2A-112: *On-farm immunoassay test kit for inflammatory diseases to test the immune status of a herd*

Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

There are increasing concerns about the immunity of pigs and its impact on efficiency of pork production in the commercial environment. While nutritional and veterinary strategies can be adopted to combat this, "up-specification" and medications can be expensive and do not necessarily translate into superior production in animals that are already healthy. Current knowledge therefore clearly indicates that there is a need to adopt separate nutritional strategies depending on the immune status of the individual herd. Further to this, pork industries worldwide are describing a need for pen-side diagnostics for early detection of health challenges and sub-clinical health issues.

A recent breakthrough in diagnosis of herd immunity is the use of oral fluid-based diagnostics for the assessment of health and diagnosis of disease. Oral fluid is relatively easy to collect on a commercial farm by hanging cotton rope in a pen and allowing the pigs to chew on it for a period of time. Based on the current knowledge and technologies available, this project aimed to develop an oral fluid diagnostic kit using lateral flow technology (similar to a kit used for pregnancy test) that would quickly show (i.e. within 1-2 hours from collection of samples to diagnosis) the immune status of a large herd from saliva samples. Based on this, decisions could then be made regarding the need for nutritional and/or veterinary intervention with the option for more testing (e.g. pathogen screening).

In order to develop such a diagnostic kit, biomarkers known to be reliable indicators of immune status in the serum or plasma had to be selected and validated as reliable indicators of immune status in the salvia. Further to this, the proposed single point measure of reactive proteins (selected biomarkers) in an oral fluid sample collected by hanging a cotton rope in pen needed to represent the mean of the selected reactive protein concentration determined by individually sampling the oral fluid of all the pigs in the same pen (i.e. does a group saliva sample represent the mean of individual samples in the same group).

Data for this project was collected from seven commercial farms with and without major health issues. C-reactive protein and haptoglobin, two of the major acute phase proteins in the pig were selected as good candidates for the reactive protein biomarker to be used in the immunoassay kit since levels in the plasma were positively correlated to levels in the saliva. Data indicated that the one point oral fluid sample collected by hanging a cotton rope in a pen did represent the mean concentration determined by collection of oral fluid samples from individual pigs in the same pen for haptoglobin but not for C-reactive protein. Results therefore suggest that haptoglobin could potentially be a suitable marker for the development of an immunoassay kit used to detected inflammatory disease from oral fluid. However, results from the project also highlighted a number of limitations to consider before progressing to stage 2 (the development of the immunoassay kit).

While the selected farms varied in their health status, all were receiving veterinary intervention at the time of sampling. Therefore, the only increases in salivary haptoglobin were in relation to management issues (e.g. dirty pens). As a result, the collection of more data is required to i) establish a direct relationship between salivary haptoglobin and health status and ii) strengthen the correlation between group and individual saliva samples in immune-challenged animals. Further to this, the concentration of reactive protein in the saliva is also likely to be influenced by drinking/chewing behaviour, dehydration, local inflammation, bleeding in the oral cavity, feeding systems and oral fluid flow rates of individual pigs. Therefore, a method of standardisation for the oral fluid

samples is needed to negate these other factors which may be influencing oral fluid flow rate and the concentration of reactive proteins in the saliva.

Table of Contents

1. Introduction

There are increasing concerns about the immunity of pigs and its impact on efficiency of pork production in the commercial environment. In a recent review of the immune system in pigs for APL, Black and Pluske (2011) identified that pigs grown in commercial production systems grow approximately 20% more slowly than pigs in a clean environment, such as a research facility. The review also identified that immune system activation increases the pigs' maintenance energy requirement by 10-15% and the protein requirement by 7-10%. Additionally, the requirements for tryptophan, sulphur amino acids and threonine are increased by a further 10%. In a recent Pork CRC-funded project (4B-109), it was demonstrated that increasing sulphur amino acids from 55% to 75% of lysine significantly increased daily muscle protein deposition rate from 58 g to 66 g in immune system-activated finishing pigs (Kim et al., 2012). However, such an improvement was not observed in healthy pigs, indicating that additional nutrient supply will not be translated to superior production when an "up-specification" strategy is used in a healthy herd. Current knowledge, therefore, clearly indicates that there is a need to adapt separate nutritional strategies depending on the immune status of the individual herd.

As part of the acute innate immune response, pro-inflammatory cytokines stimulate heaptic uptake of amino acids leading to an increase in production of acute phase proteins (APP) in the liver (Kim et al., 2013). Each APP serves a different physiological function for the immune system, however, in the pig, C-reactive protein (C-RP) and pig major acute phase proteins (PigMAP) are major APPs with levels increasing 100 to 1000-fold on stimulation, peaking at 24-48 hours after and then declining rapidly. Haptoglobin (Hp) is a moderate APP, increasing 5-10-fold on activation, peaking at 2-3 days and then decreasing more slowly in concentration over time (Eckersall and Bell, 2010). One of the major limitations of using acute phase proteins as a method to diagnose the immune status in a large herd is the need to collect representative blood samples from 1,000 to 10,000 pigs in a commercial farm, which is practically impossible and can create errors (e.g. selecting pigs that are not representative of the whole herd immune status). Therefore, the current diagnostics for herd immunity may only be applicable to a very small herd (i.e. <100 pigs). In saying this however, blood sampling can be somewhat invasive and stressful for the animal and not all that convenient for farm staff.

A recent breakthrough in diagnosis of herd immunity is the use of oral fluid-based diagnostics for the assessment of health and diagnosis of disease. (Prickett and Zimmerman, 2010). Spanish researchers recently demonstrated that haptoglobin can be detected in a sample of oral fluid, with statistically significant differences between healthy and unhealthy pigs (samples collected from pigs with diarrhoea, gastric ulcer, bronchopneumonia and multiple abscessation) (Gutierrez et al., 2009a,b; Gomez-Laguna et al., 2010; Soler et al., 2013). These researches suggested that there was a possibility of developing a non-invasive diagnostic kit suitable for testing the immune status of larger herds by collecting a representative oral fluid sample from each pen. A representative oral fluid sample can be collected in a large pen by hanging a cotton rope to the height of the pigs' head, and then letting them chew it for a short time period (e.g. 20-40 minutes). The oral fluid sample from the group is then squeezed into a collection tube and assessed for levels of selected APPs (e.g. Hp), which would indicate immune

status. Using this method, 4-5 pens selected at random in a large herd (>100 pigs) would provide a sound diagnosis of herd immunity on commercial farms.

Based on the current knowledge and technologies available, this project aimed to develop an oral fluid diagnostic kit using immunoturbimetric or lateral flow technology (similar to a kit used for pregnancy test) that would quickly show (i.e. within 1-2 hours from collection of samples to diagnosis) the immune status of a large herd. Based on this, decisions could then be made regarding the need for nutritional and/or veterinary intervention with the option for more testing (e.g. pathogen screening).

The development of such an immunoassay kit is a complicated process including selection of the correct antibodies, detection reagents and membranes. The first step in this project was to validate the proposed concept before initiating the development process. Therefore, two major hypotheses were tested to validate selected reactive proteins as biomarkers for herd immunity. The two major hypotheses were:

1. Oral fluid levels of reactive proteins are strongly correlated to plasma levels of the reactive proteins.
2. The proposed single-point measurement in an oral fluid sample collected by hanging a cotton rope in a pen represents the mean levels of reactive proteins determined by individual sampling of the reactive proteins.

2. Methodology

Stage 1 - Validation of oral fluid reactive proteins as biomarkers for herd immunity.

The selected reactive proteins in this project included the three main APPs for pigs: Hp, C-RP and PigMAP as well as soluble cluster of differentiation 14 (sCD14) and neopterin which are pro-inflammatory marker proteins released by macropharges upon stimulation by cytokines.

The completion of stage 1 of the project was a stop/go milestone for the development of a prototype for the pen-side immunoassay kit.

To test the hypotheses, we selected seven commercial farms with and without major health issues in Western Australia. The selection was done in conjunction with the consultation of swine veterinarians in the local area (Table 1). Five pens per farm were randomly selected for oral fluid and blood sample collection from individual pigs. A total of 340 grower pigs were sampled for this part of the project. Blood samples were taken via jugular venepuncture using lithium heparin coated tubes, which were then placed on ice until they could be processed further. Blood samples were centrifuged at 2800 x *g* for 15 minutes at room temperature. Plasma was collected and stored at -20 degrees C. Oral fluid samples were collected from the same individuals using Salivette® tubes by placing four sterile sponges in a sack and attaching the sack to a fishing line which was then dangled in front of the individual pig to be chewed until thoroughly moist. The sponges were placed in the Salivette® tubes and centrifuged at 3000 x *g* for 10 minutes and stored at -20 degrees C until analysed.

Two additional grower pens on each of the farms were randomly selected and an individual oral fluid sample (via the same sampling method as above) and a single point group oral fluid sample were collected by hanging a cotton rope in the pen for 40 min. A total of 110 individual and 12 group pens oral fluid samples were collected during this part of the sampling process. The cotton rope was then placed in a plastic sleeve and squeezed. The bottom corner of the plastic sleeve was cut and the group saliva sample collected and stored at -20 degrees C.

Table 1. Selected farms for sampling and herd health status information

Date	Farm	Remarks
August 6	Farm 1	Healthy
August 13-14	Farm 2	Actinobacillus pleuropneumonia (APP), swine dysentery, tail biting
August 20-21	Farm 3	Mycoplasma, APP
August 27-28	Farm 4	Mycoplasma
September 3-4	Farm 5	Mycoplasma and Mange, temporary lack of water supply in pens 2 (group sample), 3,4,5.
September 9-10	Farm 6	Mycoplasma
September 24	Farm 7	Healthy

The plasma and oral fluid samples from the commercial farms were used to select the most reliable reactive proteins to be used in the development of a herd immunity detection kit. The both plasma and saliva samples were tested for Hp (Aviva haptoglobin ELIZA kit), C-RP (Aviva C-RP ELISA kit), sCD14 (Bluegene porcine sCD14 ELISA kit), PigMAP (Cusabio porcine PigMAP ELISA kit), and Neopterin (HPLC, Eisenhunt, 2013). A correlation and simple linear regression analyses were conducted using Genstat 15 (VSN International, UK) for each of the blood measures.

3. Outcomes

3.1 Plasma and salivary C-reactive protein and haptoglobin values.

Data from the 340 pigs that were individually sampled for blood and oral fluid showed C-RP and Hp levels in plasma and saliva were positively correlated (p = 0.001) (See section 3.2), making C-RP and Hp good candidate reactive proteins for the development of the diagnostic kit. Correlations for the other blood measures (sCD14, PigMAP and neopterin) were not significant and the results have not been included in this report. Figure 1 details the individual results for C-RP oral fluid and plasma and Figure 2 details the individual results for Hp oral fluid and plasma. Arrows indicate the cut-off points to discriminate healthy from unhealthy pigs. For C-RP, the cut-off value was 50 ng/mL in oral fluid and 200 µg/mL in plasma (Figure 1) and for Hp it was 1.14mg/ml for plasma and 1.5µg/ml for oral fluid. These values have been derived by visually assessing the graphs and selecting a point of inflection. These cut-off points for Hp are comparable to the oral fluid and serum Hp cut-off points of 1.5mg/ml and 1.8 µg/ml for oral fluid determined in pigs infected with porcine reproductive and respiratory disease, which are slightly higher, but comparable to cut-off values in the current project for Hp

(Gomez-Laguna et al. 2010). In contrast, the cut-off points for plasma C-RP in the current project are substantially higher than what has been previously reported (Gomez-laguna et al., 2010; Heeguard et al., 2011).

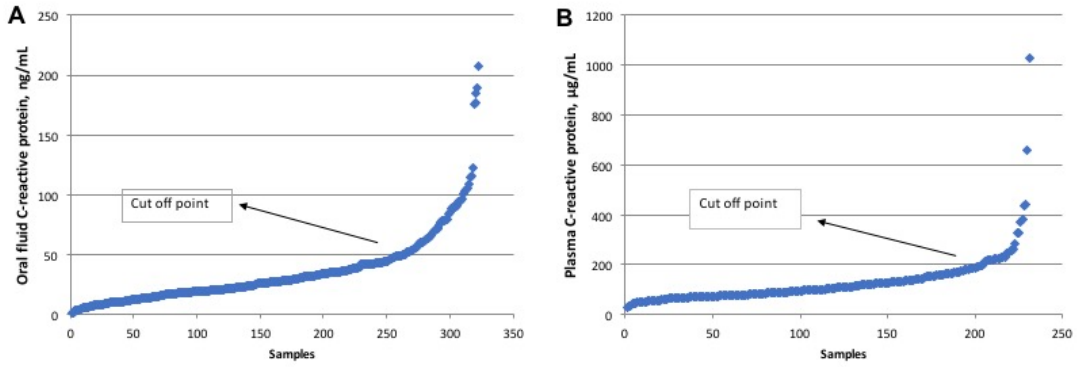

Figure 1. Cut-off points to discriminate healthy and unhealthy pigs for C-reactive protein concentrations in oral fluid (A) and plasma (B) samples.

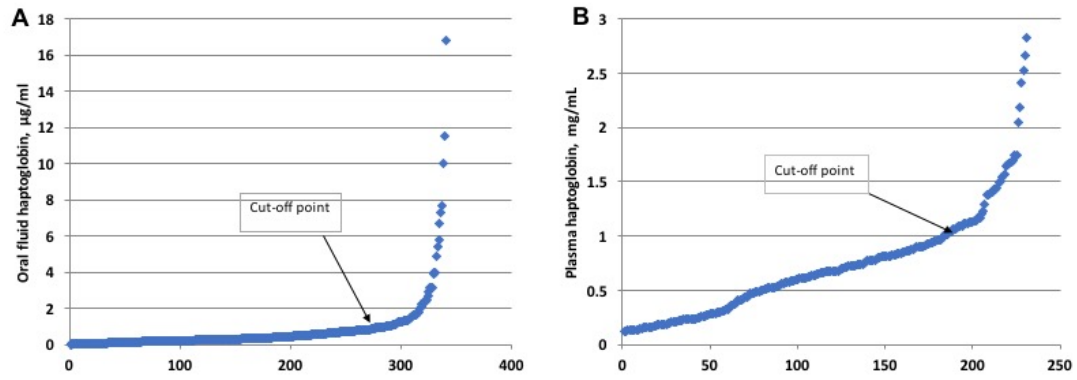

Figure 2. Cut-off points to discriminate healthy and unhealthy pigs for haptoglobin concentrations in oral fluid (A) and plasma (B) samples.

Further to this, closer examination of the salivary Hp data revealed that the two farms with obvious management issues (farm 1: very dirty pens, farm 2: cut off water supply for a period of time) dominated the high proportion of high Hp saliva samples (>1.5µg/ml; Table 1), which is reassuring, but at the same time highlights that no direct relationship between salivary Hp and health status was established in the current project.

Table 1. Number of individual saliva samples between 1-1.5microgram/ml for haptoglobin

Hp >1.5 µg/ml	No. cases	% of total sample	% of farm samples	no. samples per farm
Farm 1	**22**	**6.11**	**46.81**	**47**
Farm 2	2	0.56	2.78	72
Farm 3	1	0.28	1.61	62
Farm 4		**1.67**	**12.24**	**49**
Farm 5		0.00	0.00	42
Farm 6			0.00	38
Farm 7	1	0.28	2.00	50
Total	32	8.89	8.89	360

Farm 1 = dirty pens, Farm 2 = cut off water supply for a period of time in 3/5 sampled pens

3.2 Correlations between plasma and salivary C-reactive protein and haptoglobin values.

Correlations between plasma and oral fluid C-RP are presented in Table 1. Apart from Farms 3 and 5, C-RP concentration in oral fluid was positively correlated with C-RP concentration in plasma for five farms ($p < 0.05$) and also in the combined data ($r = 0.373$, $p < 0.001$). The strength of the correlation coefficient between oral fluid and plasma C-RP found in the present study is generally comparable to other reported values. For example, Soler et al. (2013) reported correlation coefficient of 0.59 ($p = 0.004$) between serum and oral fluid C-RP in pigs and Gomez-Laguna et al. (2010) reported correlation coefficient of 0.618 ($p = 0.025$) between serum and oral fluid C-RP concentrations.

Table 1. Mean (± SEM) C-reactive protein concentrations in plasma and oral fluid samples and correlation coefficients between plasma and oral fluid C-reactive protein concentrations in seven farms.

Farms	Mean (SEM) in plasma, µg/mL	Mean (SEM) in oral fluid, ng/mL	Correlation coefficient (r=)	Significance
Farm 1	127 (17.3)	57 (6.5)	0.542	0.025
Farm 2*	126 (9.8)	28 (4.0)	0.576	0.001
Farm 3	105 (9.7)	48 (4.0)	0.083	0.616
Farm 4	86 (5.9)	19 (1.7)	0.312	0.099
Farm 5	132 (15.3)	40 (4.6)	0.270	0.173
Farm 6	126 (10.6)	17 (2.0)	0.432	0.022
Farm 7	148 (14.9)	33 (3.5)	0.757	0.001
Overall	121 (4.7)	33 (1.7)	0.373	0.001

*3/5 pens sample had interrupted water supply during sampling.

Correlations between plasma and oral fluid Hp are presented in Table 2. Apart from Farms 1 and 6, Hp concentration in oral fluid was positively correlated with

Hp levels in plasma ($p < 0.05$) and also in the combined data ($r = 0.44$, $p < 0.001$). The strength of the correlation coefficient between oral fluid and plasma Hp found in the present study is generally comparable to other reported values. For example, Soler et al. (2013) reported correlation coefficient of 0.49 ($p = 0.02$) between serum and oral fluid Hp in pigs and Gomez-Laguna et al. (2010) reported correlation coefficient of 0.626 ($p = 0.005$) between serum and oral fluid Hp concentrations. Hiss et al. (2003) also reported a positive correlation between plasma and saliva Hp ($r = 0.35$; $p < 0.001$).

Table 2. Mean (± SEM) haptoglobin concentrations in plasma and oral fluid samples and correlation coefficients between plasma and oral fluid C-RP concentrations in seven farms.

Farms	Mean (SEM) in plasma, mg/mL	Mean (SEM) in oral fluid, µg/mL	Correlation coefficient (r=)	Significance
Farm 1	0.69 (0.10)	0.86 (0.14)	0.19	0.372
Farm 2*	0.92 (0.06)	0.61 (0.07)	0.56	0.001
Farm 3	0.49 (0.06)	0.42 (0.07)	0.42	0.014
Farm 4	0.63 (0.09)	0.29 (0.04)	0.61	0.001
Farm 5	0.92 (0.10)	0.86 (0.14)	0.39	0.038
Farm 6	0.42 (0.04)	0.18 (0.03)	0.31	0.141
Farm 7	0.93 (0.08)	0.32 (0.07)	0.83	0.001
Overall	0.73 (0.03)	0.51 (0.04)	0.44	0.001

*3/5 pens sample had interrupted water supply during sampling.

3.3 Relationship between oral fluid C-reactive protein concentration estimated by average of individual sampling in a group pen and one point sampling via cotton rope method.

One group pen oral fluid sample was removed from the data set for both C-RP and Hp due to dehydration, because the water supply was interrupted on the sampling day. The relationship between oral fluid C-RP concentration determined by the average of individual oral fluid samples and a one point group sample using the cotton rope method is presented in Figure 3. Data indicate that the one point oral fluid sample collected by hanging a cotton rope in a pen does not correlate with the mean C-RP concentration determined by collection of oral fluid samples from individual pigs in the group ($p > 0.05$). Based on the sensitivity test in Figure 1, the cut-off point for the C-RP concentration in the oral fluid samples that discriminate between healthy and unhealthy pigs is 50 ng/mL in saliva. In the Figure 2, the C-RP concentrations within the red-rectangle represent healthy pigs, while C-RP concentrations outside of the red-rectangle represent unhealthy pigs. The pigs in the three pens with higher than 50 ng C-RP/mL are categorized as unhealthy pens when the oral fluid sample was collected from individual pigs in the pen. However, one-point group sampling via hanging a cotton rope in the same pens could not diagnose these pens as unhealthy. These results suggest that C-RP would not be a reliable reactive protein to use for the diagnostic immunoassay being developed in this project.

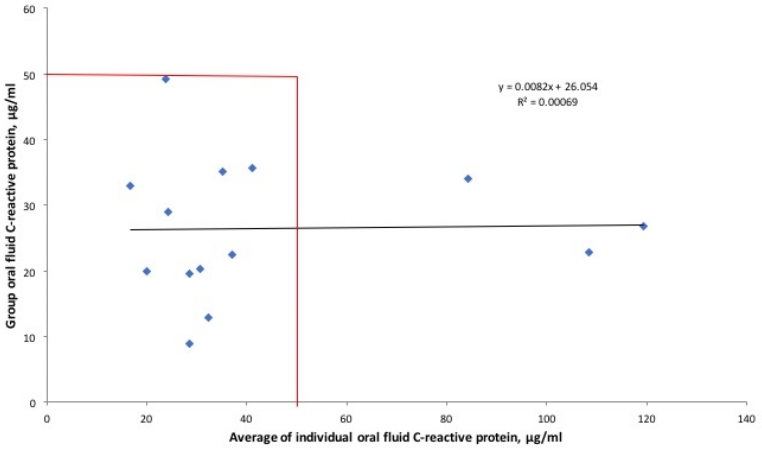

Figure 3. Correlation between oral fluid C-reactive protein concentration determined by the average of individual oral fluid sampling and one-point group sampling using cotton rope (r=0.026, P=0.929). The C-reactive protein concentrations within the red-rectangle represent healthy pigs, while C-reactive protein concentrations outside of the red-rectangle represent unhealthy pigs.

3.4 Relationship between oral fluid haptoglobin concentration estimated by average of individual sampling in a group pen and one point sampling via cotton rope method.

The relationship between oral fluid haptoglobin concentration determined by the average of individual oral fluid samples and one point group sample using the cotton rope method is presented in Figure 4. Data indicate that one point oral fluid sample collected by hanging a cotton rope in a pen reasonably represents the mean haptoglobin concentration determined by collection of oral fluid samples from individual pigs in the group (r = 0.676, p < 0.01). Based on the sensitivity test in Figure 2, the cut-off point for the haptoglobin concentration in the oral fluid samples that discriminate between healthy and unhealthy pigs is 1.5 µg/mL. In the Figure 4, the Hp concentrations within the red-rectangle represent healthy pigs, while Hp concentrations outside of the red-rectangle represent unhealthy pigs. In this data, one pen was deemed unhealthy in the group sample while the average of the individual samples indicated that the pen was healthy. However, as the slope indicates in the regression equation, the Hp concentration in the group oral fluid sample was higher by 2.56µg/ml per unit of Hp than the average of individual oral fluid samples. This suggests that an adjustment for the cut-off point may be required when single point group oral fluid samples are used to evaluate the inflammatory status of the herd.

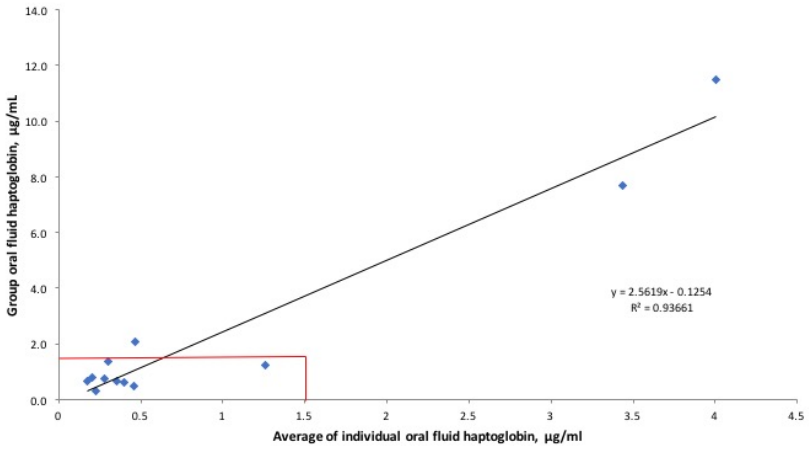

Figure 4. Correlation between oral fluid haptoglobin concentration determined by average of individual oral fluid sampling and a one-point group sampling using cotton rope (r = 0.676, p = 0.008).

4. Application of Research

The preliminary outcomes of the first stage of this project supported the first hypothesis that the concentration of selected reactive proteins (C-RP and Hp) in the oral fluid would be positively correlated to that of the concentration in the plasma. However, with regard to the second hypothesis, results demonstrated that only Hp concentration in the oral fluid sample collected by a one-point group sampling technique was positively correlated with the mean Hp concentration determined by oral fluid sampling from individual pigs in the group. The lack of relationship between C-RP concentrations in group versus individual oral samples could have been due to a number of reasons including feed system (wet versus dry) affecting oral fluid flow rates, increased production of C-RP by the salivary glands in response to local inflammation and veterinary intervention on the selected farms reducing the range of C-RP concentrations on what was deemed to be "healthy" and "unhealthy" farms. Nevertheless, results suggest that oral fluid sampling from a group of pigs is a potentially useful sample for measuring the degree of immune system activation in a herd depending on the reactive protein measured and under the experimental conditions of the current project, Hp seems to be a good candidate for this.

The project was planned to proceed to Stage 2 (development of the lateral flow pen-side immunoassay kit) if the biomarkers and methodology successfully validated the two assumptions. While results are promising with respect to using Hp as a reactive protein, there are a number of limitations and risks to consider (please see section 6 for more detail) before proceeding to stage 2.

5. Conclusion

In conclusion, concentrations of selected reactive proteins, C-RP and Hp can be detected in both saliva and plasma with clear cut-off points discriminating between healthy and unhealthy pigs. There was a positive correlation between C-RP and Hp oral fluid and plasma levels, however only a relationship between the group oral fluid sample and the mean of the individual samples existed for Hp.

6. Limitations/Risks

As mentioned above, the cut off points for salivary Hp used to discriminate between a healthy and unhealthy herd was determined at 1.5µg/ml. However, this was derived by visually assessing the graph and selecting the point of inflexion. Methods exist to determine true cut off points (reference ranges) in biological data (Katayev et al. 2010) and it is recommended that the Hp data are analysed in a similar way to determine a true cut off point. Further to this, closer examination of the salivary Hp data revealed that the two farms with obvious management issues (farm 1: very dirty pens, farm 2: cut off water supply for a period of time) dominated the high proportion of high Hp saliva samples (>1.5µg/ml), which is reassuring, but at the same time highlights that no direct relationship between salivary Hp and health status has been established in the current data. While six out of the seven commercial farms selected for this project had on-going disease issues, veterinary intervention had already occurred before the sampling could take place. This means that the collected samples likely contained a limited range of reactive proteins due to the effect of medication. The lack of differences in plasma CRP and Hp concentration support this notion with only management issues causing an increase in Hp oral fluid concentration. Further to this, more data is required to strengthen the correlation between group saliva and individual saliva samples. More specifically, more data points between 1-5µg/ml for Hp are recommended which again relates to finding farms with sufficient disease challenge.

The use of oral fluid samples for detection of presence or absence of disease through RT-PCR technique has been a successful diagnostic tool because detection of viral or bacterial genes in an oral fluid sample are not affected by altered concentrations. However, the attempt to use oral fluid samples for diagnosis of herd immunity using a lateral flow technology is highly dependent on the concentration of reactive proteins in the oral fluid sample. Therefore, the use of lateral flow technology in oral fluid samples collected on a commercial farm is unlikely to be robust as the level of reactive protein encrypted in the detection kit to discriminate between healthy and unhealthy herd is affected by the altered concentration of the reactive protein in the oral fluid samples and this can be influenced by drinking/chewing behaviour, dehydration, local inflammation, bleeding in the oral cavity, feeding systems and oral fluid flow rates of individual pigs.

7. Recommendations

The finding that Hp could potentially be a suitable marker for the development of an immunoassay kit used to detect inflammatory disease from oral fluid is a valuable outcome, however further validation of these findings is required involving more sampling on commercial farms experiencing herd health problems before veterinary intervention.

Finally, it is important to consider measuring compounds such as calcium along with protein in the saliva for calculation of oral fluid flow rates as a way to standardise the oral fluid samples and adjust the reactive protein concentration accordingly.

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