in blood collection

and as it can potentially sample a much larger proportion of animals it should provide better surveillance of the population on a property at a particular time point.

During the course of this project a large collection of sera and oral fluids have been accumulated. These samples have been stored at -20°C and can be used for future studies to evaluate real-time PCR or serology assays for detection of pathogens or the stimulated immune response. Oral fluids have been used to detect APP in Australia (Dron, Pork CRC honours student), but detection of other respiratory pathogens such as Mycoplasma hyopneumoniae has not been possible in any laboratory in the world to date. Care is required in the interpretation of results that detect pathogens. The detection of pathogens may only indicate passive carriage, not active infection.

Porcine oral fluids for use in diagnostics is an evolving area and there are a number of avenues for future research into the use porcine oral fluids some of which include:

- Sample collection (i.e. pig behaviours associated with optimal sample collection; optimal ratio of ropes to pigs in a pen);
- Sample preservation and processing for optimal PCR and serology results;
- Adaptation of assays developed for other sample matrices to oral fluid sample (i.e. determining if there is sufficient pathogen or antibody present in oral fluid for detection by these assays; if present can the pathogen/antibody be detected; can multiplex assays be developed for platforms such as Luminex® and MAGPIX® that are both sensitive and able to test for a range of antibodies or pathogens in one test).

In addition to the use of oral fluid for diagnostics, oral fluid could also be used to monitor stress and welfare in pigs. Cortisol, acute phase proteins and salivary alpha amylase can all be detected in oral fluid and have been used as markers of stress in pigs. This non-invasive method of detecting stress may have an application in evaluating group housing of gestating gilts and sows, separation of piglets from sows during lactation and at weaning and for management practices such as tail docking.

Monitoring the partitioning of nutrients away from muscle deposition towards the immune response may also be possible using oral fluid (Jae Kim, personal comm.). Reduction in prostaglandin E2 or an increase in pro-inflammatory cytokines in blood signal partitioning of nutrients away from growth and muscle deposition towards mounting an immune response. Developing non-invasive techniques to monitor nutrient partitioning could allow the efficacy of omega-3 fatty acids, boron, antioxidants and probiotics to be tested in growing pigs.

5. Conclusion

This project has successfully undertaken an evaluation of oral fluid samples for herd health monitoring of pathogens and the immune response in pigs. The study was able to detect PCV2 and *L.intracellularis* DNA in oral fluid samples collected from the field and successfully adapt a commercially available PCV2 antibody ELISA kit for detection of PCV2 antibodies in field samples. A correlation was identified between oral fluid samples and mean serum results for the two PCV2 assays and oral fluids could be utilised in the field for PCV2 surveillance instead of blood sampling as long as the limitations of this sample type for assessing viral load are considered when interpreting the results. While field investigations for the detection of Bungowannah virus were not undertaken, laboratory studies demonstrated that detection in oral fluids is possible.

Optimal transport and storage conditions for oral fluid samples were evaluated and it was shown that samples must be stored and transported at ≤4°C for optimal detection of nucleic acid and antibodies.

The use of oral fluids as a diagnostic sample is still evolving and there are still limitations of this sample matrix for PCR and serology and these need to be considered when developing and optimising assays. Results investigating potential inhibition of PCR suggested these issues were occurring prior to the PCR step possibly due to viral nucleic acid degradation, virus binding to materials that are lost during clarification or an inability to retrieve all the nucleic acid present at the nucleic acid extraction step. Overcoming these limitations will be important to achieve optimal sensitivity from oral fluids for disease surveillance. The limitations of oral fluid as a diagnostic sample were realised in this study when it was not possible to successfully adapt Lawsonia serological assays for the oral fluid matrix. An improvement in test sensitivity without compromising test specificity appears to be required.

6. Limitations/Risks

The limitations of using oral fluid samples for diagnostic testing are discussed in more detail in section 5. The limitations principally relate to the intrinsic difficulties with this sample type such as low antibody levels in oral fluids and effects on sensitivity of PCR assays. In addition, to achieve optimal sensitivity for both pathogen and antibody detection, samples should be stored and transported frozen (extended duration) or well chilled (short periods).

7. Recommendations

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

- Ongoing support should be provided to evaluate and validate diagnostic assays for disease surveillance through use of the oral fluid sample matrix;
- Oral fluid sampling should be further evaluated as a means of surveillance for notifiable and emergency animal diseases in the Australian piggery;
- Interest by producers and veterinarians in using oral fluids samples for diagnostic sampling should be investigated and their priority pathogens determined;
- Optimal transport of oral fluid samples to the laboratory is critical. Samples should preferably be frozen prior to submission and transported to the laboratory in a well insulated container packed with chilling blocks. Samples should ideally reach the laboratory within 1-2 days of dispatch to avoid the adverse effect of sample warming. Inappropriate storage or transport will adversely impact on detection of pathogens and antibodies.
- Porcine oral fluid samples can be used to measure the infection status, viral load and serological status for PCV2 within a group of pigs;
- The oral fluid samples and respective sera collected as part of this project have been stored at -20°C and could be used for evaluation/validation of other assays;
- As oral fluid samples are a challenging sample matrix, to assess any effect on the sensitivity of a PCR assays, exogenous nucleic acid (internal control) should routinely be added to each sample prior to nucleic acid extraction
- Use of oral fluid samples for surveillance of endemic diseases would provide a familiarity with this sample type should wider surveillance for a notifiable or emergency animal disease be required.

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