Final Report for BPEX Project: Evaluation of a PCR assay for *Porcine Reproductive and Respiratory virus* in oral fluids from growing pigs and its applications for diagnosis and surveillance in the UK pig industry.

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1. Executive Summary

Following reports from the US that oral fluids may prove a useful and convenient diagnostic measure of group disease status in pigs, the aim of this study was to investigate the potential of this methodology in the UK herd, using Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) as the target pathogen. Proof of principle that PRRSv RNA can be detected in oral fluids from pigs viraemic with PRRSv was successfully achieved. However the sensitivity of the technique needs to be improved before it can be more widely applied in the commercial setting. Techniques for collection and handling of samples are described, along with the methods of optimising RNA extraction from oral fluids, and modification of the RT-PCR for PRRSv RNA for this diagnostic sample. Some targets for further research and possible reasons for apparently suboptimal test sensitivity are suggested.

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2. Introduction

The use of porcine oral fluids as a diagnostic and monitoring sample for infectious diseases in pigs was first reported in 2008 by researchers at Iowa University (Prickett *et al.* 2008a & c), and Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) was detected in oral fluids in 1997 (Wills *et al* 1997). Experimental studies have confirmed the potential of oral fluids as a diagnostic sample in pigs (Prickett *et al*. 2008c). The use of oral fluids as a diagnostic sample in other species, including man, has a longer history, which was recently reviewed by Prickett and Zimmerman (2010). The premise is that oral fluid samples are collected from a group of pigs by hanging a rope in the pen, on which they chew and deposit oral fluids (comprising saliva and oral crevicular fluid), which represents a group sample to which a proportion of individuals will have contributed, and can then be assayed for the presence of, or evidence of exposure to, infectious pathogens. The collection of a group sample is attractive because more individuals are sampled per unit cost, and a group disease status is ascertained. This is the principle used in bulk milk testing of dairy cattle, which is now used routinely in measuring their health status (Pritchard

2001). Oral fluid samples are also attractive because they do not require blood sampling of pigs, which has potential animal welfare benefits. Samples will be able to be taken by farmers, making them accessible and user-friendly. Biosecurity risks of external visitors (to blood sample pigs) are reduced.

3. Aims and Objectives

The purpose of this project was to investigate the potential of porcine oral fluid samples as a means of diagnosing and monitoring infection with PRRSv in UK pigs. PRRSv was chosen because it is an economically significant enzootic pathogen in the UK pig herd contributing to impaired performance, disease and compromised animal welfare (Richardson 2004). Detection of pathogen RNA by PCR was chosen as it is a sensitive and specific technique resulting in a discrete (positive/ negative) result suitable for determining current group status. If the technique could be validated, potential uses for commercial producers include:

- Regular economical monitoring of supposed PRRSv-negative pig flows to confirm continuing freedom from infection.
- Diagnosis of acute infection where maternal or vaccinal antibodies may interfere with diagnostic methods. At present, individual blood samples are used for this purpose. This is expensive and involves blood sampling pigs.
- Determining the presence of PRRSv among pigs suffering from diseases (*e.g.* respiratory disease in growing pigs, reproductive disease in sows), and thus investigating the possibility that PRRSv is involved in the pathogenesis of such disease
- Monitoring PRRSv disease dynamics within pig flows so that appropriate interventions (vaccination, pig flow alterations, ventilation) can be implemented. Thereafter, the impact of such interventions on disease dynamics can be measured.
- Evaluating costs of disease, by comparing sufficient numbers of pig flows with and without infection (*e.g.* PRRSv) as determined by oral fluid samples throughout growth.

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The project aimed to address the following aspects of oral fluid diagnostics in UK pigs:

- Optimal methods of rope presentation, sample collection and handling.

- Some assessment of pig responses to the rope; factors affecting interactions with the rope, some idea of the proportion of pigs contributing to the sample.
- Optimisation of the RT-PCR assay for PRRSv RNA detection, comprising RNA extraction and the RT-PCR on the resulting RNA.
- Identification of problems encountered, to inform further research in this field.

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4. Materials and Methods

4.1. Presentation of rope. Source and type of rope.

First priority was to design and test user-friendly brackets/ fixtures from which to suspend the rope. These would have to be sterilisable, adaptable to the many different types of pig accommodation in the UK, and capable of withstanding the considerable rigours of (potentially destructive) chewing pigs. Two designs were used and were found to be capable of dealing with all situations encountered.

Fig 1. Adjustable wall bracket for suspending rope

Fig 2. Corner bracket for suspending rope

The rope used (as recommended by researchers in Iowa [Jeff Zimmermann, personal communication]) was made from pure cotton rope with a diameter of either 14mm or 18mm, depending on the size of the target pigs. It was sourced from Outhwaites Ropemakers, Hawes, North Yorkshire. No other types or sizes of rope were used in this study.

4.2. Establishing sample pig disease status as determined by oral fluids and by gold standard (blood sampling a representative sample of pigs).

As the assay was designed to measure viraemia by PCR, pigs likely to be viraemic were deliberately targeted for sampling. Historical diagnostic data (usually the Intervet Herd Check cross sectional serological sampling) and the experience of each unit's private veterinary surgeon were used to identify these pigs. Pens to be sampled were chosen at random from the identified population of pigs. Oral fluid samples were collected from each selected pen of pigs, and then a representative sample of pigs were blood sampled in order to assess the true status of the pen with respect to PRRSv, as outlined in figure 1.

<table>
<tr><th>Status of pen</th><th>Definition</th><th>How to establish true status (i.e. Gold Standard against which to compare oral fluids PRRS PCR Assay)</th></tr>
<tr><td>POSITIVE</td><td>At least one pig in the pen PRRS-viraemic (i.e. serum PCR positive)</td><td rowspan="2">Bleed a representative sample of pigs. Sample size is chosen based on epidemiological calculations: to be 95% certain that at least one viraemic pig will be detected at that prevalence in the population. Expected prevalence of viraemia: 30%</td></tr>
<tr><td>NEGATIVE</td><td>Every pig in the pen non-viraemic (i.e. serum PCR negative)</td></tr>
</table>

Fig. 3. Sampling strategy for determination of pen PRRSv 'gold standard' status against which to compare the oral fluids results.

The following information was also recorded for each set of target pigs:

- Age (range) of pigs
- Floor type
- Unit type (FF, WF, GF), size, system.
- Feed type

4.3. Optimisation of molecular methods of RNA extraction and PCR.

Samples were received at VLA Weybridge the day after they were taken, and in most cases processed on the day they were received. Results of experiments described below were used to inform the optimised Standard Operating Procedure (SOP) for the detecting PRRSv in oral fluids, see Appendix A.

4.3.1. Optimisation of PRRSv RNA extraction methods using known negative oral fluids spiked with supernatant from virus culture.

Based on previous communications with researchers in Iowa, it was expected that *extraction* of PRRSv RNA from the oral fluid samples was likely to be a major challenge in this project. We therefore compared various commercially available kits designed to extract RNA from a range of samples, for their ability to extract RNA from porcine oral fluid samples. Particular consideration was given to methods performing well at typical RNA concentrations in oral fluids collected from pigs in the field as part of this study. Initially known PCR-negative oral fluids were used, and spiked with various concentrations of PRRSv obtained from the supernatant of virus cultures, obtained from Mammalian virology at VLA Weybridge.

Four different extraction approaches were compared, as outlined in table 1.

Kit	Manufacturer	Starting volume	Elution volume	method	Target sample
Magnapure Total NA	Roche	200	100	Magnetic glass particles	General
QiAmp viral RNA**	Qiagen	140	60	Silica gel based membrane spin column.	Cell free body fluids
DNeasy Protect saliva*	Qiagen	200	14	Stabilisation reagent	Saliva
Magmax Viral RNA	Ambicon	300	40	Paramagnetic beads	Nasal fluid

Table 1. Summary of RNA extraction methods evaluated

*The RNA protect product was added to spiked (uncentrifuged) saliva which was then serially diluted and stored at 4^0C for 18 hours before extraction.

**Modified method to include extended lysis time.

4.3.2. Optimisation of sample handling conditions for optimal RNA preservation.

Optimal sample handling was identified early as likely to be critical to the success of this technique. These studies were performed on known positive field samples, using an optimised RT-PCR technique. Oral fluid samples were collected from each of nine pens of 45 pigs on a PRRSv-positive indoor farrow to finish unit. These pens had been sampled

the week before and the resulting oral fluid samples determined to be PRRSv positive by (optimised) PCR. Each sample was divided into two aliquots of equal size. One set of samples was shipped to VLA Weybridge on ice (having been chilled immediately after collection), while the other was shipped at ambient temperature. This experiment was not repeated.

4.3.3. Comparison of serum and oral fluid sample Ct values.

It was anticipated that oral fluid samples would contain smaller quantities of PRRSv RNA than serum samples, partly as a result of dilution, and partly as a result of anticipated problems with RNA extraction. We sought to confirm this hypothesis, in the following ways:

- Where positive oral fluid samples were obtained, Ct values for serum samples taken from pigs in pens which gave a positive oral fluids sample, were compared with the corresponding oral fluid sample.
- The Ct values for *all* positive serum samples and *all* positive oral fluid samples were plotted graphically, to compare amounts of RNA in the two samples.

These studies would help to optimise extraction methods, by pinpointing likely amounts of RNA in typical field samples and then selecting extraction methods best at working around these values.

4.3.4. Degradation of RNA in oral fluids over time and storage conditions.

Small experiments were carried out in this area. 12 (likely positive) oral fluid samples were collected in September 2009 and stored at –80^{0}C until they were tested in February 2010, and again in May 2010. If samples were positive, it would imply that they could be collected over a period of time and tested at a later date, which may prove helpful if the test was applied commercially in the UK.

Limited experiments wherein positive samples were tested on consecutive days were performed, with samples stored at room temperature between testing, were performed. However, these were limited so as to minimise duplication with 4.3.2.

4.3.5. The use of a beta actin PCR as a positive control to assess the success of nucleic acid extraction methods.

Given that RNA *extraction* from oral fluid samples was anticipated to be the most challenging aspect of the technique, attempts were made to develop a positive control method of assessing the success of the extraction, using a primers designed to detect genetic material coding for beta actin, a ‘housekeeping’ protein secreted into porcine oral

fluids. This has been reported as a useful internal control in assays for viral disease in other species (Li *et al*. 2005). The premise was that if the extraction method could be shown to detect beta actin genetic material (mRNA), this would demonstrate that the extraction method had overcome the potentially inhibitory effects of the oral fluids. Thus primers for beta actin were obtained and this PCR was run alongside the PRRSv PCR for field samples.

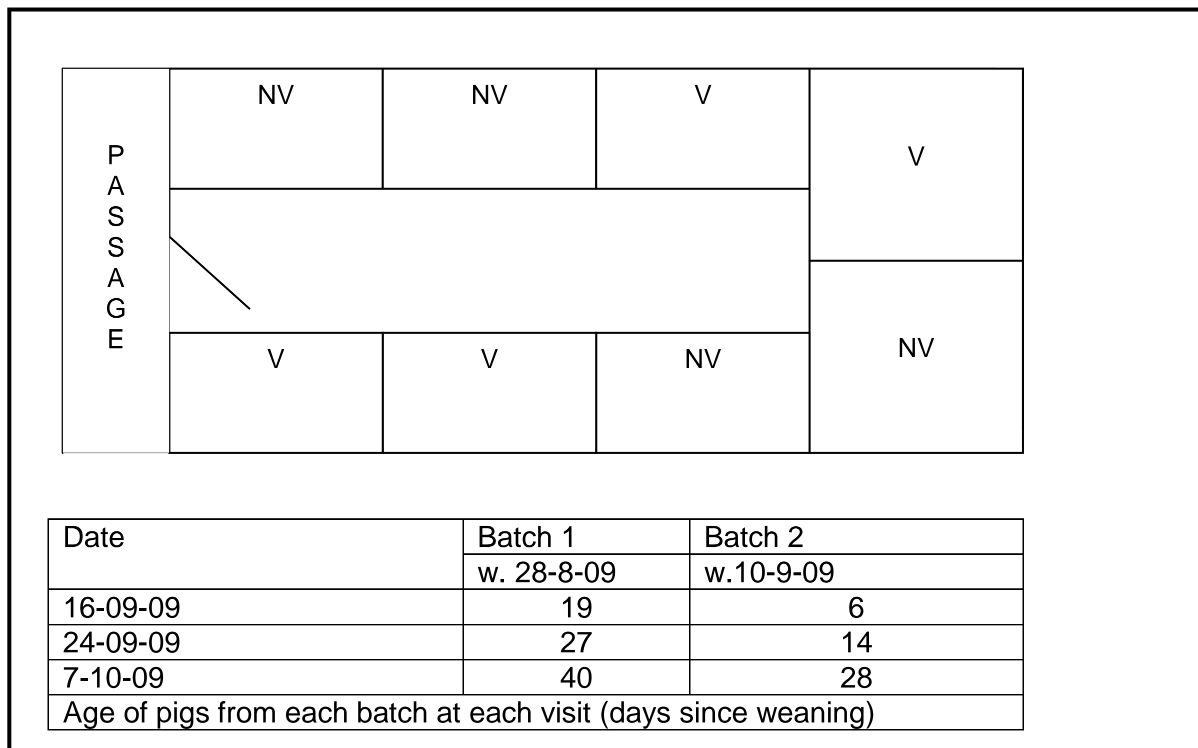
4.3.6. Electron Microscopy as a method of detecting PRRSv virus, in cases where the oral fluids PCR was negative from (serum) positive pigs.

In a continuation of the logic in 4.3.5, it was considered important to explore all possible avenues of investigation in cases where the oral fluid result was negative, despite the sample having been collected from known viraemic (PCR positive) pigs. A small sample of oral fluid samples (n=7) were therefore sent to VLA Weybridge for electron microscopy to see if virions with the morphological appearance of PRRSv could be detected.

4.3.7. Oral fluid samples from pigs previously vaccinated with PRRSv modified live vaccine.

A small controlled trial was performed using two batches of pigs, half of each batch being vaccinated at weaning with *Porcilis PRRS* while the other half (of each batch) was not. The aim was to take sequential samples from these pigs throughout the growing phase to model virus dynamics using the PCR on oral fluids. Pigs were not blood sampled at any stage. Vaccinated and unvaccinated pigs were co-mingled in the same room, but were separated by pen (see figure 4). The first trial batch was weaned on 28-8-2009, the next one 2 weeks later on 10-9-2009. Thus there was an unvaccinated batch between them. This trial was done on a weekly farrowing, indoor 1200 sow farrow to finish unit, weaning approximately 650 pigs/ week. Each trial batch comprised 360 pigs, housed in pens of 45. Thus 180 pigs were vaccinated in each of the two weekly weanings.

Visits were planned throughout the growing stage (4-10 weeks), which had previously been identified as the point of seroconversion in this pig flow. Crude measures of performance were recorded (ADG from 4-10weeks, mortality), by group.



Date	Batch 1	Batch 2
	w. 28-8-09	w.10-9-09
16-09-09	19	6
24-09-09	27	14
7-10-09	40	28
Age of pigs from each batch at each visit (days since weaning)		

Figure 4. Layout of pens and age of pigs at visits for PRRSv MLV trial. 45 pigs/ pen.
V= vaccinated. NV=Non-vaccinated.

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5. Results.

5.1. Rope presentation and pig interactions

In the majority of cases, grower pigs (aged 8-14 weeks) were targeted as these were the pigs where viraemia was suspected or identified on each holding tested. In all cases, pigs rapidly identified the rope as something new and began to investigate it by chewing within 5 minutes. No formal recording of the proportions of pigs within each pen which contributed to the sample, as pigs were not individually identified. However, it was estimated that at least one third of the pigs contributed to the oral fluids sample in each case. The exception to this rule was freshly weaned pigs, which did not approach the rope even if it was left hanging for prolonged periods, seemingly owing to timidity. Further work on rope presentation to this class of pig would be indicated. Further detailed work on pig-rope interactions have been performed by Yolande Seddon at Newcastle University, under a BPEX sponsored PhD.

Typically, 20-30ml of oral fluids were obtained from each rope.

Figure 5. Pigs interacting with rope

Figure 6. Pigs interacting with rope ten minutes after placement.

5.2. Optimisation of PRRSv PCR: Sample handling, RNA extraction and PCR.

5.2.1. Optimisation of PRRSv RNA extraction methods using known negative oral fluids spiked with supernatant from virus culture.

Negative oral fluid samples were obtained from pigs which were known already to have seroconverted to PRRSv (data not shown) (Farm 1, P153-5, table 8), and were therefore no longer viraemic. These samples were spiked with PRRSv, obtained from the supernatant of virus culture, and serially diluted using a logarithmic scale. Kits and methods compared in this way are described in table 1 (above). We ignored the small theoretical possibility that (neutralising) antibodies potentially present in the oral fluid samples from these immune pigs would interfere with the RT-PCR, on grounds of biological plausibility. Results obtained are described in table 2.

	RNA extraction method*			
PRRSv dilution ($-1 = 10^{-1}$)	**MagNA Pure (total)**	**Qiamp Viral RNA**	**RNeasy protect saliva**	**MagMAX viral RNA**
Method ID (see graph)	**A**	**B**	**C**	**D**
-1	11.1	10.0	8.7	11.4
-2	14.8	13.4	12.7	15.6
-3	18.0	16.6	15.3	17.7
-4	21.3	**19.8**	18.2	21.7
-5	36.2	**23.0**	36.3	NT
Starting vol**	200	140	200	300
Elution vol**	100	60	14	50
Concentration§	2x	2.3x	14x	6x

Table 2. Ct values for spiked saliva using different RNA extraction methods.
NB. Low Ct value = more RNA
*See Table 1a.
** The higher the starting volume, the greater the potential amount of virus RNA present. The smaller the elution volume, the higher the concentration of virus and (potentially) the lower the Ct value, as RMNA is detected earlier
§ This is the ratio of starting volume to elution volume

Wide variations are seen in the performance of the different methods of RNA extraction.. RNA Protect© (used in method C) is a commercial reagent designed to preserve nucleic acid in saliva samples (Park *et al.* 2006), and in our hands the Ct values for the samples extracted having been pre-treated with this reagent were slightly lower (*i.e.* more viral RNA) than other kits, by about 2 Ct values, but considering that the concentration in the sample was much higher than in the other extractions from the other methods (*i.e.* 14x [see table 2]), the improved Ct values did not reflect this. All in all, the addition of RNA protect did not confer a large advantage in sensitivity to our results

The QiAmp kit looked to have performed best as much lower Ct values were obtained at the higher dilutions (less concentrated samples). These results are interesting as they highlight which kits and extraction approaches work best at different concentrations of virus. We know from other data in this trial (figure 7) that Ct values in oral fluids are in general higher than those for serum, implying lower quantities of viral RNA. The aim was therefore to select an extraction method which worked best at extracting lower concentrations of virus. A further experiment was conducted with a finer dilution range to examine the Qiamp kit extraction efficiency (see table 3). This experiment demonstrated that the Qiamp kit could extract RNA at much lower concentrations when compared to the magNAPure extraction method. From this work, it can be seen that the QiAMP method (QIA cube) (B, red oval) works best.

PRRSv dilution	MagNAPure (High performance)	Qiamp (QIA cube)
	(A)	(B)
1/5	11.8	11.0
1/25	14.7	13.6
1/125	17.1	15.8
1/625	19.4	17.9
1/3125	21.7	20.3
1/15625	24.3	**22.8**
1/78125	No Ct	**25.4 (2/3)**
1/390625	No Ct	**27.0 (1/3)**
1/1953125	No Ct	No Ct
1/9765625	No Ct	No Ct
Starting volume	200	140
Elution volume	50	50

Table 3. More detailed analysis of the limits of detection of PRRSv RNA using two extraction methods

Again, the QiAMP method performed better when concentrations of viral RNA were low. This was in spite of an expected lower concentration, based on the starting volume: elution volume ratio. This method of RNA extraction was used for all subsequent work on field samples of oral fluids.

5.2.2. Optimisation of sample handling conditions for optimal RNA preservation.

With the extraction and PCR optimised, these trials were done on oral fluids taken from pigs which had been positive on oral fluid testing the week before, on the first farm for which positive oral fluid samples were obtained (farm 2, P12-6, table 8). The *same* pigs were resampled (in the expectation that they would remain positive), and sent to VLA Weybridge for PCR under different conditions. Everything else was identical for the two sets of samples. Results shown in table 4. Clearly, shipping the samples at ambient temperature had a detrimental effect on our ability to detect viral RNA. The recommendation must be that samples should be chilled as soon as taken, and shipped on ice. This may present logistical difficulties if the technique gains wider use in commercial pigs, and is an area of potential further research (see Discussion).
Based on these results, all further oral fluid samples were chilled as soon as possible after collection and shipped on ice for PCR testing.

Pen	Oral fluids handling conditions	Ct Values	Call	Oral fluids handling conditions	Ct Values	Call
1	1RT*	29.00	**POSITIVE**	1 C**	28.63	**POSITIVE**
2	2RT	No ct	Negative	2 C	30.94	**POSITIVE**
3	3RT	No ct	Negative	3 C	28.59	**POSITIVE**
4	4RT	No ct	Negative	4 C	No ct	Negative
5	5RT	No ct	Negative	5 C	36.91	**POSITIVE**
6	6RT	No ct	Negative	6 C	26.82	**POSITIVE**
7	7RT	No ct	Negative	7 C	28.39	**POSITIVE**
8	8RT	No ct	Negative	8 C	No ct	Negative
9	9RT	No ct	Negative	9 C	No ct	Negative

Table 4. Effect of different oral fluid shipping/ handling conditions on PRRSv PCR results (Testing done on 6/7/09, Farm 2, testing performed on 30/6/09. Ambient temp = 19°C).
RT= Room/ Ambient temperature (i.e. no chilling)
C = Chilled (samples put on ice soon after collection and shipped on ice)

5.2.3. Comparison of serum and oral fluids Ct values

Figure 7 clearly shows that Ct values are higher for oral fluids than for serum sample, implying either that a smaller amount of viral RNA is present in this sample, or that less viral RNA is able to be extracted from this sample.

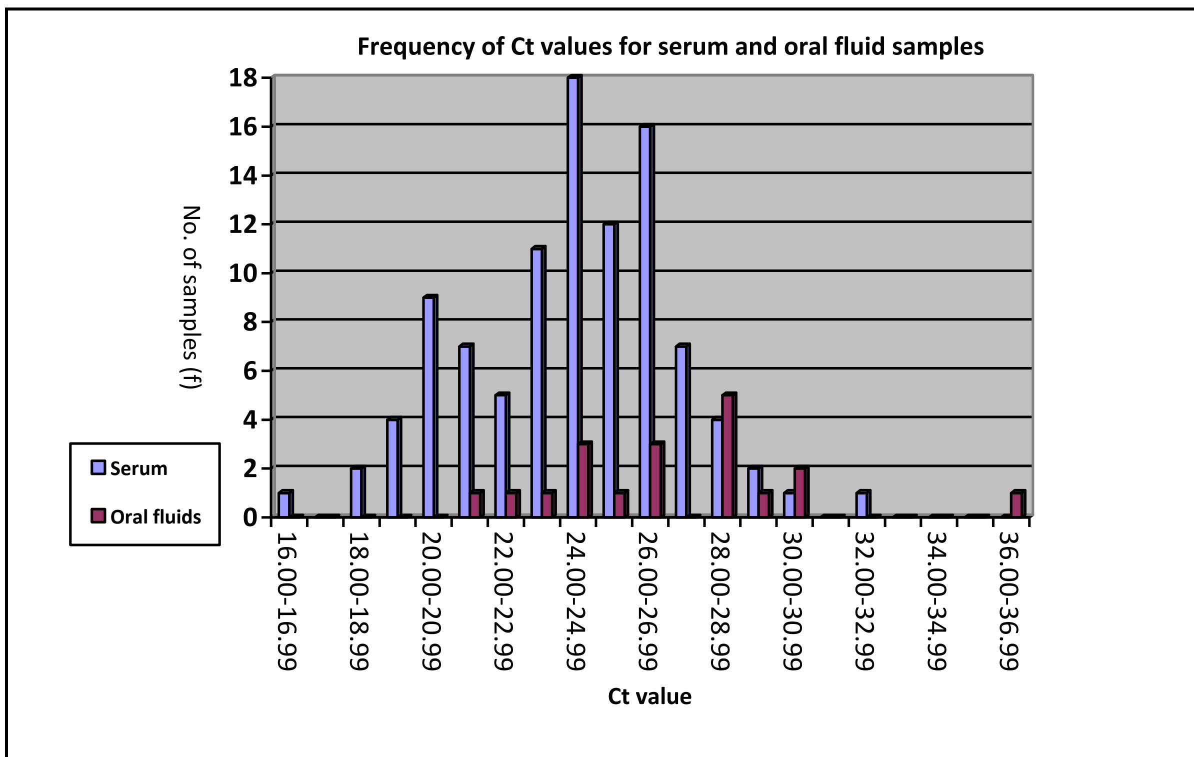

Figure 7. Distribution of Ct values for serum and oral fluid samples taken throughout the project.

These results proved useful in selecting the appropriate RNA extraction method (see above).

Figure 8 describes the correlation between (mean) *serum* PRRSv PCR Ct values, and the corresponding *oral fluid* sample from the same pen. This graph includes both occasions where at least one oral fluid sample was positive and serum samples were collected (farms 2 and 6, tables 8-10). If only results from pens where the oral fluids sample and at least one serum sample was positive, are considered, the correlation between pen Ct and oral fluids Ct is not statistically significant. However, the sample size is extremely small.

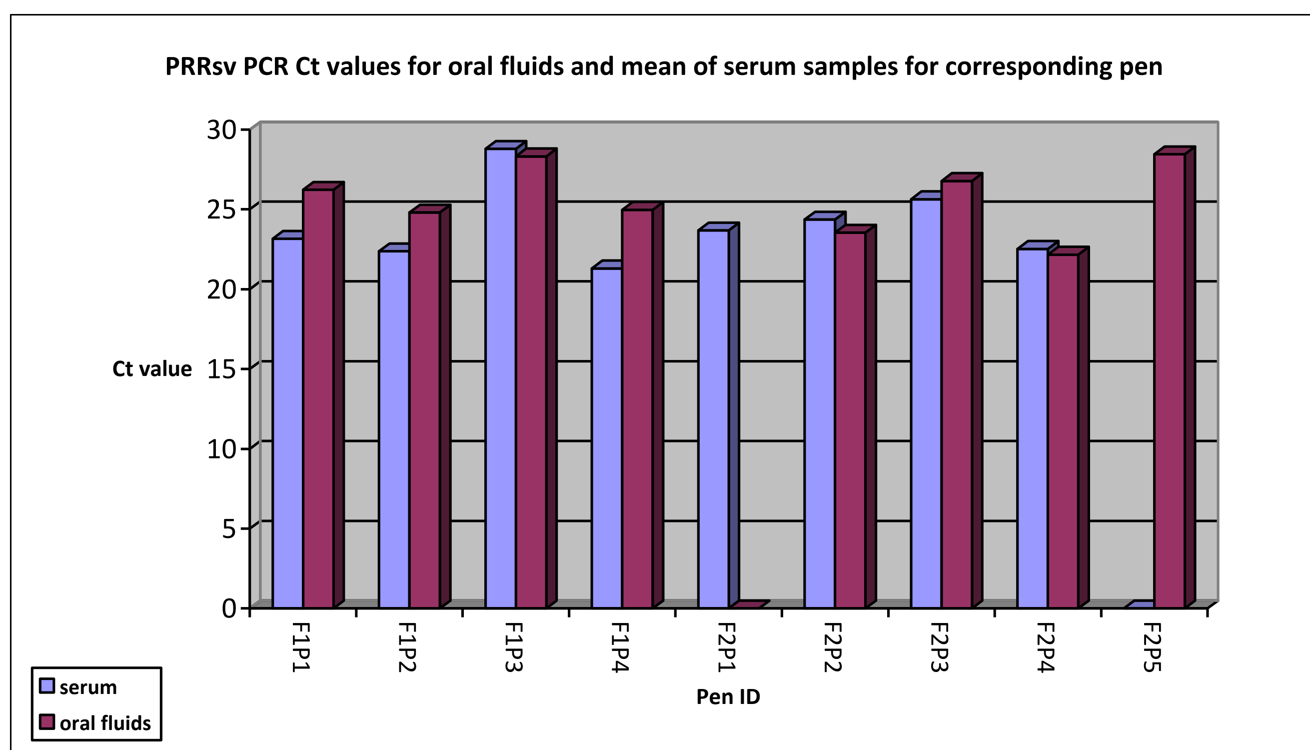

Figure 8. Comparison between mean serum PRRSv PCR Ct value (from positive individual pigs) and corresponding oral fluids sample.
F1P1 = Farm 1 pen 1, *etc.*

5.2.4. Degradation of RNA in oral fluids over time

Results for the oral fluid samples kept at –80^{0}C for 5 months and 9 months between collecting and testing are described in table 4:

Sample	Date collected	Date Tested	Ct values		
1	24/9/2009	15/02/10	Negative	Negative	Negative
2			24.46	24.36	24.36
3			Negative	29.00	Negative
4			Negative	Negative	Negative
5			25.79	25.23	25.48
6			22.17	21.75	22.00
7		18/05/10	27.43	28.00	29.56
8			Negative	Negative	Negative
9			22.82	22.46	22.53
10			Negative	Negative	Negative
11			Negative	Negative	29.28
12			Negative	Negative	Negative

Table 4. Results of PRRSv PCR on oral fluids stored at -80^{0}C for 5 and 9 months.

These results prove that samples can be kept under these conditions and viral RNA remains detectable. This is a useful feature of the testing methodology.
Limited testing was done on positive samples on consecutive days, because it was already firmly established that samples would become negative if left at ambient temperature (5.2.2.). However, the relative instability of oral fluid samples was confirmed: Of five samples, one was positive one day and negative the next (table 4).

Sample ID	04/08/09	05/08/09
P4-8-09.1	Negative	Negative
P4-8-09.2	POS (Ct=27)	Negative
P4-8-09.3	Negative	Negative
P4-8-09.4	Negative	Negative
P4-8-09.5	Negative	Negative

Table 5. Testing of same oral fluid samples on consecutive days.

5.2.5. Development of a beta actin control RT-PCR

The beta actin PCR worked well on porcine oral fluid samples from the field in that it was usually strongly positive. This proved that (mammalian) nucleic acid was being extracted from these samples. Experiments were conducted wherein the beta actin PCR was used to evaluate the different extraction methods but it was found that there was so much beta actin nucleic acid in the samples that Ct values were similar for all methods. The samples with the highest Ct values for the beta actin PCR were simply the ones where the elution: starting volume was high, *i.e.* where the extractions were more concentrated, regardless of extraction method. Thus the beta actin was not considered a good model for the efficacy with which *PRRSv* nucleic acid was being detected because of the wildly different absolute

amounts of the two types of nucleic acid. Indeed in the field samples where oral fluid samples proved negative (in our hands) despite having come from known viraemic pigs (*e.g.* Farms 4 & 5, Table 9), the beta actin PCR on these oral fluid samples was always positive. This was not considered to rule out the possibility that the sample might contain some PRRSv RNA, which had been the intention.

The potential usefulness of beta actin as an internal control was also diminished by the fact that some (cytoplasmic) beta actin *D*NA was probably being detected by the assay, (DNA being a more robust and less labile molecule than RNA) meaning that the comparison with PRRSv was even worse. Thus, while beta actin may provide a useful internal control for other infectious agents where large quantities of nucleic acid are likely to be present (e.g. porcine circovirus type 2 (PCV2), to ensure that the PCR is not inhibited by oral fluids, unfortunately it did not prove useful for this purpose with respect to PRRSv.

5.2.6. Electron microscopy for detection of virus particles in saliva.

<table>
<tr><th>Sample ID</th><th>Farm of origin/ Ref</th><th>Pen status (as determined by sera)</th><th>Oral fluids PRRSv PCR</th><th>EM Results</th></tr>
<tr><td>1</td><td rowspan="3">P128-11</td><td>POSITIVE</td><td>Negative</td><td rowspan="3">Virus particles seen but NOT morphologically typical of PRRSv</td></tr>
<tr><td>3</td><td>POSITIVE</td><td>Negative</td></tr>
<tr><td>7</td><td>POSITIVE</td><td>Negative</td></tr>
<tr><td>2.1</td><td rowspan="4">P91-11</td><td>POSITIVE</td><td>POSITIVE</td><td rowspan="4">No viral particles seen.</td></tr>
<tr><td>2.2</td><td>POSITIVE</td><td>POSITIVE</td></tr>
<tr><td>1.2</td><td>POSITIVE</td><td>Negative</td></tr>
<tr><td>3.1</td><td>POSITIVE</td><td>Negative</td></tr>
<tr><td colspan="5">Table 6. Results of electron microscopy on oral fluid samples from known (serum) PRRSv positive pigs (from two farms)</td></tr>
</table>

Electron microscopy (EM) did not prove helpful in resolving the true status of these oral fluid samples. It is estimated that a concentration of 10^6 virus particles/ ml is required to be able to visualise viruses using this method. If such a sample had been tested with the RT-PCR we would have expected lower Ct values, probably less than 20. This is based on the calculation that if a Qiamp extraction was conducted on a sample containing 10^6 virus particles/ ml the substrate for the RT-PCR would have contained 8000 particles. Ct values in field samples were much higher than this, suggesting that the oral fluids contained virus loads of less than 10^6 virus particles/ ml.

Thus we conclude that EM is not useful because of the low virus load.

5.2.7. Oral fluid samples from pigs previously vaccinated with PRRSv modified live vaccine.

In the event, the two batches were each sampled only three times, because pigs were moved to finisher accommodation sooner than planned because of pressures on pig flow on the unit. On the first occasion, the younger batch of pigs did not interact with the rope very well and samples were only obtainable from 4 of the 6 target groups.
Nevertheless, results were obtained from the study, as shown in table 7. The onset of viraemia can be identified, and this happens earlier in the older batch, as expected. However, viraemia can be detected in both batches.
Tests are ongoing to establish the identity of this viral RNA, by genetic sequencing I order to determine whether or not it is PRRS MLV virus.

Visit 1: 16-9-09			Visit 2: 24-9-09			Visit 3: 7-10-09		
P212-9			P288-9			P48-10		
Batch	Pen ID & Vax status	Oral fluids PRRSv PCR results	Batch	Pen ID & vax status	Oral fluids PRRSv PCR results	Batch	Pen ID & vax status	Oral fluids PRRSv PCR results (Ct)
1 w.28-8	1.1NV	Negative	1 w.28-9	1.1NV	Negative	1 w.28-9	1NV	Negative
	1.2NV	Negative		1.2NV	**POSITIVE (24.30)**		2NV	**POSITIVE (28.33)**
	1.3V	Negative		1.3V	Negative		3V	Negative
	1.4V	Negative		1.4V	Negative		4NV	Negative
	1.5V	Negative		1.5V	**POSITIVE (25.50)**		5V	Negative
	1.6NV	Negative		1.6NV	**POSITIVE (21.97)**		6V	Negative
2 w.10-9	3.1V	NS	2 w.10-9	3.1NV	Negative	2 w.10-9	7NV	Negative
	3.2V	Negative		3.2NV	Negative		8NV	Negative
	3.3NV	Negative		3.3V	Negative		9NV	**POSITIVE (22.6)**
	3.4V	Negative		3.4V	Negative		10NV	Negative
	3.5NV	NS		3.5V	Negative		11V	Negative
	3.6V	Negative		3.6NV	Negative		12V	Negative

Table 7. Results of oral fluids PCR on each of two visits to vaccinated pigs
V= vaccinated at weaning with PRRSv MLV. NV= Not vaccinated at weaning with PRRSv MLV
NS= No sample collected (pigs did not chew rope (too young?)

These results suggest:

- Oral fluids may be a useful method for modelling disease dynamics in a longitudinal fashion, in pig flows, including following interventions such as vaccination.
- Viral RNA was found in both vaccinated and unvaccinated pigs. Interpretation of this finding may rely on the identity of these viruses.

There were no significant differences between the groups in wean to 10 week old mortality or average daily gain, but this was a very small trial and groups were co-mingled.

5.3. Establishing sample pig status as determined by oral fluids and by gold standard (blood sampling a representative sample of pigs).

Tables 8-10 describe the results of the field sampling carried out as part of this study, using an optimised oral fluids PCR and using blood sampling of pigs within each pen as the gold standard defining pen status.

In all cases, the aim was to target viraemic pigs. This was successful on 5 of 7 units (71%) and a total of 61 pens were sampled, of which 30 (51%) were positive as determined by serum sampling the pigs for PRRSv PCR. A total of 383 blood samples and 102 oral fluid samples were collected throughout the study.

Farm	Pens Sampled	Pigs bled/ pigs in pen	% Bled	Pen result (Bloods)	Sample Prevalence viraemia	Pen result (Saliva)	Agreement?	Total Pens
1 P153-4	1	6/ 20	30	Negative	0	Negative	Y	1
	2	6/ 19	32	Negative	0	Negative	Y	2
	3	6/ 18	33	Negative	0	Negative	Y	3
	4	6/ 21	28	Negative	0	Negative	Y	4
	5	6/ 16	37	Negative	0	Negative	Y	5
	6	6/ 18	33	Negative	0	Negative	Y	6
2 P12-6	1	6/44	13	Positive	100	Positive	Y	7
	2	6/45	13	Positive	100	Positive	Y	8
	3	6/ 43	13	Positive	8	Positive	Y	9
	4	6/ 44	13	Positive	100	Positive	Y	10
	5*	6/ 45	13	Negative*	0	Positive	N	11
	6	6/ 44	13	Negative	0	Negative	Y	12
	Finishers 7	6/15	40	Negative	0	Negative	Y	13
	Finishers 8	6/16	37	Negative	0	Negative	Y	14
2a (P438-6)	5	20/45	44	Positive	65	Negative	N	15
3 P407-	R1R1	5/20	25	Negative	0	Negative	Y	16
	R1L1	4/22	18	Negative	0	Negative	Y	17
	R2R1	5/21	24	Negative	0	Negative	Y	18
	R2R2	5/18	28	Negative	0	Negative	Y	19
	R3L1	5/23	22	Negative	0	Negative	Y	20
	R3L2	5/22	22	Negative	0	Negative	Y	21
	R4R1	5/19	26	Negative	0	Negative	Y	22
	R4L1	5/20	25	Negative	0	Negative	Y	23

Table 8. Results of serum and oral fluid sampling for PRRSv RT-PCR: first three farms.

Of the farms where pens were determined to be positive by blood sampling (5 farms), positive oral fluid samples were obtained on two (40%). On the other farms, all oral fluid samples were negative, despite a reasonable proportion of the pens from which these samples were taken, being identified as positive (by PRRSv RT-PCR on sera). This may imply a ‘farm effect’ in the success of the technique, whereby on some farms it will work and on others it will not. The reasons for the latter case need to be further explored. Possible explanations in this case include:

- Strain variation in the PRRSv virus. PRRSv is a very mutable virus and farm strains, possibly with different epidemiological and biological properties, may exist. Workers in the US trying to experimentally infect groups of pigs have found that the success of this infection is strain dependant. Different strains may be differently excreted into oral fluids and hence more difficult to detect in this sample. This has been shown to be the case with other RNA viruses in pigs *e.g.* Classical Swine fever (Weesendorp *et al* 2009)
- Ambient temperature. We have shown that success of RNA extraction becomes less likely if samples are shipped at ambient temperatures rather than chilled. The

positive oral fluid samples were obtained in April and November, while the negative ones were taken in July. It is possible that RNA degradation may have happened faster at the higher July temperatures.

- Pressure of infection/ virus load. Differences in individual pig Ct values, within prevalence, between pen and between farm prevalences of PRRSv viraemia were observed (table 13), but based on a limited amount of data analysis, there is no clear evidence that the reason for the negative samples was that the level of infection was below the threshold of detection. This possibility is considered unlikely.

Farm	Pens Sampled	Pigs bled/ pigs in pen	% Bled	Pen result (Bloods)	Sample Prevalence viraemia	Pen result (Saliva)	Agreement?	Total Pens
4 P261-7	WGH 32.2	6/ 6	100	Negative	0	Negative	Y	24
	WGH 32.7	6/ 8	75	Negative	0	Negative	Y	25
	WGH 32.3	6/ 6	100	Negative	0	Negative	Y	26
	WGH 32.5	6/ 6	100	Negative	0	Negative	Y	27
	WGH7 31.8	6/ 9	67	Negative	0	Negative	Y	28
	WGH7 31.7	6/ 7	85	Negative	0	Negative	Y	29
	WGH7 31.3	6/ 7	86	Negative	0	Negative	Y	30
	WGH7 31.4	6/ 7	86	Negative	0	Negative	Y	31
	8Sec1 31.1	6/ 10	60	Positive	67	Negative	N	32
	8Sec1 31.2	6/ 10	60	Positive	67	Negative	N	33
	8Sec1 31.3	10/ 10	100	Positive	80	Negative	N	34
	8Sec1 30.4	6/ 6	100	Positive	50	Negative	N	35
	8Sec2 30.3	5/ 8	63	Positive	100	Negative	N	36
	8Sec3 30.3	5/ 8	63	Positive	60	Negative	N	37
	BH2 30.7	6/ 8	75	Positive	33	Negative	N	38
	BH2 30.3	7/ 7	100	Positive	71	Negative	N	39
5 14-P617-7	1	13/56	23	Positive	38	Negative	N	40
	2	13/58	22	Positive	23	Negative	N	41
	3	7/17	7/17	Positive	29	Negative	N	42
	4	7/17	41	Negative	0	Negative	Y	43
	5	7/16	43	Positive	14	Negative	N	44
	6	7/17	41	Negative	0	Negative	Y	45
	7	7/17	41	Negative	0	Negative	Y	46
	8	7/18	39	Positive	14	Negative	N	47

Table 9. Results of serum and oral fluid sampling for PRRSv RT-PCR: farms 4 & 5..

Farm	Pens Sampled	Pigs bled/ pigs in pen	% Bled	Pen result (Bloods)	Sample Prevalence viraemia	Pen result (Saliva)	Agreement?	Total Pens
6 P91-11	1.1	6/30	20	Positive	25	Negative	N	48
	1.2	6/35	17	Negative	0	Negative	Y	49
	2.1	5/35	14	Positive	60	Positive	Y	50
	2.2	5/30	17	Positive	80	Positive	Y	51
	2.3	5/35	14	Positive	60	Positive	Y	52
	3.1	5/32	16	Negative	0	Positive	**N**	53
	3.2	6/37	16	Positive	20	Negative	N	54
7 P128-11	1	5/43	11	Positive	40	Negative	N	55
	2	5/44	11	Positive	40	Negative	N	56
	3	5/45	11	Positive	60	Negative	N	57
	4	5/42	12	Positive	40	Negative	N	58
	5	6/42	14	Positive	40	Negative	N	59
	6	5/42	12	Positive	40	Negative	N	60
	7	4/45	9	Positive	40	Negative	N	61

Table 10. Results of serum and oral fluid sampling for PRRSv RT-PCR: farms 6&7.

5.3.1. Diagnostic parameters of oral fluid testing: Sensitivity and Specificity.

To summarise the results in tables 8-10, a test sensitivity and specificity were calculated using blood sampling to determine pen status as a gold standard. Results in table 11.

		'True Status' i.e. Blood Result		**Totals**
		Positive	Negative	
'Test' status *i.e.* oral fluids result	Positive	7	2*	9
	Negative	23	29	52
Totals		30	31	61

Table 11. Interpretation of results so far: All Farms

* Suspect that these are true positives

Sensitivity = 7/30 = **23.3%**

Specificity = 29/31 = **93%**

Sensitivity is unacceptably low at 23.3%, implying that oral fluid sampling has missed positive pens as determined by blood sampling. However, problems with low sensitivity are reported by other researchers in this field, as described in a recent abstract (Chittick *et al*

2010), where diagnostic sensitivity (using experimentally infected animals and an optimal PCR/ extraction) was around 43%.

However, if we make the large assumption that the technique (for now) just *does not work* on some units, for reasons which remain to be determined, and only include figures from the farms where one or more positive oral fluids sample was obtained (*i.e.* Farms 2 & 6), different figures for test performance can be obtained.

<table>
<tr><td colspan="2" rowspan="2"></td><td colspan="2">'True Status' i.e. Blood Result</td><td rowspan="2">Totals</td></tr>
<tr><td>Positive</td><td>Negative</td></tr>
<tr><td rowspan="2">'Test' status i.e. oral fluids result</td><td>Positive</td><td>7</td><td>2</td><td>9</td></tr>
<tr><td>Negative</td><td>3</td><td>4</td><td>7</td></tr>
<tr><td colspan="2">Totals</td><td>10</td><td>6</td><td>16</td></tr>
<tr><td colspan="5">Table 12. Interpretation of results so far: Only oral fluids positive farms
* Suspect that these are true positives</td></tr>
<tr><td colspan="5">Sensitivity = 7/10 = 70%
Specificity =4/6 = 66%</td></tr>
</table>

Table 12 suggests that the test can be used with a moderate degree of accuracy on farms where it has been shown that PRRSv RNA *can* be detected in oral fluid samples by PCR.

5.3.1. Further potentially useful data on PRRSv infection dynamics.

This study involved taking a large number of blood samples from different units. The data is broken down in table 13.

The data in table 13 is useful because it is often necessary to estimate the expected prevalence when calculating what proportion of a pen needs to be sampled in order that at least one positive animal is sampled to a pre-defined degree of confidence. These figures are, of course, highly dependant on pig flows and timing of sampling.

The main determinant of pigs' first exposure to PRRSv is the pattern of pig movements within a farm. Clearly these are farm-specific. All these farms operate an All in All out policy by room/ yard only. On the basis of this evidence, the point of first exposure to PRRSv can be anticipated by analysis of the pig flow and in the majority of cases in this study, was 3-5 weeks into the growing period (*i.e.* 7-12 weeks of age).

Oral fluids were positive in samples from farms 2 and 6. There are no patterns in within pen prevalence of viraemia, or between pen prevalence, which would explain this observation.

Farm (see tables 8-10)	Housing Type	Age of pigs	% Positive pens	Within pen prevalence range (sample) (Positive pens only)	Mean pen Prevalence (positive pens only)	Mean farm prevalence (positive pens only)
2	Fully slatted grower house	7-11 w	66% (4/6)	17-100%	76%	76%
4	Grower House	8-12w	100% (6/6)	50-100%	68%	60%
	Bacon House	12-20w	100% (2/2)	14-28%	52%	
5	Straw Yard Growers	8-14w	100% (2/2)	23-38%	31%	25%
	Part Slatted Growers	10-14w	50% (3/6)	14-28%	19%	
6	Fully slatted grower house	8-12w	71% (5/7)	17-80%	50%	50%
7	Fully slatted grower house	7-12w	100%	40-60%	42%	42%

Table 12. Recorded prevalence of viraemia in positive pens tested (based on serum PCR).

7. Conclusions and suggested direction of further work

We have shown that oral fluid sampling can under some circumstances be used to determine the PRRSv status of a pen of pigs. However, sensitivity remains poor to moderate even on farms where the technique has been shown to work. Suggestions for further work include:

- Further refinement and optimisation of the extraction method, concentrating on those units featured in this report where oral fluid samples from known positive pigs have proved negative.
- Genetic material from all PRRSv isolates collected during this study has been retained. Genetic sequencing of these isolates may be of use in determining whether virus strain variation can account for differences in results between units.
- Consideration may be given to experimental infection of pigs with different PRRSv strains to model excretion patterns. This may be tacked on to other experiments, *e.g.* at VLA Weybridge.
- Further work on the physical characteristics of the oral fluids themselves, *e.g.* pH, viscosity, presence of degradative enzymes, degree of particulate matter

contained within, *etc.*, to investigate whether these properties influence the survival of PRRSv, which is quite a labile virus known to be sensitive to pH changes. By extension, such features of oral fluids may be able to be linked to diet.

- A user-friendly method of chilling samples soon after collection and then shipping them to the laboratory on ice, is required. This would best take the form of a kit containing all necessary equipment.
- Other tests for PRRSv on oral fluid samples (e.g., antibody testing) have been reported as feasible (Wong *et al* 2010), and should be investigated.

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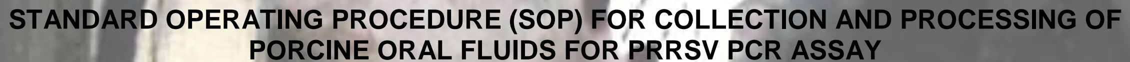
Appendix A

See below for the SOP for oral fluid collection, sample handling, RNA extraction and RT-PCR.



STANDARD OPERATING PROCEDURE (SOP) FOR COLLECTION AND PROCESSING OF PORCINE ORAL FLUIDS FOR PRRSV PCR ASSAY

A. Collection of Oral fluid samples.

1. Select pigs suspected to be viraemic. If not known, adopt a cross sectional approach and target pigs of different ages
2. Suspend a length of pre-sterilsed pure cotton rope in an accessible location for all pigs. The rope should be:
   i. Presterilised in lengths of 90cm in individual bags. Applying autoclave tape to one end of the rope will prevent fibres splaying and will assist mounting rope on bracket (see iv., below). Presterilised ropes available from VLA or private veterinary surgeons.
   ii. Aim for rope to be about pig shoulder height, will need to adjust depending on age/ size of pigs.
   iii. Diameter of rope should be 18mm for grower and finisher pigs, 14mm for weaners.
3. To suspend rope:
   i. Don a pair of nitrile disposable gloves.
   ii. Feed taped end of rope through hole in bracket *from underneath*, while keeping other end within bag.
   iii. Keeping hold of top half, remove bag and allow bottom half of rope to unfurl. Try to avoid touching this part of the rope.
   iv. Tie a knot in the rope above the hole to fix it securely in place, ensuring that the bottom end of the rope hangs at approximately pig shoulder height.
   v. Usually pigs will investigate rope and begin chewing immediately. If not, it may help to wiggle the rope enticingly.
4. Leave the rope in place for 20-30 minutes.
5. To extract oral fluids from the rope:
   i. Don a pair of disposable nitrile gloves. Use a separate pair for each rope dealt with.
   ii. Keeping hold of the lower portion, cut the rope just below the bracket.
   iii. Insert the (wet) distal end of the rope in a plastic bag.
   iv. With the wet rope in the bag, and keeping hold of the bag, milk the fluids into the bag using wringing motions starting at the top (dry end) and working downwards, so that fluids collect in the bag. Two people are helpful for this task.
   v. Pour the collected oral fluids into a sterile plastic universal container and chill immediately.
6. For best results, oral fluid samples should be transported to the testing laboratory **on ice** (*i.e.* at around 4^0C).

B. Protocol for PRRSv PCR on oral fluid samples

1. RNA is extracted from saliva samples as soon as possible after receipt. Alternatively, they are stored at –80C until tested.
2. 1ml of saliva is centrifuged at 12000 rpm for 10 minutes to remove the larger fragments. Supernatant is used for RNA extraction.
3. RNA is extracted from 140μl of saliva using the QIAmp Viral RNA kit. 35ul of RNA is eluted from the kit.
4. The extracted RNA is tested using the real time Taqman© PCR for the detection of PRRSv RNA*. Double the normal amount of Reverse Transcriptase enzyme and Qiagen Hotstart Taq enzyme is added to the PCR.

*This PCR detects an RNA sequence comprising part of Open Reading Frame (ORF) 7, which encodes the viral nucleocapsid protein. It is relatively conserved. A commercial one step RT-PCR mastermix is used which allows both reverse transcription (RT) and PCR to take place in a single tube. Both US and EU PRRSv are detected.