Comparing the Mucosal and Systemic Immune Response after APP Vaccination with Natural Challenge

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Executive Summary

APP-Alive is a live intranasal vaccine to prevent Actinobacillus pleuropneumoniae (APP) in pigs. When vaccine failures became apparent, we developed IgA based ELISA tests to investigate the immune response at the mucosa of the respiratory tract. The antibody responses found in blood and lungs of a serovar 5 vaccinated herd were compared to those of an apparently APP-free herd and two herds with endemic serovar 7 infection.

During test development, the vaccine at the collaborating farm was changed from APP-Alive to intramuscular immunisation (IM), with a killed whole cell (bacterin) vaccine which was unlikely to stimulate the same level of mucosal IgA response. However, we continued with the project to develop the tests as potential diagnostic tools and to examine the mucosal and serum antibody profiles in the different herds.

To develop APP serovar specific IgA based tests, seven different APP antigens were isolated. We found that lipopolysaccharides (LPS) were the most specific antigens for the serovars of interest when tested against anti-sera to Serovars 1, 7 and 15.

On-farm results from these two new ELISA tests poorly supported the APP classification status originally given to each farm. Therefore, test results from the EMAI ELISA's were compared to results from two other IgG ELISA tests, Swinecheck® APP (5a-5b) and ID Screen ® APP 4-7 Indirect using the same samples. The EMAI ELISA's for both serum IgG and IgA were rated as good for serovar 5 and excellent for serovar 7 at detecting allegedly truly positive animals using Swinecheck® and ID Screen® APP results as the "gold standards". As these gold standards were not developed for use with lung washings a similar assessment of the EMAI ELISA tests using lung mucosal samples was not possible. We could only conclude, on the basis of the serum sample correlations and a single vaccinated pig with a high level of IgG present in Bronchoalveolar Lavage(BAL), that if elevated levels of antibodies to either serovar were present in BAL they would be detected by the EMAI IgA ELISAs.

The EMAI ELISAs, Swinecheck® and ID Screen® test results all supported the assertion that there were Serovar 7 negative pigs in the Serovar 7 herd; Serovar 7 positive pigs in the serovar 5 vaccinated herd; one Serovar 7 positive sample in the APP free herd and serovar 5 negative pigs in the serovar 5 vaccinated herd. The results of all tests were compared with lung damage scores obtained at abattoir sampling. There was no correlation between EMAI ELISA antibody levels in individual pigs and their pneumonia score at slaughter.

Neither vaccination nor infection appeared to produce a consistent detectable rise in mucosal IgA in the herds. However, the triple IM vaccination protocol, followed by an 8 week interval prior to sampling, was too long for the detection of the IgA mucosal response which recent work has shown occurs in the initial stages of vaccination/infection. However, the sera IgA response, which occurs in the later stages, was evident and more reliable than the induced IgG response for detecting antibody response.

The EMAI ELISA tests will be suitable for future live vaccination efficacy studies but more frequent collection of blood and /or mucosal fluid samples, pre and post immunisation will be necessary to better define and quantify the specifics of the immune response to vaccination.

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1. Introduction

Actinobacillus pleuropneumoniae (APP) is the causative agent in porcine pleuropneumonia. It can have sudden onset and significant mortality. Chronic pneumonia has negative effects on pig growth rates and feed efficiency, resulting in production losses. In addition, production costs are increased by antibiotic treatments, which may be of limited efficacy due to the development of bacterial resistance (Dayao et. al. 2014). The control measures for APP available to producers include medication, vaccination, improved animal husbandry, disinfection and the adoption of management strategies such as “all in and all out” stocking. Often outbreaks still occur despite the implementation of these approaches.

A number of APP vaccines, both live and dead, are marketed in Australia. These include a) an inactivated whole cell of serovars 1 and 15, b) outer membrane protein and toxoids APXI, II and III (serovars 1, 7 and 15); c) killed and live autogenous vaccines and formerly d) APP-Alive. The APP-Alive vaccine was a live autogenous vaccine produced from a serovar 5 isolate (ACE#189). It was delivered intra-nasally to piglets prior to weaning. Despite successful trials of the APP-Alive vaccine, a number of mortalities occurred in the field.

The availability of an effective vaccine against APP is desirable, not only to decrease mortality, but also to improve control of chronic disease and reduce the prevalence of carriers who intermittently shed causing outbreaks. Live vaccines, delivered orally or intra-nasally, have proved the most successful in protecting animals from mucosal infections. The active invasion and infection at the mucosal surface prolongs antigen exposure and elicits a higher level of antibody response compared to an inactivated vaccine. In fact, it has been reported that vaccination with APP bacterin is unable to prevent initial infection and colonization (Ramjeet et al 2008). Mucosal immune responses to vaccination and/or natural challenge can be detected in the blood (systemically) and at the site of infection (mucosal secretions). For respiratory pathogens like APP, useful secretions include nasal swabs, oral fluid and Bronchi-Alveolar Lavage (BAL).

The development of serovar specific APP ELISA’s would allow producers to differentiate vaccination from natural infection, enabling evaluation of vaccine efficacy. The APP antigens initially presented to the immune system of the pig are the components of the outer cell wall. These make good candidates for ELISA development and include lipopolysaccharides (LPS), outer membrane proteins (OMP) and capsular polysaccharides (CPS). APP serovar specific ELISA’s have previously been developed using both CPS’s and LPS’s as the antigen to which pigs IgG antibodies bind.

The aim of this project was to develop an ELISA that can be used to detect immune responses to vaccination in pig sera and at the site of infection (BAL). In order to differentiate the immune response to vaccination from natural challenge, we developed serovar specific ELISA’s.

2. Methodology

2.1. Herd Selection and Sample Collection.

Dr Hugo Dunlop (Chris Richards and Associates) collected paired sera and BAL from an APP negative herd (F0), two serovar 7 positive herds (F7) and a serovar 5 positive herd (F5) that had undergone three immunizations with a serovar 5 killed autogenous APP vaccine (ACE#189). BAL was collected by infusing 20 mL of 0.9% sterile saline into a caudal diaphragmatic lobe. The fluid was then aspirated out of the lung into a sample tube. Samples were collected from herds on different days using new equipment to eliminate the likelihood of cross contamination between the APP serovars. Observation of the lungs at slaughter enabled Enzootic Pneumonia (EP) scoring according to Pointon et al (1999) and Pleurisy lung scores. **Table 1** contains herd details, the number of samples collected per herd, the number of pigs tested and the associated lung scores. Samples were transported at 4°C to EMAI by courier where they were stored at -20°C. When samples from all four farms were available testing was conducted.

2.2. Serovars, Strains and Reference Sera.

Serovar 1 (HS54 strain), Serovar 5 (K17 strain), Serovar 7 (WF83 strain) and Serovar 12 (1096 strain) were aseptically revived from the lyophilised culture collection stored at EMAI. A serovar 5 strain (V5 = APP-Alive isolate ACE#189) was supplied on plates by ACE Laboratories.

Porcine sera, positive to serovars 1, 7 or 15, were obtained from Dr Graeme Eamens (EMAI). These had been collected during pen trials conducted at EMAI and were known to be serovar specific. A pooled APP negative serum was supplied by Ms Bernadette Turner (EMAI). An APP serovar 5 positive serum was not available.

2.3. Antigen Isolation and Assessment

A variety of S5 and S7 antigens (7) were isolated and examined for serovar specificity by SDS PAGE and Western Blotting. Cross blotting of the antigen preparations were performed using sera positive to either Serovars 1, 7 or 15 which were visualised using both alkaline phosphatase conjugated anti pig IgG and IgA antibodies. Lipopolysaccharides (LPS) were the most serovar specific antigen investigated. In particular, one technique of LPS isolation (Rezanin et al 2011) was selected to produce the ELISA antigens. Later, the V5 strain (ACE#189) became available and its LPS's were extracted for use as antigens in the EMAI ELISA.

2.4. ELISA Development

Direct ELISA's were developed (antigen bound to the micro plate) using alkaline phosphatase conjugated detection antibodies from Bethyl Laboratories. Soluble BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium) alkaline phosphatase substrate was used for colour development (KPL). Plates were read at 620µm using a Molecular Devices SpectraMax M2® microplate reader and the data were analysed with SoftMax Pro 5.4® software. The S5 and S7 ELISA's were optimised with the same positive serovar specific sera used in western blotting.

Assays were conducted on the samples collected from farms over several days. However, an individual pig's samples were all processed on a single day. The sera samples were diluted 1/200 and the optimised BAL dilution of 1/5 was replaced by a 1/6 dilution because of the limited sample volume. For sera the positive and negative controls were those used in western blotting while BAL controls were supplied by Dr Hugo Dunlop. The number of sera and BAL samples collected from each herd and tested with the EMAI V5 and S7 ELISA's can be seen in **Table 1.**

2.5. Gold Standards

Commercially available, serovar specific tests were used as "Gold Standards". Swinecheck® APP 5a,5b (Biovet Inc.®) and ID Screen® APP 4-7 Indirect (IDvet®) commercial IgG ELISA kits were used according to the manufacturer's instructions. ELISA Ratios (ER) were calculated by dividing the Mean OD of the sample by the Mean OD of the Positive control. The negative control required an ER of less than 0.15 and the mean OD of the positive control had to be greater than 0.8 for the Swinecheck test to be valid. The ID Screen test was valid if the mean OD of the positive control was greater than 0.35 and the ER of the negative control was less than 0.3. The ER decision points for each test were Swinecheck: >0.5: positive, 0.4-0.5: suspicious, <0.4: negative. ID Screen: >0.4: positive, 0.3-0.4: suspicious, <0.3: negative. The number of pigs per herd which were tested with these gold standards can be found in **Table 1**. BAL was not tested using the ID screen APP4-7 kit. The results obtained from these gold standards were used to define true positive and negative pigs and for comparison with the results obtained for V5 and S7 EMAI ELISA's.

2.6. Data Analysis

It was assumed that discrete single serovar infections occurred on the sampled farms. Scatter plots were constructed to demonstrate the separation between farms by plotting the average

OD's for each immunoglobulin class (IgG or IgA), each antigen (V5 or S7) and the type of sample fluid used (sera or BAL) versus farm (F0, F5 or F7) in the EMAI ELISA's. In addition, the serovar positive/negative statuses of individuals were defined by the "Gold Standard" assays, Swinecheck (S5) and ID Screen (S4-7) for sera from all four farms. Scatter plots were also produced using the "Gold Standard" ELISA ratios plotted for each farm.

Receiver Operating Characteristic (ROC) curves were constructed after the gold standard assays defined individual pigs as true negatives or positives. These ROC curves estimated the sensitivity, specificity and accuracy of the EMAI ELISA's by comparing the true positive rate against the false positive rate for different possible cut points. It shows the trade-offs between sensitivity and specificity i.e. any increase in sensitivity will be accompanied by a decrease in specificity. The closer the curve follows the left-hand and top borders of the ROC space, the greater the calculated Area Under the Curve (AUC) and the more accurate the test. The AUC is defined using the standard traditional academic point system: 0.90 – 1.00 = excellent, 0.80 - 0.90 = good, 0.70 - 0.80 = fair, 0.60 - 0.70 = poor, <0.6 = fail (Kleinbaum and Klein 2010). ROC curves were produced using ROC function in the Epi library in R (Carstensen et al. 2013). All computations were performed using the R statistical environment (R Core Team. 2013).

Kendall's correlation coefficients were calculated as a nonparametric measure of statistical dependence between the two variables of EP Lung Score and the mean OD measured in each of the EMAI ELISA's. This analysis was also repeated using Pleurisy Lung scores. Correlation coefficients were calculated for each sample type, (serum and BAL) and immunoglobulin class (IgG and IgA).

3. Outcomes

3.1. Farm Selection, Sample Collection and Testing.

The number of samples collected per farm by Dr Hugo Dunlop, according to sample type and the tests conducted are listed in **Table 1** below with the overall EP and Pleurisy Lung scores.

Table 1 - The Total Number of Pigs Tested per Farm According to Serovar, Sample Type and Assay.

Farm ID (code)	Herd Serovar (Source herd)	S5 & S7 ELISA - IgG and IgA		Swinecheck APP5a-5b		ID Screen APP 4-7 (Sera only)	EP[a] Lung Scores - No. of Pigs	Pleurisy[b] Lung Scores - No. of Pigs
		Sera	BAL	Sera	BAL			
F0 (CFPZ)	Nil (S12)	24	21	24	14	18	Clear	Clear
F7 (CBW)	S7 (S15)	30	22	29	12	29	2-**2**, 1-**1**	P2-**1**
F7 (CCI)	S7 (S15)	20	17	20	10	20	Clear	P1-**1**, P2-**3** P3-**2**
F5 (SHEA)	S5	18	19	24	7	24	7-**1**, 5-**1**, 4-**4**, 3-**1**, 2-**1**, 1-**3** 0-**12**	P3-**7**
TOTALS		**92 x 4 = 368**	**79 x 4 = 316**	**97**	**43**	**91**		

[a] EP scores. Scored 0-15 according to Pointon et al (1999).
[b] Pleurisy score – P1 = Fibrous tag between lobes of lungs. P2 = Fibrous tag between viscera and parietal pleura. P3 = Severe pleurisy, some lung missing.

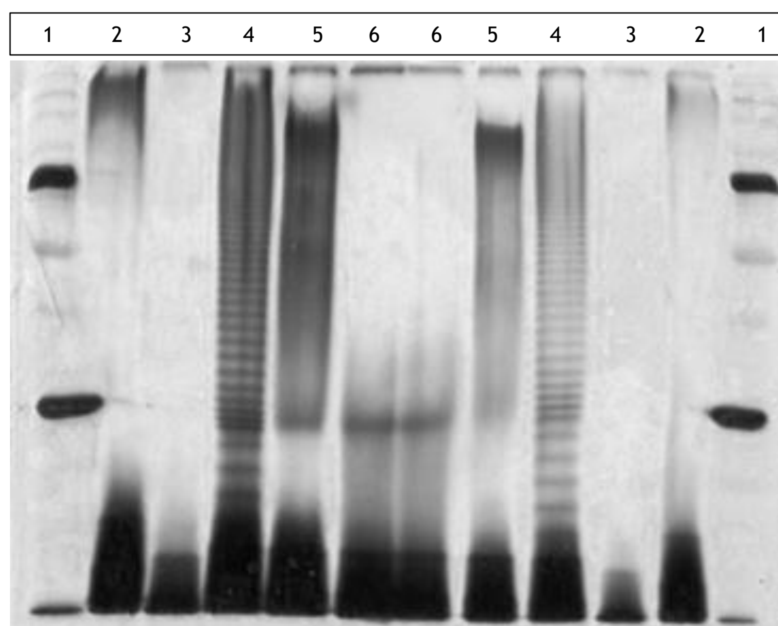
3.2. Serovar Specificity of Antigen Preparations.

SDS-PAGE and Western blotting were conducted on LPS antigens isolated from serovar 1, 5, 7 and 12 isolates. Anti-serum specific for serovars S1, S7 or S15 did not contain antibodies of either the IgG or IgA class that recognized the Serovar 5 (strain K17) antigen S5. The S1 anti-serum recognised only S1 LPS; the S7 anti-serum recognised the Serovar 7 and 12 LPS antigens and the S15 anti- serum recognised the Serovar 7 LPS's from both immunoglobulin classes as anticipated (Blackall et.al 2002). A Serovar 5 specific anti-serum was not available at the time of test development. Therefore the ability of the S5 LPS antigen to bind, and S7 LPS antigen not to bind, to serovar 5 specific antibodies could not be established.

The antigenicity demonstrated by the serovar 7 LPS and the lack of cross-reaction of the S5 (strain K17) LPS isolate with the S1, S7 and S15 positive sera, suggested their incorporation into an ELISA may be successful. When the serovar 5 strain ACE#189 (V5) later became available, its LPS antigen was extracted and used in the ELISA system, initially developed with the S5 (K17) strain. The culture characteristics and performance of the V5 antigen in the ELISA's prompted further investigation of the V5 (ACE#189) LPS's by electrophoresis and cross blotting. Despite the same extraction technique and pellet mass, differences were apparent in SDS-Urea electrophoresis (**Figure 2a below)** between the LPS extracted from the Serovar 5a strain K17 (lanes 3) and the V5 ACE#189 strain (lanes 6). Altman et. al (1990) described K17 as R-type only (single repeating monosaccharide) while other serovar 5 isolates have the more usual oligosaccharide repeating units (S and RS type).

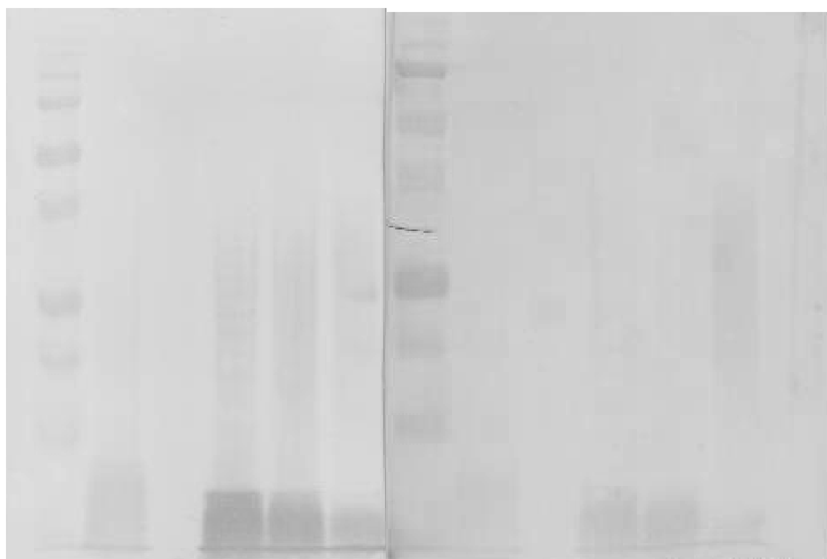
Western blotting showed the LPS extracted from the V5 strain bound IgG antibodies from the V5, S7 and S15 anti-sera while the LPS extracted from the S5 K17 strain did not**. (Data not shown).** Conversely, IgG and IgA antibodies in the V5 anti-serum bound to the LPS from all but the S5 (K17) strain, with IgG antibodies being more prevalent than IgA. (**Figure 2b).**

Figure 2a - Silver Stained Urea PAGE of LPS



Lanes 1 = Protein Molecular weight marker
Lanes 2 = Serovar 1 (LHS54) LPS antigen extract.
Lanes 3 = Serovar 5 (K17) LPS antigen extract.
Lanes 4 = Serovar 7 (WF83) LPS antigen extract.
Lanes 5 = Serovar 12 (1096) LPS antigen extract.
Lanes 6 = Serovar 5 (ACE#189) LPS antigen extract.

Figure 2b - Western Blots- IgG and IgA Antibodies from V5 Anti-Sera against Serovar S1, S5, S7, S12 and V5 LPS'S.



IgG 1	2	3	4	5	6	1	2	3	4	5	6 IgA

Lane 1 = Protein Molecular Weight Marker
Lane 2 = Serovar 1 (LHS54) LPS extract.
Lane 3 = Serovar 5 (K17) LPS extract
Lane 4 = Serovar 7 (WF83) LPS extract.
Lane 5 = Serovar 12 (1096) LPS extract.
Lane 6 = Serovar 5 (ACE#189) LPS extract.

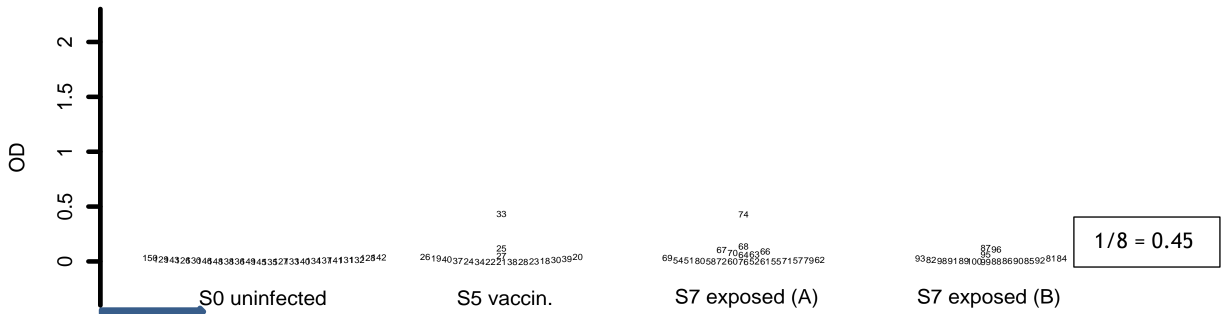
BAL samples were used in western blots and ELISA's to demonstrate that a 1/5 dilution contained detectable levels of both IgG and IgA antibodies.

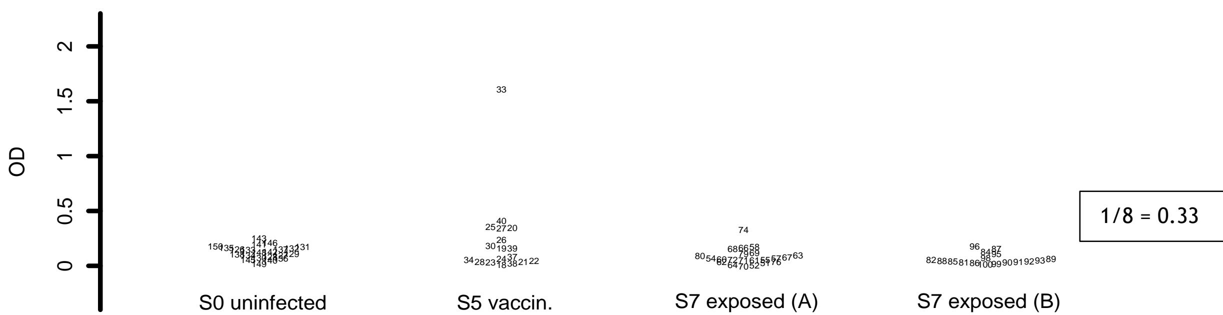
3.3. ELISA Results

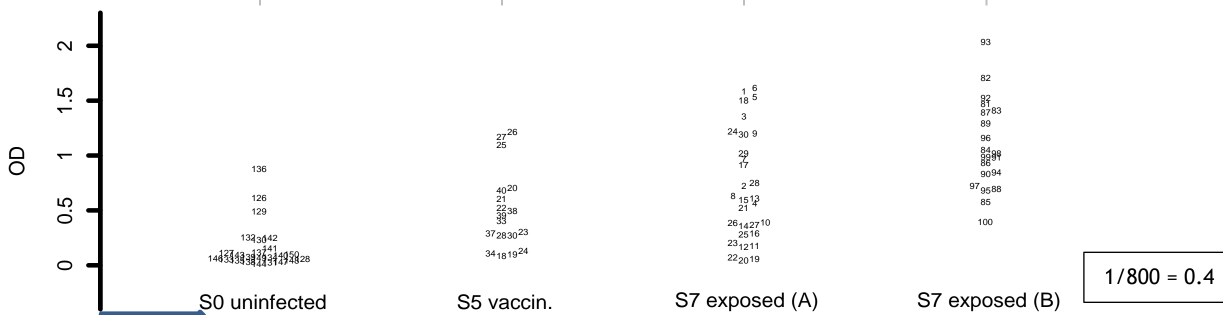
The average OD obtained for the EMAI ELISA's according to farm (F0, F7 and F5), antigen (V5 or S7), sample type (BAL or Sera) and type of immune response (IgG or IgA) were assessed by scatter plot analysis. The average OD for each pig is plotted for each farm using the individuals number as a marker. These can be seen in **Figure 3** for the IgG response and **Figure 4** for the IgA response. **Figure 3** shows little separation exists between the farms for any of the IgG responses. The exceptions being pig 33 from the F5 farm in the V5 BAL ELISA and some evidence of a systemic difference between sera from the two Serovar 7 positive farms (CBW and CCI) for the EMAI S7 ELISA.

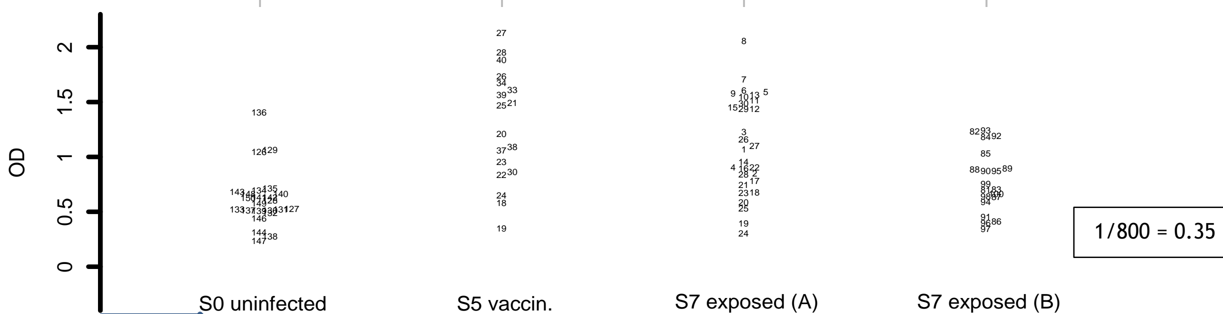
The level of IgA antibodies appears to be a superior measure, compared to IgG, at differentiating sera and BAL samples according to their farm of origin (**Figure 4)**. Unfortunately, the overall response of pigs from the V5 vaccinated farm in the V5 EMAI ELISA is diminished by the small number (4) of high responding pigs. The highest responding vaccinated pig had an OD (approximately 1) that was twice that of the pigs from the F7 farm (less than 0.4) in the S5 IgA sera ELISA.

Figure 3 - The IgG Response, Expressed as Average OD, of a S0 Control (CFPZ), V5 Vaccinated (Shea) and S7 Exposed Farms A (CBW) and B (CCI) in Serovar V5 Specific and Serovar 7 Specific EMAI ELISA's Utilising Sera or Bronchial Alveolar Lavage (BAL)



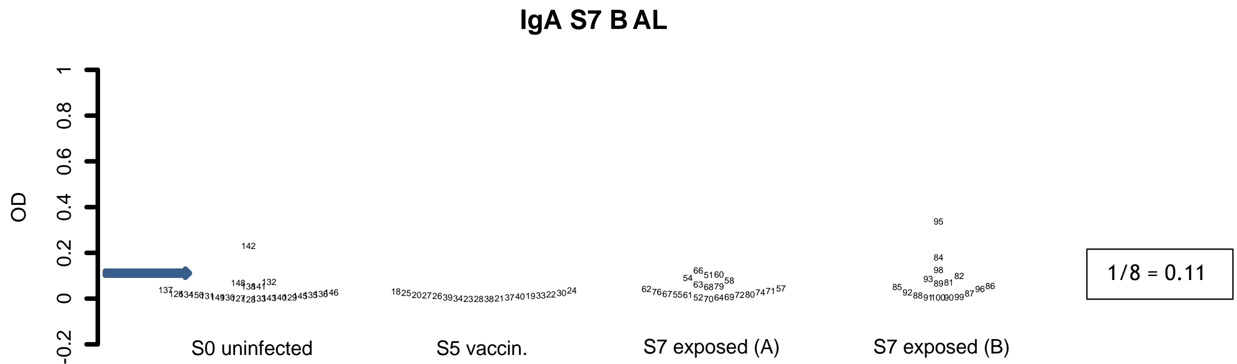


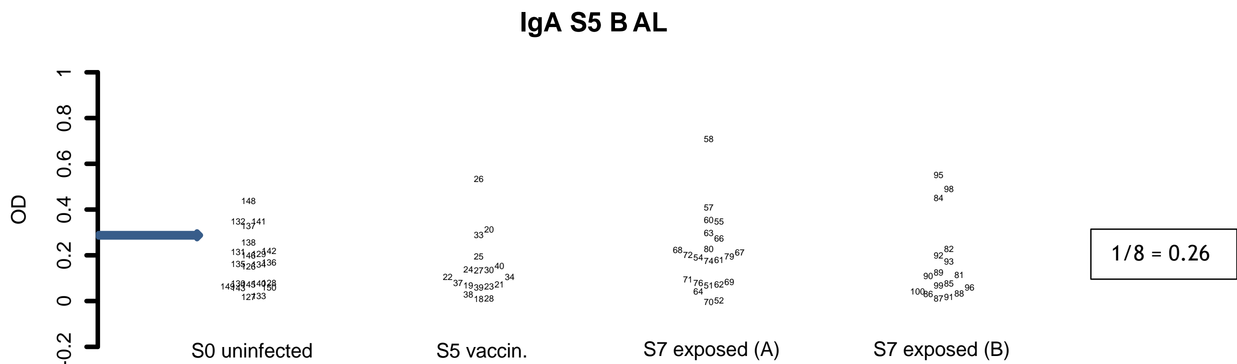


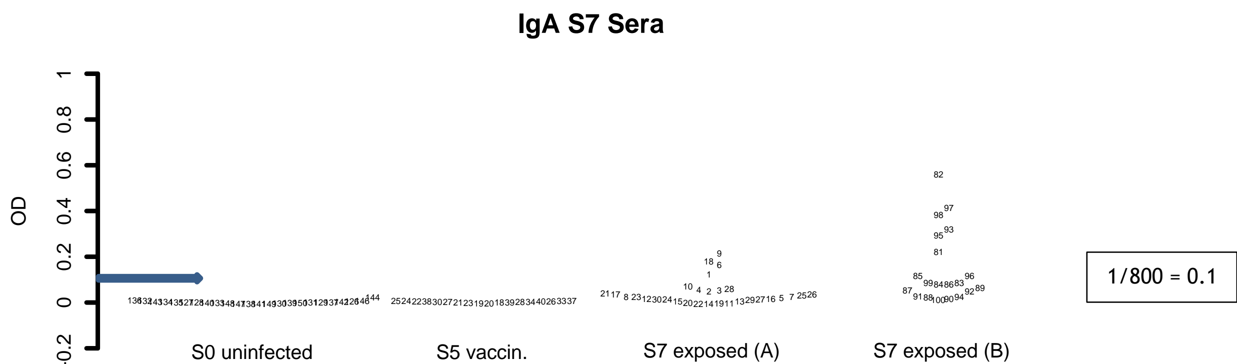


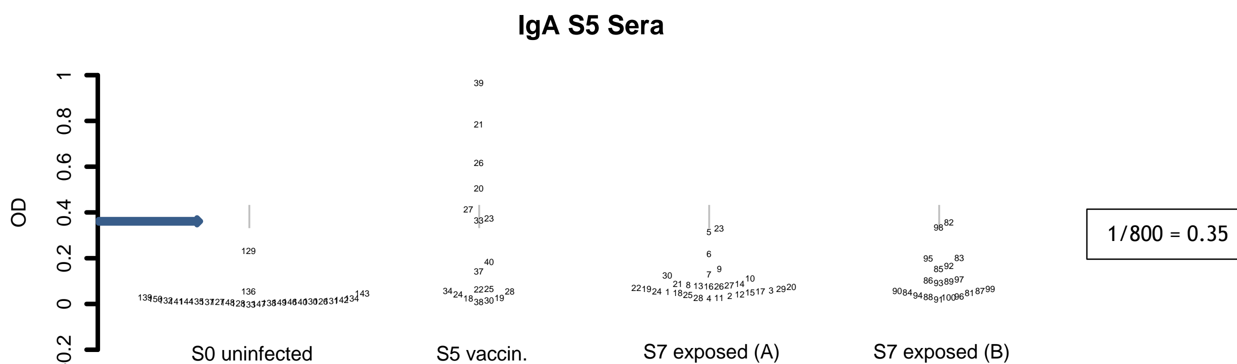
1/800 = 0.35 - Relative OD and Dilution Factor of Known Positive Standards used to obtain OD Cut -Off values.

Figure 4 - The IgA Response, Expressed as Average OD, of a S0 Control (CFPZ), V5 Vaccinated (Shea) and S7 Exposed Farms A (CBW) and B (CCI) in Serovar V5 Specific and Serovar 7 Specific EMAI ELISA's Utilising Sera or Bronchial Alveolar Lavage (BAL)







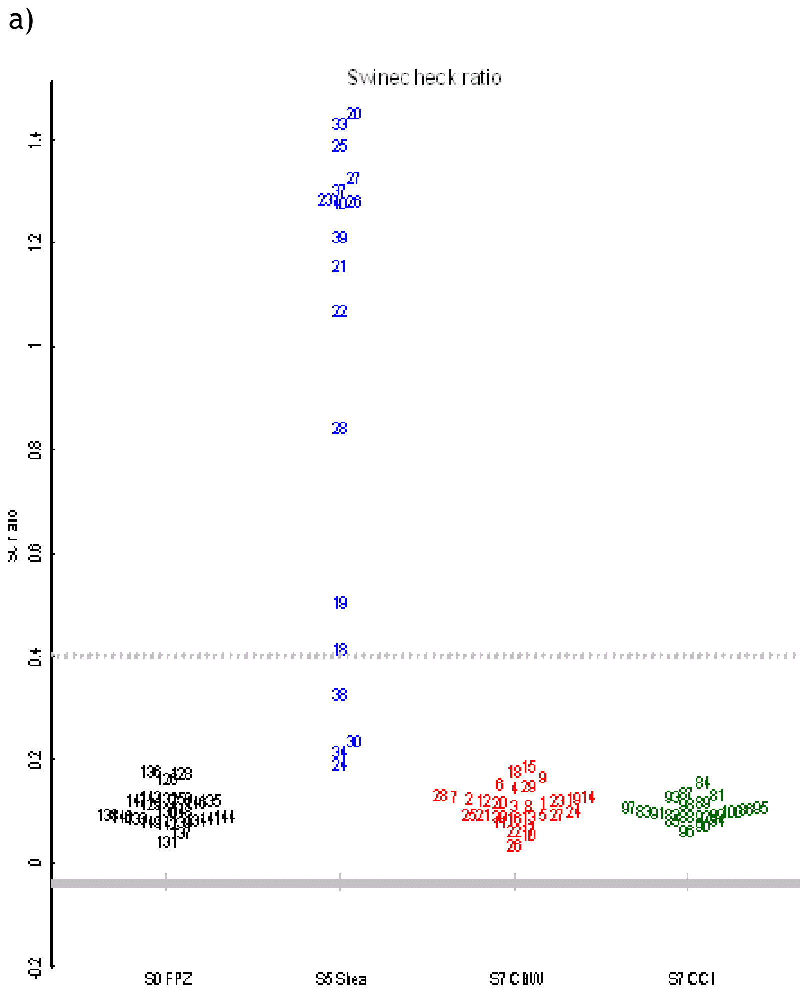


1/800 = 0.35 - Relative OD and Dilution Factor of Known Positive Standards used to obtain OD Cut - Off Values.

ID Screen® APP 4-7 Indirect and Swinecheck® APP 5a,5b commercial IgG ELISA kits were used as gold standards to determine the serovar status of individual pigs. The Swinecheck® APP 5a,5b ELISA confirmed that 5/24 pigs on the vaccinated F5 farm were negative with respect to Serovar 5, as were all of the pigs from the F0 and F7 farms (**Figure 5**). The ID Screen® APP (4-7) test indicated that 1 pig on the APP free farm (F0) was considered Serovar 7 positive and then suspicious when retested, (both results appear in **Figure 6**). Three pigs from the vaccinated F5 farm tested positive to Serovar 7. All of the pigs at one F7 farm (CCI) tested positive while 9 pigs from the second F7 farm (CBW) were negative with respect to Serovar 7. These results were compared with those obtained with the V5 and S7 EMAI ELISA's.

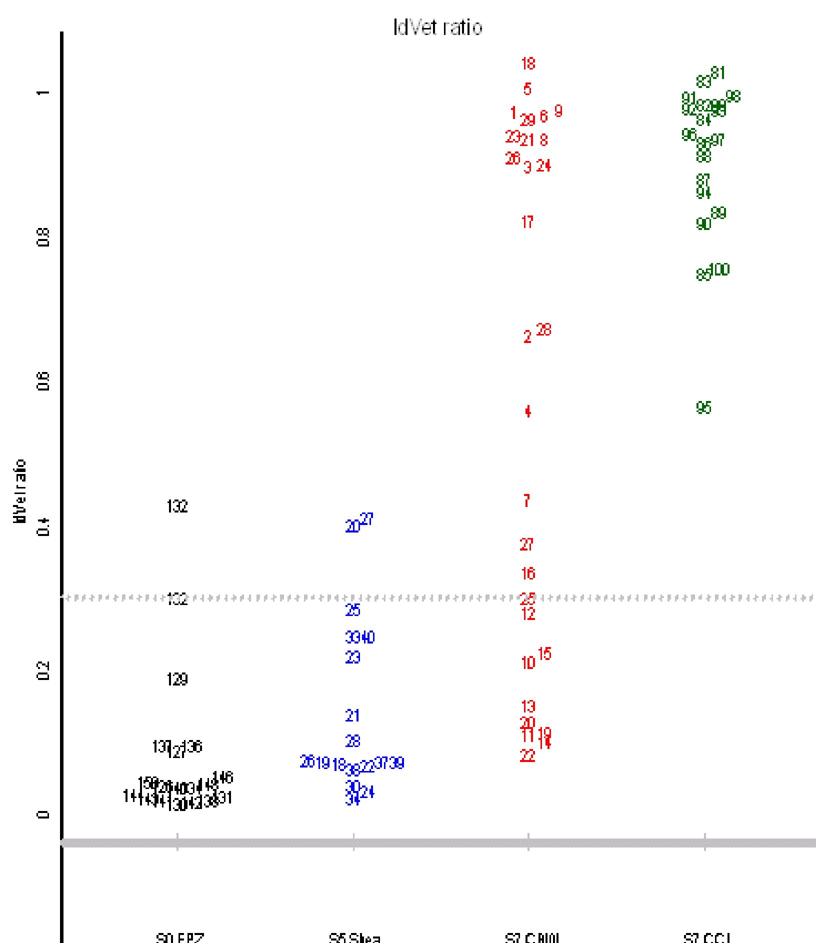
Figure 5 - ELISA Ratios of Sera Samples Tested Using the Swinecheck® APP 5a,5b ELISA kit. Grouped According to their Farm, with each farm a different colour.

a)



The plot character used is the pig number. The dotted lines show the recommended decision points for Swinecheck: >0.5: positive, 0.4-0.5: suspicious, <0.4: negative.

Figure 6 - ELISA Ratios of Sera Samples Tested Using the ID Screen® APP 4-7 Indirect ELISA kit. Grouped According to their Farm, with each farm a different colour.



The plot character used is the pig number. The dotted lines show the recommended decision points for ID Screen: >0.4: positive, 0.3-0.4: suspicious, <0.3: negative

Receiver Operating Characteristic (ROC) curves were constructed to determine three parameters (specificity, sensitivity and accuracy) which allowed comparison of the V5 and S7 antigens as well as the IgG and IgA immune responses in the EMAI ELISA's **(Appendix 9)**. The V5 EMAI sera ELISA's displayed good accuracies of 88 and 86%, sensitivities of 75 and 92% and specificities of 86 and 67% respectively for the IgA and IgG responses. The EMAI sera S7 ELISA's gave superior results with excellent accuracies of 96 and 92%, sensitivities of 95 and 82% and specificities of 87 and 89% respectively for IgA and IgG. ROC curves were also constructed for the BAL samples collected **(Appendix 10)**. The results of this ROC Curve analysis are summarised in **Table 2.**

Table 2 - Summary of the ROC Curve Analysis Parameters of Sensitivity, Specificity and Accuracy of the V5 and S7 LPS ELISA's (IgG and IgA) against the Serovar Status of Individuals as Determined by Swine Check ™and ID Screen ™ ELISA kits. (Appendix 9 & 10).

Immunoglobulin	LPS Serovar	Sample type	Cut Point OD	Sensitivity %	Specificity %	Area Under Curve	AUC Rating
IgA	V5	Sera	0.130	75.0	86.1	0.881	Good
	S7	Sera	0.011	94.9	87.2	0.955	Excellent
	V5	BAL	0.061	91.7	23.3	0.513	Fail
	S7	BAL	0.039	57.9	85.3	0.738	Good
IgG	V5	Sera	0.957	91.7	67.1	0.857	Good
	S7	Sera	0.616	82.1	89.4	0.917	Excellent

Immunoglobulin	LPS Serovar	Sample type	Cut Point OD	Sensitivity %	Specificity %	Area Under Curve	AUC Rating
	V5	BAL	0.210	50.0	97.7	0.676	Poor
	S7	BAL	0.007	84.2	38.2	0.576	Fail

3.4. Lung Scores

The Enzootic Pneumonia (EP) and Pleurisy (Pl) scores for each pig sampled are listed in **Appendix 5-8**. The highest EP scores of 7 and 5 were seen on the F5 vaccinated farm. Both of these pigs displayed positive readings for sera IgG antibodies in the V5 EMAI ELISA (0.839 and 1.475) and Swinecheck™ (1.065 and 1.385). Only one of these pigs had elevated levels of both IgG (0.196) and IgA (0.358) antibodies detected in BAL by the V5 EMAI ELISA and BAL IgG antibodies by the S7 EMAI ELISA.

The highest level of IgG antibodies found in BAL samples using the V5 (1.610) and S7 (0.436) EMAI ELISA's were displayed by pig 33 from the F5 vaccinated farm. This pig and five others from the F5 farm had zero EP and PL scores while testing V5 positive indicating positive vaccination responses. Only pigs from the F0 farm (CFPZ) were clear of lung lesions with zero EP and Pl scores. The F7 CBW Farm had 3/30 individuals that recorded EP scores of 1 or 2 and one PL score of 2 despite 21/29 being positive or suspect according to ID Screen. The F7 farm CCI had zero EP scores but 6/17 with Pl scores despite being all positive according to ID Screen (**Table 1).**

Kendall Correlation Coefficients were calculated firstly for EP Lung score and mean OD for each EMAI ELISA. This was repeated for Pleurisy Lung scores. For a 100% correlation the Kendall coefficient is equal to 1.0. This analysis found little statistical dependence between both lung score and the EMAI ELISA Mean OD for both the IgG or IgA responses on the vaccinated farm (F5) or the F7 farms. The highest coefficient (0.38) was observed between the Pleurisy scores and the F7 IgA BAL ELISA results from farm F7 (CCI). The lowest coefficient (0.02) occurred between EP lung score and the S7 IgG sera ELISA on the S7 (CBW) Farm. Two slightly negative correlations were obtained but were also insignificant. (**Table 3).**

Table 3 - Kendall's Correlation Coefficients Calculated for EP and Pleurisy Lung Scores Versus the EMAI V5 and S7 ELISA's for the Variables of Sample Type (BAL and Sera) and Immunoglobulin Class (IgA and IgG).

FARM	Assay	BAL IgA	BAL IgG	Sera IgA	Sera IgG
EP Lung Scores					
F5 SHEA	V5 ELISA	0.1	0.25	0.08	0.1
F7 CBW	S7 ELISA	0.07	-0.11	0.14	0.02
Pleurisy Score					
F5 SHEA	V5 ELISA	0.04	0.06	-0.04	0.04
F7 CCI	S7 ELISA	0.38	0.25	0.15	0.08

4. Application of Research

4.1. Serovar Specificity

Serovar 7 APP cell walls contain lipopolysaccharide (LPS) which are long with branched o-antigens that are known as "smooth". Exposure of a host to smooth o-antigens is known to induce highly specific antibodies. This specificity made APP serovar 7 positive farms excellent candidates for differentiation from serovar 5 APP-Alive vaccinated farms.

It was observed during large scale liquid culture that the APP serovar 5 reference strain (K17) was quite different to the V5 APP-Alive strain (ACE#189). The APP-Alive V5 strain was more robust, adherent and formed large "curtains" of cells. This was consistent with reports that wild type strains often have additional saccharides present in the lipopolysaccharide (LPS)

complex (Trembly et al 2013) and that variation between serovar 5 isolates had been observed and structurally determined by Altman et al (1990). For this reason LPS from the V5 APP-Alive strain (ACE#189) were used as ELISA antigen in preference to those purified from the S5 (K17) strain. Electrophoresis and highly sensitive cross blotting demonstrated that the V5 LPS antigen was detected by the V5, S7 and S15 anti-sera and that the V5 anti-sera contained antibodies which bound to the LPS antigen extracts from serovars 1, 7 and 12. These results suggested that the serovar V5 LPS antigen was less serovar specific than anticipated. In addition, ELISA results demonstrated that some pigs on the F5 farm were positive to both serovars 5 and 7.

The EMAI ELISA's developed with antigens from S7 and the V5 isolates differentiated between farms based on serovar with varying accuracy. The S7 EMAI ELISA was deemed as "excellent" while the V5 EMAI ELISA was only deemed "good" for both IgG and IgA antibodies. This was exacerbated by the low level vaccination response observed at 22 weeks on the V5 farm and the presence of serovar 7 positive animals.

Given the constant source of natural challenge provided by chronic carriers, the reliance on sourcing breeding stock "off-farm" and evidence of Serovars 5 and 7 infections in pigs on Farm 5, it appears the challenges faced by some producers are not based on a single APP serovar.

4.2. Comparison of Sample Type (Sera vs. BAL)

This study aimed to develop ELISA's to quantify a pigs' response to intra-nasal APP-Alive vaccination. Prior to sample collection the APP-Alive vaccine was withdrawn and was replaced with a killed autogenous vaccine delivered intramuscularly on three occasions. Despite changing the vaccination programme, samples from the mucosa of the lung, bronchial alveolar Lavage, (BAL) and serum should have differentiated between chronic or previous infection (High sera/Low BAL) and recent natural infection or vaccination (Low sera/high BAL).

It is not uncommon for an ELISA antigen, while effective against sera, to be unsuitable for detecting antibodies in BAL fluid (Marchioro et al 2013). However, this was not the case in this study. The EMAI ELISA with BAL identified a single pig (33) with a high response from the F5 farm. Sera assays showed there were S7 negative pigs on the S7 farm, S7 positive pigs on the F0 and F5 farms and V5 negative pigs on the vaccinated farm. It appears the 8 week interval between final vaccination and BAL sampling, while considered reasonable in some effective vaccination programmes, may have been too long for detection of the mucosal response. In fact, Loftager et al (1993) found that APP serovar 2 IgA antibodies were present in mucosal samples at an early stage of infection when serum antibodies were undetectable and undetectable in mucosal fluids when detected in serum later in the infection.

Bronchial Alveolar Lavage is difficult to collect, requiring a skilled practitioner. The BAL samples in this study were collected at the time of slaughter. This limits the opportunities of time course sampling to pinpoint or monitor vaccination response. However, this study shows the presence of mucosal IgA antibodies in the lower respiratory tract. This should be compared with screening for specific antibodies using fluids from the upper respiratory tract. A less stressful method of sample collection, for both the pig and the practitioner, is the use of ropes for pigs to chew upon for the collection of oral fluids. Oral fluids have proved to be extremely accurate for the detection of some bacteria and antibodies and may be useful to monitor the secretary immune response to APP vaccination over time. This type of information can also be used to integrate, assess and optimise the use of other tools like disinfectants and antibiotics, quantify disease threat and assess the level of protection or immunity.

4.3. Immunoglobulin Class

IgA is part of the primary defence mechanism against penetration of mucosal surfaces by bacteria and viruses. In particular, secretory IgA is induced by polysaccharide antigens and stops bacteria adhering to mucosal cells while neutralising them. IgA antibodies are also a secondary line of defence in serum against bacteria which have successfully breached the

mucosal barrier. The ELISA's developed in this study showed that serum IgG and IgA immunoglobulins were both considered excellent at detecting and differentiating APP serovar 7 LPS antigen, but were "good" against APP serovar 5 LPS antigen. In this study, sera IgA had superior specificity, sensitivity and accuracy compared to the IgG responses and could be considered a better predictor of protection than IgG level at 22 weeks of age and 8 weeks post vaccination.

4.4. Response to Vaccination

There are more aspects to vaccine based disease protection than the peak achieved in antibody production. Long-term protection requires antibody persistence and/or immune memory. Evidence of a positive vaccination response, 8 weeks after a course of 3 intramuscular injections of a killed autogenous vaccine, in pigs from the V5 Shea farm included five pigs with elevated IgA antibody levels in sera and one pig with elevated IgG antibody levels in BAL. While the IgA sera levels indicated that some vaccinated pigs had responded positively to vaccination, this contrasted with 7/19 pigs who had the highest pleurisy scores of P3 indicating that they had severe pleurisy with some lung missing which is associated with active APP infection. These active infections indicated that natural challenge was occurring and that vaccination had not provided adequate or long term protection against the disease. As previously stated IgA antibodies to APP are known to be present in mucosal samples at an early stage of infection but were possibly missed in this study. Conversely, the elevated IgA serum antibody levels displayed on both the Serovar 5 and 7 positive farms reflect sampling at the later stage of infection/vaccination in this study. The observed IgG levels indicate that the vaccination regime afforded some immunity but did not stimulate a consistent protective response. The dual approach of immune exclusion by a stimulated mucosal IgA response and immune elimination by a strongly induced IgG systemic response appears to be required to achieve protection against disease.

5. Conclusion

The main rational for this project was to develop tools to quantify an animals' response to an APP vaccine and distinguish it from one with a natural infection. APP ELISA's developed using Lipo-polysaccharides as antigen, found the IgA response in sera to be more sensitive, specific and accurate than the IgG response to both serovar 5 and 7. Interaction between the serovar 5 and 7 ELISA's (but not 7 and 5) suggested the LPS antigen isolated from the ACE#189 strain was less useful at differentiating serovar specific infections while V5 anti-serum (post-vaccination) contained antibodies to common LPS regions.

By using the S7 EMAI ELISA, it was possible to differentiate between vaccine response (serovar 5) and natural challenge (serovar 7). BAL samples collected from the same pigs displayed low antibody levels in the ELISA system and verify other researcher's findings about the early mucosal response to infection/vaccination. In this study, the BAL samples collected may have missed the peak mucosal response, which could also have been small and short lived. The intramuscular administration of killed whole cell vaccine should have initiated and boosted production of serum IgG antibodies to overcome any infection. Both the ELISA results and the prevalence and the level (P3) of pleurisy present in the vaccinated pigs indicate this was not the case.

Collection of BAL samples can be difficult and their value as a screening or monitoring tool on farm is very limited. The use of ropes to collect oral fluids may be a useful alternative for assessing the secretary immune response to APP. This could be done rapidly and relatively inexpensively allowing for more frequent monitoring and screening of pigs.

Anomalies on farm, such as a control pig which was S7 positive, V5 vaccinated pigs which were S5 negative and others S7 positive and S7 negative pigs on one S7 farm have highlighted the challenges posed by APP infection and its control.

6. Limitations/Risks

This project aimed to produce a diagnostic tool to replace the current challenge trials used to evaluate vaccine efficacy which are dependent on lethal sampling and close observation of pig behaviour. In addition, the importance of antigen type, vaccine type, route of delivery, vaccination and sampling intervals and the vagaries of sourcing serovar specific infected herds have been highlighted.

It appears that a whole cell approach to vaccination has not supplied sufficient levels or interval of antigen exposure to the pigs' immune system to enable a prolonged and effective immune response to be mounted. One strategy often used to overcome this problem includes a vaccine which incorporates polysaccharides to induce the primary secretary responses, backed up by a protein/toxin capable of eliciting a classic systemic IgG reaction.

The farms involved in this study source their pigs from serovar 12 or 15 farms and have disease problems with Serovars 7 and/or 5. These on-farm findings highlight the fact that single serovar problems are unlikely to exist in the production world. While single serovar screening can aid in vaccine development, targeted vaccine use and monitoring prior to transfer from known serovar status farms, the concept of single serovar disease control is possibly unrealistic given the threat posed by chronic carriers. The integration of vaccination, disinfection and the use of antibiotics for APP and other diseases need to be considered as part of the control and management of both chronic and acute disease caused by APP.

7. Recommendations

The use of a single serovar test may fail to identify infected animals and determine the true APP status of a piggery. In this study APP free status was not confirmed with a single pig testing positive to serovar 7 on the control farm. Serovar exclusivity was also not observed on the V5 vaccinated farm where some pigs proved to be positive to serovar 7 also. It appears that APP infection, vaccination and immunity cannot be measured or treated by considering only a single serovar. This would suggest that in the "real world" the induction of multi-antigen or common antigen APP immunity, of sufficient level and duration to prevent disease outbreaks, is required. However, this does not mean that serovar specific tools still don't have their place aiding in vaccine development and use, as well as for management decisions and practices concerning animal sourcing, movement, disinfection and antibiotic treatments. This project highlights the multi-faceted problem APP infection and immunity pose to the producer and the advantages to be gained by conducting studies such as this under controlled conditions.

This study demonstrated the importance of serum IgA levels in APP infections and vaccination. Given the initial site of natural challenge is at the mucous membranes; the importance of an adequately stimulated immune response at the mucosal surface followed by the IgA and IgG systemic response, is evident. In addition, *A. plueropneumoniae* produces highly immunogenic toxins whose in vivo expression or incorporation into a vaccine appears to be critical to obtain cross protection and vaccine efficacy (Shao et al 2010).

The complicated and terminal nature of BAL collection at slaughter, appears to exclude it as a useful tool for APP screening. Conversely, the ease of oral fluid collection, as pooled samples from pens, would make them ideal for APP screening and monitoring purposes. Detection of bacteria and /or antibodies in oral fluids has been successful for a number of diseases including APP (Costa et. al. 2012). By including general clinical signs such as weight loss, coughing, increased temperature, lack of appetite and lethargy, differentiation between chronic and acute disease or immunity may be possible as part of routine monitoring.

Vaccination may be used, not only to reduce the incidence of disease, but to also reduce the overall threat posed by chronic carriers and is therefore highly desirable. The level and interval of protection afforded by any vaccine needs to be considered in terms of the scheduling of antibiotic use and the degree of maturity of the immune system. A detailed study of several

combinations of these variables would be useful for the development of an effective vaccine strategy and to design an integrated disease control programme.

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9. References

Altman, E. Griffith, DG. And Perry, MB. (1990) “Structural studies of the O-chains of the Lipopolysaccharides produced by strains of *Actinobacillus (Haemophilus) pleuropneumoniae* serotype 5. *Biochem. Cell Biol.* **68:** 1268-1271.

Blackall PJ, Klaasen HL, van den Bosch H, Kuhnert P, Frey J. (2002) “Proposal of a new serovar of Actinobacillus pleuropneumoniae: serovar 15.” Vet Microbiol. 3; 84(1-2):47-52.

Carstensen, B. and Plummer, M. and Laara, E. and Hills, M., (2013) "Epi: A Package for Statistical Analysis in Epidemiology”.

Costa, G. Oliveira, S. and Torrison, J. (2012) “Detection of *Actinobacillus plueropneumonia* in oral-fluid samples obtained from experimentally infected pigs. “ *J. Swine Health Prod* **20 (2)**: 78-81

Dayao, D. E, Gibson, J.S., Blackall, P.J., and Turni, C. (2014)”Antimicrobial resistance in bacteria associated with porcine respiratory disease in Australia”. *Veterinary Microbiology* 2014 June 25, **171** (1): 232-5.

Kleinbaum, D.G. and Klein, M., (2010). "Logistic Regression: A Self-Learning Text", Springer. Page 357.

Loftager, M.K. and Eriksen, L. (1993) “Antibodies against *Actinobacillus plueropneumoniae* serotype 2 in mucosal secretions and sera infected pigs as demonstrated by an enzyme-linked immunosorbent assay”. *Research in Veterinary Science.* **54**: 57-62.

Marchioro, S.B, Maes, D. Flahou, B. Pasmans, F., Sacristan, R. Vranckx, K., Melkebeek, V. Cox, E., Wuyts, N., and Haesebrouck, F. (2013) “Local and systemic immune responses in pigs intramuscularly injected with an inactivated *Mycoplasma hyopneumoniae* vaccine.” *Vaccine.* **31**: 1305-1311.

Pointon AM, Davies PR, Bahnson PB. (1999) “Disease Surveillance at slaughter”. In: Straw B, D’ Allaire S, Mengeline W, Taylor D, eds. Diseases of Swine. Ames, Iowa: Iowa State University Press, pages: 1111-1132.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/

Ramjeet, M., Deslandes, V., Goure, J. and Jacques, M. (2008) “*Actinobacillus plueropneumoniae* vaccines: from bacterins to new insights into vaccination strategies”. *Animal Health Research Reviews.* **9(1):** Pages 25-45.

Rezania, S., Amirmozaffari, N. Tabarraei, B., Jeddi-Tehrani, M., Zarei, O., Alizadeh, R., Masjedian, F. Zarnani, A.H. (2011). “Extraction, Purification and Characterization of Lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*.” *Avicenna J. Med. Biotech.* **3 (1):** 3-9

Shao, M., Wang, Y., Wang, C., Yang, G., Peng, Y. Liu, J., Li, G., Liu, H. and Liu, S. (2010) “Evaluation of multicomponent recombinant vaccines against *Actinobacillus pleuropneumoniae* in mice” *Acta Veterinaria Scandinavica* 2010, **52**:52 doi:10.1186/1751-0147-52-52

Tremblay, Y.D.N., Deslandes, V. and Jacques, M. (2013). “*Actinobacillus pleuropneumoniae* genes expression in biofilms cultured under static conditions and in a drip-flow apparatus”. *BMC Genomics* **14:**364 -378

Appendices

Appendix 1

The Average OD Obtained for Sera collected from Farm HD 130521 CFPZ – APP Free – Source S12 (F0) tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	Average OD V5 LPS		Swinecheck Ratio (S5)	Average OD S7 LPS		ID Screen Ratio (S7)
		IgA	IgG		IgA	IgG	
	126	0.014	1.050	0.159	0.006	0.616	0.035
	127	0.01	0.529	0.082	0.002	0.116	0.083
	128	0.003	0.607	0.165	0.001	0.063	-
	129	0.234	1.068	0.112	0.003	0.495	0.185
	130	0.013	0.518	0.097	0.000	0.233	0.012
	131	0.016	0.524	0.036	0.001	0.031	0.022
	132	0.350	0.164	0.049	0.075	0.02	0.425**
CFPZ	132	0.018	0.492	0.121	0.008	0.259	0.296**
	133	0.000	0.525	0.080	0.000	0.055	-
HD	134	0.026	0.696	0.076	0.004	0.076	0.034
130521	135	0.012	0.716	0.117	0.003	0.047	-
	136	0.057	1.410	0.170	0.011	0.879	0.091
APP Free	137	0.011	0.518	0.051	0.003	0.122	0.092
F0	138	0.006	0.278	0.09	0.000	0.033	0.017
	139	0.030	0.511	0.072	0.000	0.080	-
Source	140	0.011	0.668	0.087	0.000	0.093	0.033
S 12	141	0.006	0.636	0.082	0.000	0.158	0.015
	142	0.02	0.637	0.072	0.004	0.253	0.014
	143	0.047	0.684	0.126	0.004	0.097	0.018
	144	0.013	0.318	0.087	0.024	0.017	0.023
	146	0.01	0.446	0.112	0.009	0.065	0.049
	147	0.002	0.240	0.116	0.000	0.034	-
	148	0.009	0.665	0.095	0.000	0.050	0.039
	149	0.009	0.580	0.074	0.000	0.069	-
	150	0.002	0.628	0.121	0.000	0.102	0.040

* Positive   ** Suspicious   - Only matched BAL and Sera sets assayed

Appendix 2

The Average OD Obtained for Sera collected from Farm HD 130419 CBW – Source S15 (F7) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	Average OD V5 LPS		Swinecheck Ratio (S5)	Average OD S7 LPS		ID Screen Ratio (S7)
		IgA	IgG		IgA	IgG	
	51	0.055	1.072	0.112	0.124	1.590	0.97*
	52	0.037	0.853	0.120	0.050	0.728	0.660*
	53	0.060	1.231	0.106	0.055	1.361	0.895*
	54	0.027	0.910	0.143	0.057	0.570	0.557*
	55	0.315	1.596	0.086	0.022	1.538	1.003*
	56	0.221	1.608	0.149	0.166	1.622	0.965*
CBW	57	0.130	1.710	0.121	0.027	0.969	0.432*
	58	0.084	2.059	0.106	0.027	0.634	0.932*
HD	59	0.154	1.584	0.163	0.215	1.203	0.973*
130419	60	0.113	1.550	0.048	0.073	0.396	0.207
	61	0.029	1.521	0.075	0.000	0.179	0.107
APP Infect	62	0.044	1.439	0.118	0.019	0.173	0.277**
S7	63	0.080	1.569	0.086	0.007	0.613	0.148
	64	0.090	0.957	0.125	0.000	0.361	0.096
Source	65	0.053	1.454	0.183	0.007	0.598	0.220
S 15	66	0.079	0.898	0.081	0.017	0.294	0.330**
	67	0.056	0.786	0.058	0.037	0.918	0.818*
	68	0.049	0.680	0.172	0.181	1.504	1.037*
	69	0.067	0.400	0.118	0.000	0.062	0.108
	70	0.077	0.590	0.114	0.000	0.047	0.125
	71	0.090	0.748	0.09	0.043	0.529	0.932*
	72	0.072	0.910	0.057	0.000	0.078	0.080
	73	0.332	0.675	0.117	0.026	0.208	0.938*
	74	0.057	0.308	0.098	0.016	1.222	0.897*
	75	0.041	0.535	0.091	0.060	0.287	0.297**
	76	0.079	1.162	0.032	0.014	0.388	0.908*
	77	0.084	1.103	0.09	0.016	0.373	0.371**
	78	0.029	0.844	0.128	0.060	0.755	0.669*
	79	0.067	1.438	0.146	0.014	1.025	0.959*
	80	0.127	1.493	0.087	0.016	1.198	-

Appendix 3

The Average OD Obtained for Sera collected from Farm HD 130424 CCI - Source S15 (F7) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	Average OD V5 LPS		Swinecheck Ratio (S5)	Average OD S7 LPS		ID Screen Ratio (S7)
		IgA	IgG		IgA	IgG	
	81	0.051	0.702	0.128	0.224	1.473	1.026*
	82	0.358	1.233	0.087	0.563	1.711	0.979*
	83	0.203	0.708	0.095	0.088	1.415	1.012*
	84	0.053	1.184	0.152	0.081	1.057	0.960*
	85	0.156	1.036	0.076	0.118	0.582	0.746*
	86	0.105	0.415	0.099	0.081	0.935	0.928*
CCI	87	0.058	0.637	0.133	0.054	1.396	0.877*
	88	0.033	0.888	0.084	0.025	0.700	0.910*
HD	89	0.100	0.899	0.114	0.064	1.298	0.830*
130424	90	0.059	0.878	0.066	0.018	0.837	0.816*
	91	0.021	0.456	0.093	0.029	0.985	0.991*
APP Infect	92	0.168	1.195	0.086	0.051	1.528	0.976*
S 7	93	0.094	1.243	0.125	0.321	2.040	0.972*
	94	0.039	0.591	0.078	0.027	0.851	0.860*
Source	95	0.202	0.878	0.101	0.295	0.687	0.562*
S 15	96	0.035	0.403	0.058	0.118	1.165	0.939*
	97	0.108	0.349	0.102	0.416	0.728	0.933*
	98	0.337	0.644	0.107	0.384	1.021	0.993*
	99	0.067	0.759	0.091	0.086	0.990	0.981*
	100	0.030	0.664	0.095	0.014	0.399	0.753*

* Positive ** Suspicious - Only matched BAL and Sera sets assayed

Appendix 4

The Average OD Obtained for Sera collected from Farm HD 130408 SHEA (F5) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	Average OD V5 LPS		Swinecheck Ratio (S5)	Average OD S7 LPS		ID Screen Ratio (S7)
		IgA	IgG		IgA	IgG	
	17	-	-	1.302*	-	-	0.299**
	18	0.026	0.584	0.409**	0.001	0.092	0.067
	19	0.028	0.352	0.500**	0.000	0.101	0.069
SHEA	20	0.506	1.214	1.447*	0.000	0.710	0.397**
	21	0.788	1.496	1.141*	0.002	0.610	0.134
HD	22	0.065	0.839	1.065*	0.007	0.529	0.065
130408	23	0.376	0.959	1.269*	0.000	0.308	0.215
	24	0.045	0.652	0.185	0.009	0.134	0.029
APP Vac.	25	0.067	1.475	1.385*	0.010	0.109	0.282**
S 5 (V5)	26	0.617	1.735	1.268*	0.007	1.219	0.071
	27	0.416	2.132	1.311*	0.004	1.174	0.406*
Vaccinated	28	0.057	1.953	0.838*	0.004	0.275	0.100
	29	-	-	1.132*	-	-	0.244
Wk 7	30	0.017	0.868	0.231	0.004	0.275	0.037
Wk 10	31	-	-	0.382**	-	-	0.029
Wk 14	32	-	-	1.32*	-	-	0.227
	33	0.366	1.611	1.428*	0.009	0.406	0.244
Sampled	34	0.059	1.675	0.212	0.005	0.110	0.018
Wk 22	35	-	-	0.204	-	-	0.025
	36	-	-	0.221	-	-	0.036
	37	0.145	1.064	1.300*	0.01	0.293	0.068
	38	0.010	1.092	0.323	0.006	0.499	0.058
	39	0.968	1.569	1.195*	0.003	0.458	0.069
	40	0.185	1.887	1.275*	0.006	0.685	0.244

* Positive ** Suspicious - Only matched BAL and Sera sets assayed

Appendix 5

The Average OD Obtained for BAL from Farm HD 130521 CFPZ – APP Free – Source S12 (F0) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	BAL Average OD V5 LPS		Swinecheck Ratio (S5)	BAL Average OD S7 LPS		Sera ID Screen Ratio (S7)	EP	PL
		IgA	IgG		IgA	IgG			
	126	0.152	0.155	0.050	0.023	0.014	0.035	0	
	127	0.021	0.100	0.065	0.003	0.004	0.083	0	
	128	0.081	0.084	-	0.000	0.038	-	0	
	129	0.208	0.112	0.083	0.007	0.023	0.185	0	
	130	0.079	0.075	0.076	0.006	0.013	0.012	0	
	131	0.217	0.177	0.063	0.013	0.016	0.022	0	
	132	0.350	0.164	0.049	0.075	0.02	0.425**	0	
CFPZ	132						0.296**(R)	0	
	133	0.023	0.151	0.067	0.002	0.006	-	0	
HD	134	0.162	0.097	-	0.020	0.008	0.034	0	
130521	135	0.163	0.167	0.056	0.018	0.002	-	0	
	136	0.171	0.069	-	0.022	0.004	0.091	0	
APP	137	0.330	0.152	-	0.039	0.012	0.092	0	
Free	138	0.258	0.109	0.078	0.054	0.006	0.017	0	
F0	140	0.078	0.052	0.039	0.006	0.006	0.033	0	
	141	0.349	0.200	0.053	0.055	0.014	0.015	0	
Source	142	0.221	0.130	-	0.232	0.041	0.014	0	
S12	143	0.059	0.254	-	0.004	0.016	0.018	0	
	145	0.075	0.059	0.043	0.013	0.000		0	
	146	0.202	0.21	-	0.029	0.009	0.049	0	
	148	0.439	0.128	0.056	0.070	0.006	0.039	0	
	149	0.065	0.021	-	0.006	0.003	-	0	
	150	0.062	0.178	0.034	0.017	0.038	0.040	0	

* Positive   ** Suspicious   - Only matched BAL and Sera sets assayed   (R) Repeated

Appendix 6

The Average OD Obtained for BAL collected from Farm HD 130419 CBW – Source S15 (F7) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen

Farm ID (APP)	Pig ID	BAL Average OD V5 LPS		Swinecheck Ratio (S5) (BAL)	BAL Average OD S7 LPS		Sera ID Screen Ratio (S7)	EP	PL
		IgA	IgG		IgA	IgG			
	51	0.071	0.031	0.039	0.104	0.013	0.970*	0	
	52	0.004	0.006	-	0.007	0.003	0.660*	0	
	54	0.192	0.071	0.069	0.093	0.014	0.557*	0	
	55	0.349	0.059	0.040	0.019	0.005	1.003*	0	
	57	0.410	0.070	-	0.047	0.01	0.432*	0	
	58	0.711	0.176	-	0.080	0.006	0.932*	0	
CBW	60	0.357	0.061	0.063	0.107	0.003	0.207	2	
	61	0.182	0.055	-	0.018	0.003	0.107	0	
HD	62	0.075	0.039	-	0.043	0.016	0.277**	0	
130419	63	0.300	0.100	0.054	0.064	0.066	0.148	0	
	64	0.044	0.014	0.056	0.006	0.062	0.096	1	
APP	66	0.275	0.166	0.056	0.125	0.097	0.330**	0	
S7	67	0.215	0.081	0.059	0.023	0.108	0.818*	2	
	68	0.225	0.158	-	0.052	0.140	1.037*	0	
Source	69	0.084	0.121	-	0.009	0.038	0.108	0	
S 15	70	0.000	0.000		0.000	0.08	0.125	0	
	71	0.097	0.052	0.038	0.036	0.009	0.932*	0	
	72	0.204	0.057	0.034	0.018	0.003	0.080	0	
	73						0.938*	0	P2
	74	0.178	0.332	0.050	0.029	0.431	0.897*	0	
	76	0.081	0.042	-	0.031	0.002	0.908*	0	
	79	0.197	0.113	0.050	0.054	0.013	0.959*	0	
	80	0.230	0.093	-	0.019	0.009	-	0	

Appendix 7

The Average OD Obtained for BAL collected from Farm HD 130424 CCI – Source S15 (F7) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen

Farm ID (APP)	Pig ID	BAL Average OD V5 LPS		Swinecheck Ratio (S5) (BAL)	BAL Average OD S7 LPS		Sera ID Screen Ratio (S7)	EP	PL
		IgA	IgG		IgA	IgG			
	81	0.116	0.035	-	0.073	0.031	1.026*	0	
CCI	82	0.229	0.057	0.060	0.101	0.020	0.979*	0	P1
	84	0.452	0.130	-	0.181	0.032	0.960*	0	P3
HD	85	0.078	0.044	-	0.053	0.009	0.746*	0	
130424	86	0.034	0.029	0.051	0.056	0.008	0.928*	0	
	87	0.013	0.158	0.059	0.025	0.127	0.877*	0	
APP Infect	88	0.035	0.050	0.072	0.016	0.004	0.910*	0	
S 7	89	0.127	0.065	-	0.068	0.008	0.830*	0	P2
	90	0.111	0.034	0.052	0.007	0.008	0.816*	0	
Source	91	0.020	0.04	-	0.008	0.008	0.991*	0	
S 15	92	0.202	0.045	0.043	0.029	0.013	0.976*	0	
	93	0.176	0.052	0.035	0.087	0.031	0.972*	0	P2
	95	0.552	0.111	0.090	0.339	0.067	0.562*	0	P3
	96	0.061	0.178	0.087	0.044	0.112	0.939*	0	
	98	0.493	0.076	0.046	0.126	0.009	0.993*	0	
	99	0.071	0.020		0.010	0.000	0.981*	0	
	100	0.043	0.017		0.007	0.005	0.753*	0	P2

* Positive ** Suspicious - Only matched BAL and Sera sets assayed

Appendix 8

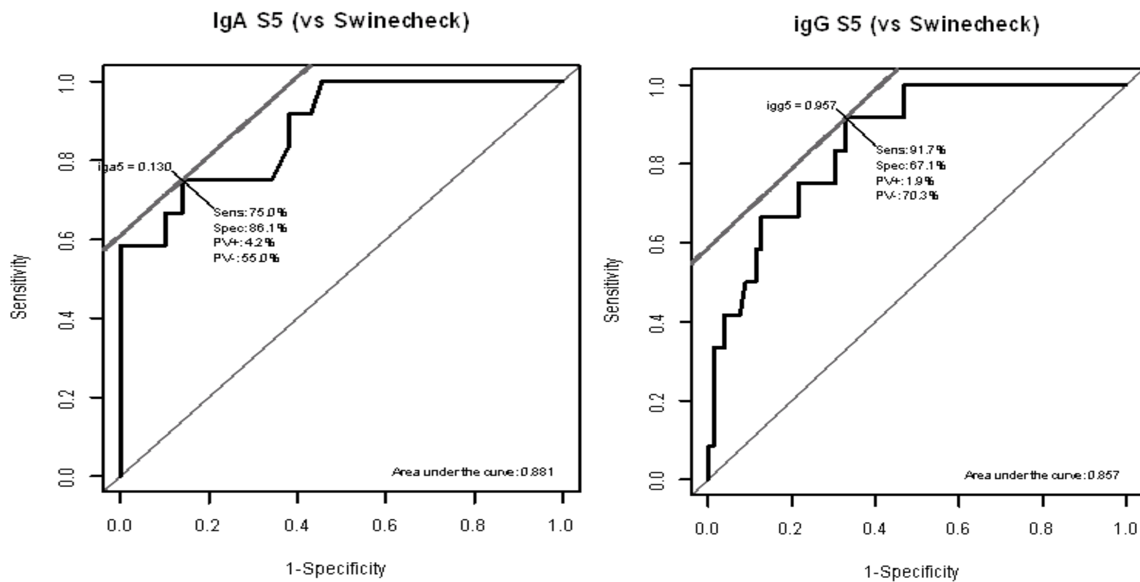
The Average OD Obtained for BAL collected from Farm HD 130408 SHEA (F5) Tested with V5 and S7 LPS ELISA's and Ratio's for Swine Check and ID Screen.

Farm ID (APP)	Pig ID	BAL Average OD V5 LPS		Swinecheck Ratio (S5) (BAL)	BAL Average OD S7 LPS		ID Screen Ratio (S7)(Sera)	EP	PL
		IgA	IgG		IgA	IgG			
	17							0	P3
	18	0.011	0.011	0.048	0.033	0.007	0.067	0	
	19	0.071	0.162	-	0.014	0.032	0.069	1	
SHEA	20	0.315	0.348	0.589*	0.017	0.044	0.397**	4	P3
	21	0.074	0.039	-	0.002	0.000	0.134	0	
HD	22	0.107	0.051	0.085	0.020	0.001	0.065	7	
130408	23	0.065	0.033	0.219	0.001	0.003	0.215	0	P3
	24	0.141	0.066	-	0.037	0.006	0.029	1	
APP Vac.	25	0.196	0.358	-	0.026	0.123	0.282**	5	
S 5 (V5)	26	0.536	0.239	-	0.013	0.045	0.071	0	
	27	0.136	0.345	-	0.016	0.053	0.406*	4	P3
Vaccinated	28	0.014	0.042	0.066	0.000	0.001	0.100	3	
	29							2	
Wk 7	30	0.139	0.186	-	0.027	0.020	0.037	0	P3
Wk 10	33	0.291	1.610	-	0.016	0.436	0.250**	0	
Wk 14	34	0.106	0.059	0.125	0.002	0.002	0.018	1	
	37	0.081	0.086	-	0.007	0.010	0.068	0	
Sampled	38	0.030	0.027	-	0.000	0.000	0.058	0	P3
Wk 22	39	0.062	0.165	-	0.007	0.025	0.069	4	
	40	0.155	0.414	0.356**	0.012	0.024	0.250**	4	P3

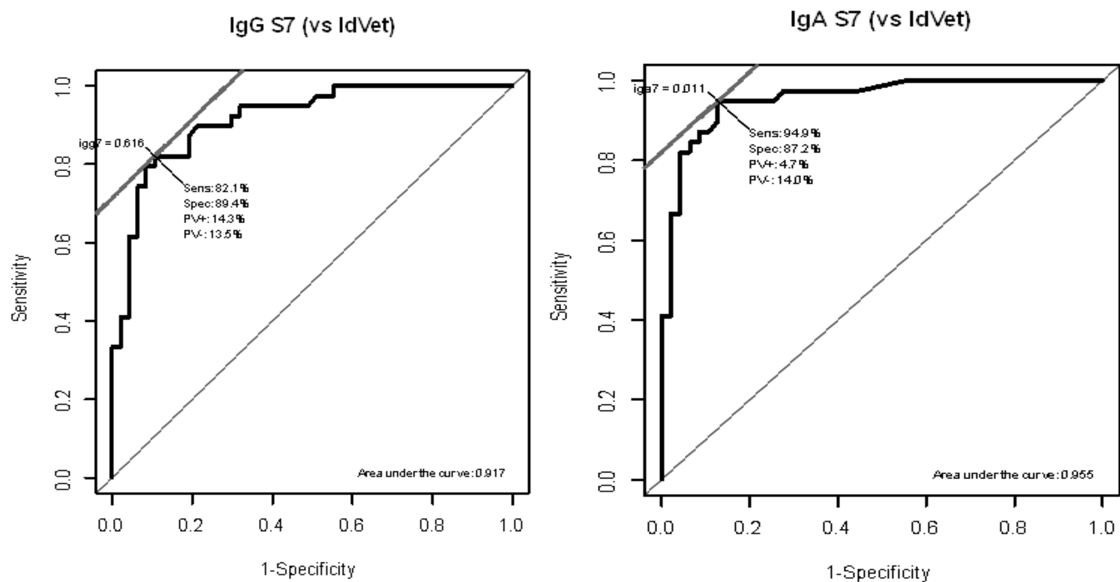
* Positive ** Suspicious - Only matched BAL and Sera sets assayed

Appendix 9

ROC Curves Calculated for Sera IgA V5 and IgG V5 EMAI ELISA using Swine Check (5a & 5b) as a Gold Standard to Classify each Pigs Serovar.(>0.4 => true positive, <0.4 => true negative).

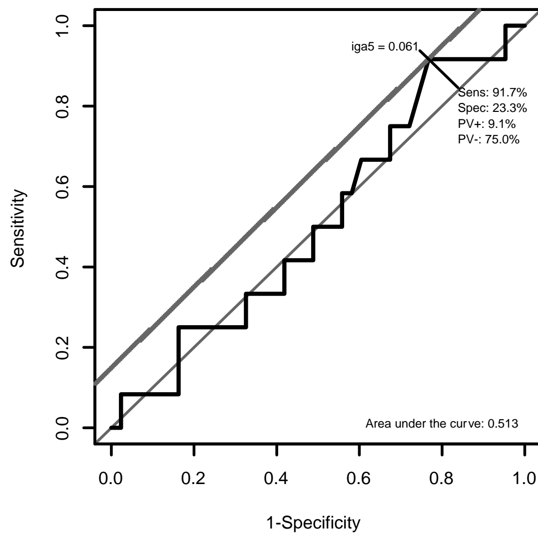


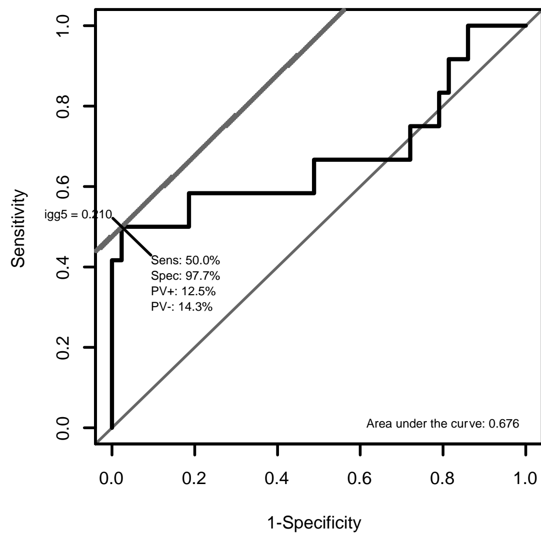
ROC Curves Calculated for Sera IgA S7 and IgG S7 EMAI ELISA using ID Screen 4-7 as a Gold Standard to classify each Pigs Serovar. (>0.4 => true positive, <0.4 => true negative).

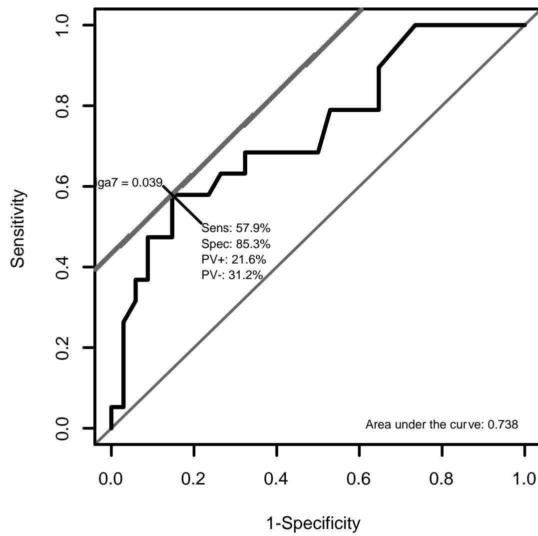


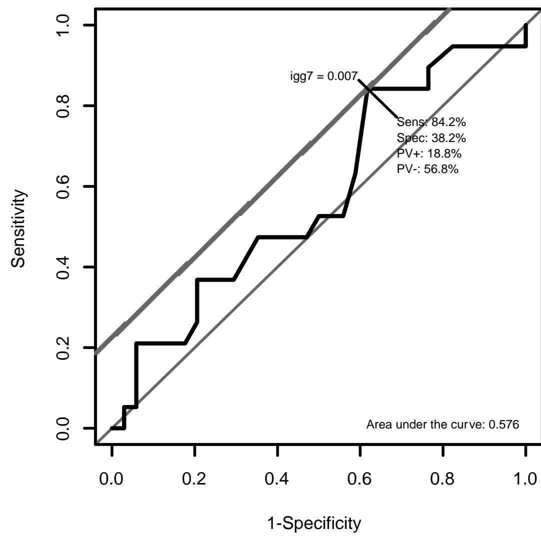
Appendix 10

ROC Curves Calculated on BAL Samples for the IgA and IgG Response in the V5 and S7 EMAI ELISA using Swinecheck and ID Screen as Gold Standards.



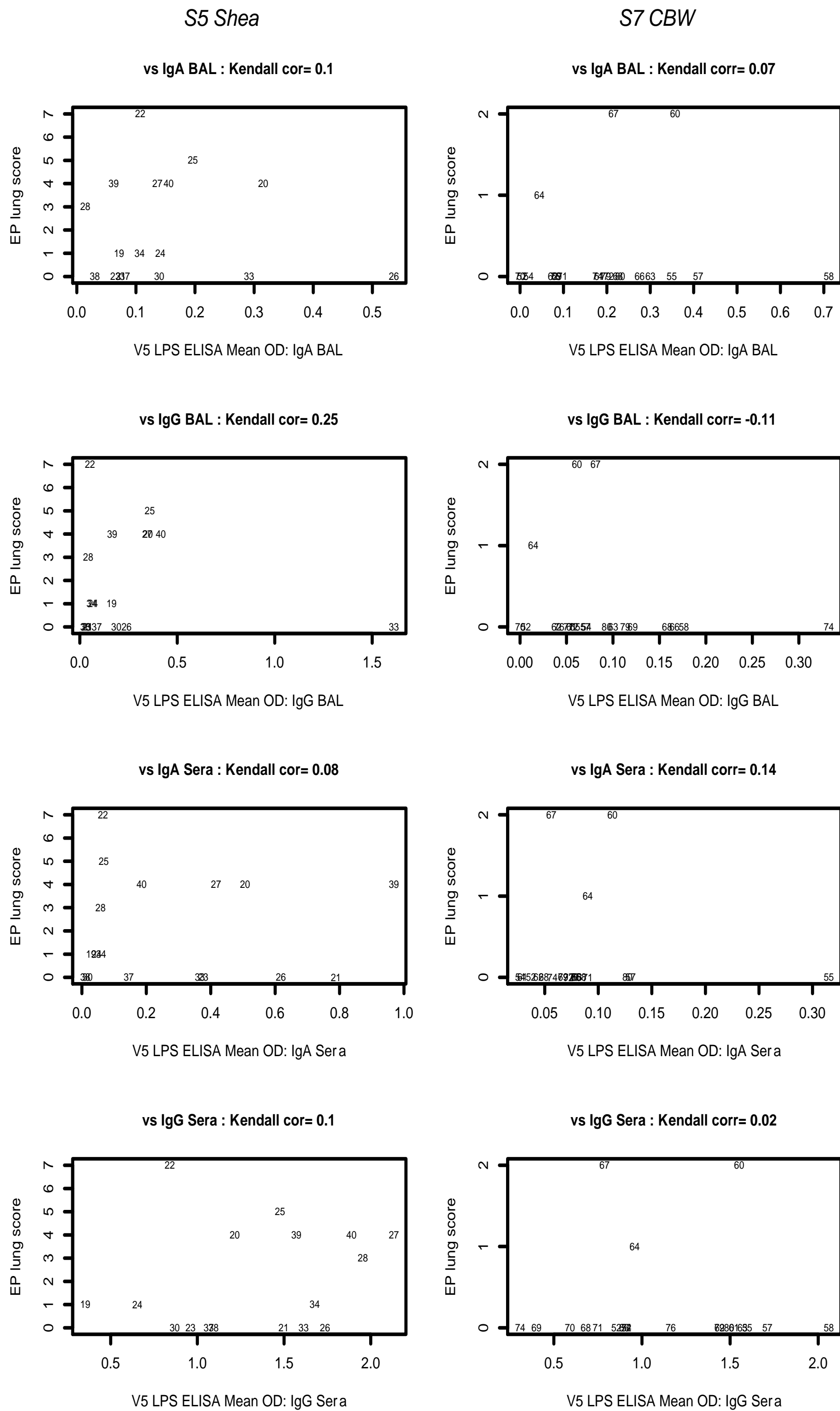






Appendix 11

Kendall Coefficients Calculated for EP Lung score versus Mean OD from the EMAI ELISA's for each Variable of Immunoglobulin class (IgG & IgA), Sample Type (BAL & Sera) and APP Serovar V5 and S7 (Farms F5 Shea and F7 CBW).



Appendix 12

Kendall Coefficients Calculated for Pleurisy Lung Score versus Mean OD from the EMAI ELISA for each Variable of Immunoglobulin class (IgG & IgA), Sample Type (BAL & Sera) and APP Serovar V5 and S7 (Farms F5 Shea and F7 CCI)