Controlled exposure as a management tool for Glässer's disease

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Executive Summary

The Problem

Glässer's disease is still a major problem in the pork industry. Key impacts of this disease are:

- Mortalities as high as 1-2%, despite the use of antibiotics. In some cases, mortality rates as high as 5.4% for post weaning and as high as 11% for pre-weaning have been reported
- Retardation in growth

The current killed vaccines, besides being expensive, only give protection if all the strains present on the farm are included in the vaccine.

The Project

The objective of this project was to reduce production costs and improve herd feed conversion efficiency by offering a solution to farmers for the control of Glässer's disease. The solution is controlled exposure of piglets to a live strain of *Haemophilus parasuis* while they are still under the protection of maternal antibodies. Controlled exposure will give the piglets protection against the serovars on the farm but will also give cross-protection to other serovars. Therefore, if another serovar is introduced onto the farm, the results will not be catastrophic.

Field trials on four farms have confirmed that the approach is safe, even when pathogenic isolates are used. However, the field trials have not been able to demonstrate the efficacy of controlled exposure. This has occurred because of the difficulty of demonstrating statistical validity for a disease that often is variable in expression.

Achievements

Even though the field trials only gave indications that the method has efficacy, the knowledge gained during the trials is currently helping pig farmers.

Major advances are a suite of diagnostic and support tools for pig veterinarians dealing with Glässer's disease.

1. A new improved PCR assay has been developed. This Real Time PCR is much more sensitive and specific than the existing conventional PCRs. The method has been validated for direct application to systemic sites and lungs and is available for use.
2. A serovar profiling service has been developed and is now available. This involves the submission of nasal swabs from pigs at weaning and also from diseased pigs. If *H. parasuis* is a problem on the farm, isolates are obtained and the serovars present on the farm are determined. A recommendation on which of the serovars should be included into an autogenous vaccine is provided. This service has now been provided for 23 farms successfully.
3. A *H. parasuis* genotyping service has been developed and is now available. This service can be used to understand on farm epidemiology (is one strain present on multiple farms? is an outbreak associated with a novel strain or the re-appearance of previously known strain?). This genotyping (and an expanding data-base of genotypes) will greatly improve our understanding and hence our ability to control outbreaks.

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1. Introduction

Glässer's disease is still a major problem in the pork industry. The latest figures from Auspork showed that, despite the use of antibiotics in preventative programs (~$0.90 per pig), the level of mortalities can still be as high as 1-2% with a cost of $0.50 to $1 per pig. The retardation in growth costs about $1 to $2 per pig, bringing the total of costs to $2.40 to $3.90 per pig. Mortality rates as high as 5.4% for post weaning, and as high as 11% for pre-weaning, have been reported from some Australian farms. Autogenous vaccines, besides being expensive, only give protection if all the strains present on the farm are included in the vaccine (Oliveira and Pijoan 2004). This project had the objective to reduce production costs and improving herd feed conversion efficiency by offering a solution for farmers to the control of Glässer's disease. The solution is controlled exposure of piglets while they are still under the protection of maternal antibodies. Controlled exposure will give the piglets protection against the serovars on the farm but will also give cross-protection to other serovars. Therefore, if another serovar is introduced onto the farm the results will not be catastrophic. This would mean that the cost of antibiotics is not necessary anymore, and mortalities plus growth retardation due to Glässer's would be avoided. The heavy use of antibiotics has a limited future. This alternative method does not rely on antibiotic use and therefore might lead to an increased demand for high-quality, niche Australian pork products. While commercial and autogenous vaccines, all of which are currently inactivated, normally induce satisfactory protection, there are key limits and constraints. Both vaccines will only give protection if all prevalent serovars in the herd are used in the vaccine and, if the vaccine is given at the right time (Oliveira and Pijoan 2004). Problems with both commercial and autogenous vaccines might arise if the prevalent serovar of the herd changes over time.

In the light of these reported failures and problems with commercial and autogenous vaccines and the cross-protection associated with live organisms, an alternative to vaccination has been proposed. Oliveira *et al*. (2001; 2004) have successfully experimented with controlled exposure of 5-day-old piglets to low doses of the live strains present in the herd. This alternative approach resulted in piglets inoculated with the herd's systemic strains having less morbidity and mortality than piglets colonised by nose-to-nose contact with inoculated sows. It should be noted that this approach has been used in a multi-valent manner, where the controlled exposure was to all genotypically different strains found in the herd. We propose to establish the controlled exposure model developed by Oliveira *et al*. (2001; 2004) for Australian herds and take this concept further by looking at cross protection.

The PCR methodology, commonly used for the detection of *H. parasuis*, cannot detect *H. parasuis* if there are less than 10^2 bacteria in the sample (Oliveira *et al*. 2001). This relative lack of sensitivity is a problem for the moderately virulent serovars of *H. parasuis*. The alternative method is the culture method. However, this method has its limitations as it depends on live bacteria. *H. parasuis* is not a very vigorous bacterium and is very susceptible to higher temperatures. Hence, if the samples can not be obtained from the pig directly after death and, if the samples can not be transported to the laboratory immediately, culturing will almost certainly fail due to the lowered viability of *H. parasuis* and the more vigorous growth of contaminants. Hence, in most scenarios, PCR methods have distinct advantages over culture. The sole drawback to PCR is the need for at least 100 cells. Therefore, we have developed a more sensitive PCR – a Real Time

PCR assay. This PCR was designed and initially validated on pure cultures. The PCR was validated on samples previously collected from pigs challenged with *H. parasuis*, which had corresponding culture results.

Work on this project extended beyond the original aims of the project, as other infected farms had to be identified and brought into the project. This required knowledge of the *H. parasuis* status on the farms. To gain this knowledge a serovar profiling method for farms had to be established. This tool could then be used to establish the serovars present on the farms and serovar profile each farm.

This report will describe all experiments conducted on the four farms involved and also report the serovar profiling method and the Real Time PCR. Detailed reports for farm experiments, the serovar profiling method and the Real Time PCR are in the appendices. The general report provides an overview of the project, methodology common to all experiments, summary of results for all experiments and newly developed methods and also provides an overview of the overall project outcomes and applications.

2. Methodology

Preliminary sampling to establish serovars present on farm

Nasal swabs were either taken from pigs from multiparous sows at weaning time or from sick pigs displaying symptoms typically seen in Glässer's disease (e.g. coughing, anorexia). The sample sizes varied as it depended on the number of multiparous sows available on the day and on the number of sick pigs that had signs that were possibly associated with Glässer's disease. Necropsies were performed on some farms at the same time as sampling was done. The pigs necropsied were extremely sick pigs displaying symptoms typically seen in Glässer's disease. Swabs of tissue were taken and brought to the laboratory. All swabs were then processed as detailed below. Sick animals for sampling were selected on the basis of showing clinical signs and there was no attempt made to definitely establish the cause of the clinical signs.

Swabs were placed in Amies transport media and kept on ice until inoculated on to BA/SN agar, prepared as previously described (Turni and Blackall, 2007) and on to blood agar; the latter being cross-streaked with a nurse colony of *Staphylococcus hyicus*. The plates were then incubated aerobically for 18 - 24 hours at 37ºC. Suspect colonies of *H. parasuis* were selected and sub-cultured on BA/SN for DNA processing, storage, identification and serotyping.

Colonies were prepared for PCR as previously described. Isolates were confirmed as *H. parasuis* by the PCR of Oliveira *et al.* (2001). The ERIC PCR, as described by Oliveira *et al*. (2003), was used to group the strains within one farm according to genotype profile. The genotype profile was compared only within each farm and the interpretation performed according to Oliveira *et al*. (2003). If two isolates had the same genomic fingerprint, i.e. an identical band pattern including location and intensity, they were assumed to be the same strain. For multiple sampling on a farm the known genotypes were run as controls in the ERIC PCR. If only small numbers of *H. parasuis* were being compared and the differences were quite obvious, then gels were analysed by eye. Otherwise the Bionumercis software (Bionumeric version 4.50, Applied Maths Inc, Sint-Martens-Latem, Belgium) was used to analyse the gels. Within each genotype detected on a farm, representative isolates were serotyped by gel diffusion (GD) testing (Turni and Blackall, 2005). If a clear cut answer could not be obtained, the isolates were

then examined by the indirect haemagglutination (IHA) test (Turni and Blackall, 2005). Isolates which did not react in any of the tests were regarded as non-typable and were termed serovar NT.

The pathogenicity of the serovars was allocated according to the classification of Kielstein and Rapp-Gabrielson (1992) with serovars 1, 5, 12, 13 and 14 being highly pathogenic, serovars 2, 4 and 15 being moderately pathogenic, serovar 8 being slightly pathogenic and serovars 3, 6, 7, 9 and 11 being non-pathogenic.

Farm 1

The serovar 4 *H. parasuis* strain from farm 1 was revived from -70°C storage three days prior to preparation onto BA/SN agar. It was sub-cultured twice on chocolate agar plates, that consisted of BBL™ Blood Agar Base (Becton Dickinson, Sparks, MD USA), 5 % defibrinated sheep blood (Bio-Lab, Melbourne VIC) and 0.0025% reduced nicotinamide adenine dinucleotide (NADH) (Roche Diagnostics, Mannheim Germany), with 18 hours incubation at 37°C. *H. parasuis* was harvested into 2 ml of phosphate buffered saline and then diluted to a concentration of 1 x 10^4 colony forming units (cfu)/ml. A sample of the prepared inoculum, as well as samples of the left over inoculum, was plated onto chocolate agar plates and a count performed after one day incubation. The inoculum was kept on ice until being used for inoculation three hours after preparation. Survival counts showed that the prepared inoculum was at the same concentration for this period if kept on ice. A 0.5 ml dose of the inoculum was sprayed into each nostril of the pigs, with the total dose per pig being 1.0 ml.

Farm 2

The *H. parasuis* strain from the farm was confirmed as serovar 6 in the GD test but was a serovar NT in the indirect IHA. Three days prior to the controlled exposure day, the strain was revived from -70°C storage three by inoculation onto BA/SN agar. It was sub-cultured onto chocolate agar plates (prepared as above) twice and was harvested after 18 hours incubation at 37°C (as described above). The *H. parasuis* was adjusted to a concentration of 7 x 10^5 cfu/ml. All other procedures for sample handling, controlled exposure and viable counting were as described above. The preparation was used within four hours. Survival counts showed that the prepared inoculum was at the same concentration for this period if kept on ice.

Farm 4

Three days before the controlled exposure day, the serovar 9 *H. parasuis* strain from the farm was revived from -70°C storage by inoculation onto BA/SN agar. It was sub-cultured on chocolate agar plates (prepared as above) twice before being harvested after 18 hours incubation at 37°C. The *H. parasuis* was harvested as above and adjusted to a concentration of 7 x 10^5 cfu/ml. All other procedures for sample handling, controlled exposure and viable counting were as described above. The preparation was used within three and a half hours. Survival counts showed that the prepared inoculum was at the same concentration for this period if kept on ice.

Experimental Set-up

Sows were inoculated with the strain of *H. parasuis* used for controlled challenge three and five weeks before farrowing. Before farrowing the sows were split into two groups (control and treatment group), which were separated by space in the farrowing sheds. Five to seven days after birth, the piglets of the treatment group

were challenged with a strain from the farm (see above), which had been used to vaccinate the sows.

A day before or on the day of weaning a subset of piglets from each group were nasal-swabbed. The effectiveness of the controlled exposure was evaluated by number of pigs displaying clinical signs possibly related to *H. parasuis* infection, number of antibiotic treatments and pig mortality. Nasal swabs of sick pigs were taken twice a week to find a correlation to *H. parasuis* infection. A sub-sample of pigs was weighed after leaving the weaning sheds.

Real time PCR

The specificity of a real time PCR amplifying the *inf*B gene was evaluated with 68 *H. parasuis* isolates and 36 strains of closely related species. As well, 239 samples of DNA from tissues and fluids of 16 experimentally challenged animals were tested with the real time PCR, and the results compared with culture and a conventional PCR. For more details see Appendix 6.

3. Outcomes

Safety of the controlled exposure method

The safety of the controlled exposure was tested with a moderately pathogenic strain on farm 1 and with non-pathogenic strains on farms 2 and 4. None of the controlled challenge pigs showed any signs of illness due to Glässer's disease. In the dosage trial performed on farm 4, piglets aged 6 days were given dosages as high as 10^9 cfu/ml and these piglets showed no sign of disease.

Farm 1

Controlled exposure

This farm has a serovar 4 disease causing strain, which was used as the controlled challenge strain and also in the vaccine used for the sows. None of the pigs, from the control group or the treatment group (controlled challenged pigs) displayed any signs of Glässer's disease during the experiment; which lasted 66 days post controlled challenge. As the sows from both groups were vaccinated with the serovar 4 strain, it is not surprising that none of the pigs displayed any signs of Glässer's disease. The farm also had a non-typable (NT) strain of *H. parasuis* that was spreading rapidly among the pigs. This strain seemed non-pathogenic as no pigs in either group, as well as, any of the pigs in the two rooms where the two groups were kept, showed any sign of Glässer's disease. The serovar 4 was present in some of the pigs that were not controlled exposed (6/23 animals from the control group) at 72 days of age. It might have been present before, but not detected due to the presence of the NT strain of *H. parasuis*.

From both the treatment and control group the non-typable strain could only be found in the nasal cavity when the pigs had entered the weaning rooms. This, and the fact that only one high parity sow out of ten transmitted the non-typable strain to her off-spring (preliminary sampling to establish serovars on farm) would suggest that the strain does not have a very high prevalence on the farm. This imbalance in the spread of different strains has not been observed previously and might be an important issue in the epidemiology of the disease. The farm had been experiencing problems with *H. parasuis* despite vaccination. However, no Glässer's disease problems were seen in these pigs. The rapid spread of the NT strain could have been linked with the absence of clinical Glässer's disease.

Farm 2

Controlled exposure trials

On this farm the controlled exposure trial was performed twice. In the first experiment the control group consisted of 193 animals, while the treatment group consisted of 207 animals. In the second trial the numbers were 199 and 217, respectively. In the first experiment more animals from the treatment group were treated with antibiotics than from the control group (26/15). In the second experiment more of the control group had to be treated (10/ 16). No post-weaning deaths were recorded in the experiments.

In the preliminary serovar profiling work on this farm, 12% of the samples did not yield *H. parasuis*. This suggests that 12% were possibly naïve and susceptible to Glässer's disease; a figure which matched the prevalence of the cases of Glässer's disease. Eight serovars were recovered on the farm in this preliminary sampling. When the controlled exposure experiments were performed, the percent of samples not yielding *H. parasuis* decreased (see Table 1) and the number of serovars recovered increased to 14 serovars with 18 genotypes. The number of naïve and susceptible pigs (% not yielding *H. parasuis*) did not match the number of pigs that needed treatment (possible cases of Glässer's disease)

Table 1. Results of the two experiments on Farm 2

Experiment	Group	Group size	% not yielding *H. parasuis* at weaning	% sick pigs after weaning
1	control	179	1.9	8.4
	treatment	186	2.1	14
2	control	176	8.7	9.1
	treatment	193	4.0	5.2

H. parasuis is a problem on most farms about 7 - 14 days after weaning. This farm seems no exception to this. In the preliminary sampling, isolates of serovars 12, 15 and NT were obtained from nasal swabs of sick picks, with the necropsy yielding an isolate of serovar 15. Experience with serovar profiling farms has indicated that if there is a problem with *H. parasuis* on a farm then nasal swabbing sick pigs seems to yield pathogenic strains as is seen in the preliminary sampling of this farm (isolates of serovars 12 and 15 are generally regarded as pathogenic). As the serovar 15 isolate was obtained from a necropsy sample, it is reasonable to assume that this serovar is causing problems on the farm. The interesting observation in both experiments was that sampling sick pigs from the control group yielded isolates of serovar 15, while none of the sick pigs from the treatment group yielded this serovar. Especially in the second trial, it became apparent that the majority of sick pigs in the treatment group did not yield *H. parasuis* after weaning and if then it was the serovar distributed by the sow with the exception of serovar 13 (Table 2). This serovar was recovered from a sick pig at weaning, while all other serovars from sick animals were recovered past weaning.

Table 2. Experiment 2 Serovars recovered from the nasal swabs of the sick pig from control and treatment group and the percent of pigs displaying the same serovar as piglets from the same mother at weaning (some pigs had more than one serovar). Serovar 6 and 9 are regarded as non-pathogenic, while serovar 10, 13 and 15 are regarded as highly pathogenic

Group (Control/treatment)	Serovar	% of sick pigs with this serovar	% same serovar as piglets at weaning from the same sow
C	-	46	
C	6	38	0
C	9/10	8	0
C	10	8	0
C	15	23	0
C	NT	8	0
T	-	70	
T	6	10	0
T	10	10	10
T	13	10	0
T	NT	10	0

- *H. parasuis* not recovered

In the first trial a lot more pigs of the treatment group were treated with antibiotics (all sick pigs received antibiotic treatment, Table 1) than in the control group. The pathogenic serovars in the treatment group were only displayed 24 days past weaning, except of the serovar 5 or 12, which was seen at 11 and 15 days post weaning. The control group, on the other hand, displayed pathogenic serovars much earlier with the majority being recovered on day 11 post weaning, which is the expected period for an *H. parasuis* outbreak to occur. It might be that these pigs at 22 days past weaning had other problems than *H. parasuis*.

Overall, the trials on this farm gave some indication that controlled exposure has the potential to work, but the concept needs to be proven on a farm that has an acute problem with *H. parasuis* and were most of the pigs are not colonized with a strain of *H. parasuis*.

Table 3. Experiment 1 Serovars recovered from the nasal swabs of the sick pig from both groups – treatment and control (T and C). Each row represents the serovar recovered from one pig at one day of sampling. Pathogenic serovars highlighted in gray.

group	serovar	same as sow	days past weaning	group	serovar	same as sow	days past weaning
C	NT	YES	0	T	9	NO	0
C	NT	YES	0	T	5 or 12	NO	11
C	NT	YES	0	T	9	YES	11
C	9	YES	0	T	9	YES	11
C	9	YES	0	T	9	YES	11
C	9	YES	0	T	9	NO	11
C	2/1	NO	4	T	9	YES	15
C	9/13	YES	4	T	5 or 12	NO	15
C	15 + 10	YES/NO	4	T	6	YES	24
C	15 + 10	YES/NO	4	T	6	NO	24

group	serovar	same as sow	days past weaning	group	serovar	same as sow	days past weaning
C	15	YES	4	T	9 + 6	YES/YES	24
C	NT	YES	4	T	9/10	NO	24
C	NT	YES	4	T	9/10	NO	24
C	NT	YES	11	T	10	YES	24
C	NT	YES	11	T	10	NO	24
C	5 or 12	NO	11	T	NT	NO	24
C	9	YES	11				
C	9	YES	11				
C	15 + 9	NO/NO	11				
C	15	NO	11				
C	15	YES	11				
C	15	NO	11				
C	13 + 10	NO/YES	15				
C	15	NO	15				
C	10	NO	24				

Farm 3

Large scale experiments were set up with a big pig company on farms in South Australia. However, the farms pulled out of the trials part way through the first experiment. So we were forced to look for more farms in Queensland. This experiment in South Australia involved the serovar profiling of three farms, establishing a transport method for the inoculum which guaranteed survival of the organism (involved trial runs and survival studies) and preparation of inoculum for four weeks (total of 3000 pigs). It also involved the processing of nasal swabs from the first weaned pigs (58 swabs in total), which were processed and the genotype established. Unfortunately, the farms pulled out of the trials and no data was available to analyse, and no report was written.

Farm 4

Preliminary exposure trial

In this trial the safety of the method was established with 71 pigs in the treatment group and 63 pigs in the control group. None of the piglets developed symptoms of disease after the controlled challenge at 5 days of age.

The problem of *H. parasuis* typically becomes apparent on this farm 10 days after weaning. At this stage many pigs have lost weight and if not given antibiotic at this stage pigs will start to die. This trial was no exception with many pigs at 10 days post-weaning looking anorexic. A sub-sample of the pigs from both groups were classed into good, average and poor condition by the farm manager and nasal swabs taken and cultured for *H. parasuis* (see Table 4).

Table 4. Results of condition classification and nasal swabbing of pigs at 10 days post-weaning

Group (number sampled)	Good condition (%)	Average condition (%)	Poor condition (%)	*H. parasuis* serovar 1/11 recovered (%)	No *H. parasuis* recovered (%)
Control (52)	54	25	21	48	46
Treatment (39)	51	36	13	69	31

As these two groups of pigs were in a large igloo with other non-trial pigs, all pigs had to be treated when pigs were showing signs of ill-health. The strain that causes disease on the farm is very pathogenic and treatment has to be given early otherwise pigs do not recover. Despite being given a conventional killed vaccine and antibiotics in the feed, the non-trial pigs were also falling back and needed treatment on day 11 (with this treatment being water medication for the entire igloo).

The aim of this experiment was to establish that the number of sick pigs and the mortality rate in the control group would not exceed the death rate observed previously on farm. It was also meant to establish the safety of the controlled challenge method.

None of the pigs died in the experiment. Four pigs were taken out into the nursery to avoid the treatment of antibiotics which had to be given to all pigs in the igloo via the water. These pigs did get the antibiotic in the feed. Therefore, the number of sick pigs taken out was only slightly higher due to our artificial intervention (6.3% compared to 3.5%). In summary, the experiment established that the death rate and the number of sick pigs would not markedly increase in pigs that are not given the normal vaccine used for the farm and the antibiotics used at weaning. As a result, the management of the farm agreed to allow further experiments on the farm.

In this experiment more pigs of the treatment group than the control group had *H. parasuis* serovar 1/11 in their nasal cavity. The igloo used in this trial was split into two pens, one being for the male and the other for the female pigs. The treatment group was together with the male pigs, while the control group was together with the female pigs. This could have made a difference in the spread of the disease and hence the detection of the organism in the nasal cavity. The presence of the organism in their nasal cavity does not mean their immune system is not mounting an active response. Rather, nasal colonization means that infection has occurred and it might even invade their lungs, but might not necessarily cause sever disease symptoms.

While both groups had nearly the same percentage of pigs classified as “good condition”, the treatment group had fewer pigs in the “poor” group (13% compared to 21% of controls) and thus more pigs in the “average” group (36% compared to 25% of controls).

One observation that suggests that the controlled challenge dose is too low is the fact that *H. parasuis* was not recovered from the nasal cavity of the treatment pigs two days before weaning. Therefore, the next step was to establish a challenge dose at which all treated pigs become colonized.

Dosage Trial

During the above preliminary trial to test the safety of controlled exposure of *Haemophilus parasuis*, it was discovered that, at weaning, the particular strain used for the controlled exposure – a serovar 9 strain – given as a controlled exposure (at 5 days of age) in the nostrils of the exposed pigs could not be retrieved from the nasal cavity at weaning. This was an unexpected result. As this could suggest that the dosage rate for the controlled exposure was too low, an experiment using a titration of higher dosage levels was performed to establish whether higher concentration of the controlled challenge inoculums could be retrieved back when sampled just before weaning.

In this experiment piglets of eight low parity sows, (the sows having been twice vaccinated with the strain used for controlled exposure), were controlled exposed at the age of 6-7 days after birth. These sows were separated by space from each other. The off-spring of two sows belonging to one group given the same concentration of inoculums. Four inoculum concentrations were trialled.

Table 5. Results of dosage experiment on Farm 4

Dosage	No. of pigs sampled	No. with *H. parasuis*	Serovar
Sampled one day before weaning			
3.6×10^8 cfu/ml	21 pigs from two low parity sow	0	N/A
4.3×10^7 cfu/ml	20 pigs from two low parity sow	0	N/A
0.9×10^8 cfu/ml	20 pigs from two low parity sow	2	9
1.08×10^9 cfu/ml	22 pigs from two low parity sow	0	N/A
Sampled 10 days after weaning			
3.6×10^8 cfu/ml	17	1	9
4.3×10^7 cfu/ml	15	0	N/A
0.9×10^8 cfu/ml	16	0	N/A
1.08×10^9 cfu/ml	16	0	N/A
0 cfu/ml	40	3	9 (2 isolates) 1/11 (1 isolate)

The dosage level did not seem to make a difference in the recovery of *H. parasuis* from the nasal cavity. In the present experiment, only a low level of colonization with *H. parasuis* was detected (two of 83 pigs pre-weaning and 1 of 64 pigs post-weaning). This level is around the level that is normally seen on this farm for the serovar 9 organism. As an example, in the preliminary trial two out of 52 control pigs were colonised with serovar 9, a prevalence of 3.8%.

As the inoculum of *H. parasuis* was still viable upon return to the laboratory, the assumption can be made that there was nothing wrong with the inoculum. Every precaution was taken not to negatively affect colonisation, including the management not using antibiotic treatment for 7 days after the controlled challenge.

The technique of nasal swabbing only samples the bacteria in the upper part of the nasal cavity. It could be that the bacteria sprayed into the nasal cavity do not

colonise in this part of the cavity. The organism might establish in the lower part of the nasal cavity or in the tonsils and even trachea.

The low prevalence of serovar 1/11 in the control pigs, which should at day 10 after weaning come down with Glässer's disease, is noteworthy. In the preliminary trial 25 pigs out of 52 control pigs had serovar 1/11, which is a prevalence of 48%.

On the farm there are now sows coming through that have been vaccinated twice again since the strain was left out of the vaccine and caused the outbreak of Glässer's disease on the farm.

Serovar profiling method

The objective of this component of the project was to investigate the diversity of *Haemophilus parasuis* serovars present in pig herds using nasal swabbing. Nasal swabs were used to obtain multiple isolates of *H. parasuis* which were grouped first by genotyping with representative isolates then being serotyped. Swabs were taken from the nasal cavity of just-weaned healthy pigs from multiparous sows from 12 farms and from post-weaned pigs of multiparous sows on one farm. On five out of these 13 farms, nasal swabs were also obtained from pigs showing clinical signs suggestive of Glässer's disease. On a further seven farms, nasal swabs were obtained only from pigs with clinical signs suggestive of Glässer's disease.

A total of 556 *H. parasuis* isolates were genotyped, while 150 isolates were serotyped. *H. parasuis* was detected on 19 of 20 farms, including two farms with an extensive history of freedom from Glässer's disease. Isolates of *H. parasuis* belonging to serovars regarded as potentially pathogenic were obtained from healthy pigs at weaning on 8 of the 10 farms with a history of Glässer's disease outbreaks. Sampling 213 sick pigs yielded 115 isolates of *H. parasuis*. Ninety-nine of these isolates belonged to serovars that were either potentially pathogenic or of unknown pathogenicity. Only 16 isolates from these 213 sick pigs were of a serovar known to be non-pathogenic. Healthy pigs contain a range of *H. parasuis*, even on farms free of Glässer's disease. Nasal swabbing of both healthy and sick pigs seems a useful tool to serovar profile farms.

Real Time PCR

The aim of this study was to validate a real time PCR test for the diagnosis of Glässer's disease, a major pig disease caused by *Haemophilus parasuis*. The specificity of a real time PCR amplifying the *inf*B gene was tested with 68 *H. parasuis* isolates and 36 strains of closely related species. As well, 239 samples of DNA from tissues and fluids of 16 experimentally challenged animals were tested with the real time PCR, and the results compared with culture and a conventional PCR.

The real time PCR gave positive results for all 68 *H. parasuis* isolates and negative results for all 36 non-target bacteria. When used on the clinical material from experimental infections, the real time PCR produced significantly more positive results than the conventional PCR (165 compared to 86). The sensitivity of the real time PCR combined with high specificity makes it a very valuable tool for the diagnosis of Glässer's disease. This new method will improve the ability of laboratories to diagnose Glässer's disease, especially in laboratories where the culture method for *H. parasuis* is not optimal.

Summary

The recently released *Actinobacillus pleuropneumoniae* vaccine method has proven that live vaccination with fully virulent field isolates can work. The same principle applied in the *A. pleuropneumoniae* is applied in the controlled challenge model for *H. parasuis*. Piglets are challenged with a strain while under the protection of the maternal antibodies. They then get colonised and develop their own immunity. The situation for *H. parasuis* is a bit more complicated than for *A. pleuropneumoniae* as most farms have more than one pathogenic *H. parasuis* strain and vaccine needs cross-protection against other strains on the farm. Farm trials seeking to prove the efficacy of a vaccination program need to be performed on farms that have an acute outbreak of the disease. The control group needs to be severely affected by Glässer's disease to give statistical significance, especially if small groups (200 animals per group) are used. As some of the Glässer's disease symptoms could also be associated with other diseases (like anorexia) it is important that the symptoms are more specific to the disease. The only other way is to have huge numbers of pigs participating in the trial. Alternatively, the trial could be conducted under pen conditions at a research facility. In such a research facility, other variables can be held constant and therefore, small groups should give statistically significant results.

Despite extensive efforts, no farm trial in the current project resulted in a frank outbreak of confirmed Glässer's disease in the control group. This absence of frank disease in the control group meant that it was impossible to demonstrate the efficacy of the controlled exposure concept.

4. Application of Research

The knowledge gained during the project is currently helping pig farmers.

Major advances achieved in the project were a suite of diagnostic and support tools for pig veterinarians dealing with Glässer's disease.

1. A new improved PCR assay has been developed. This Real Time PCR is much more sensitive and specific than the existing conventional PCRs. The method has been validated for direct application to systemic sites and lungs and is available for use.
2. A serovar profiling service has been developed and is now available. This service involves the submission of nasal swabs from pigs at weaning and also from diseased pigs. If *H. parasuis* is a problem on the farm, the organism will be isolated and the serovars present on the farm determined. A recommendation on which of the serovars should be included into an autogenous vaccine will be provided. This service has been provided for 23 farms to date.
3. A *H. parasuis* genotyping service has been developed and is now available. This service can be used to understand on farm epidemiology (is one strain present on multiple farms? is an outbreak associated with a novel strain or the re-appearance of previously known strain?). This genotyping (and an expanding data-base of genotypes) will greatly improve our understanding and hence our ability to control outbreaks.

5. Conclusion

Safety of the controlled exposure method

The experiments demonstrated without a doubt that this method is safe to use when using moderately pathogenic and non-pathogenic strains. Even at very high concentration of the inoculums, the method was safe and no signs of illness were observed. If using highly pathogenic strain, the presence of maternal antibodies is vital for the safety of the method. On a commercial piggery, not all piglets will have their fair and equal share of maternal antibodies. Hence, the use of a highly pathogenic strain as a control exposure agent might not be as safe. The research to date has shown that most farms have non-pathogenic strains or moderately pathogenic strains, suggesting that there is no need for the use of highly pathogenic strains.

Controlled exposure

The experiment on Farm 2 highlighted the diversity of serovars of *H. parasuis* that could be found on a farm (14 different serovars). This farm had the highest diversity of serovars detected on the 20 farms sampled in this project; the average across all farms being three serovars per farm. It also highlighted the changes of serovar within batches of pigs with some of the serovars that are at low frequency only appearing in some batches of pigs. This indicates the importance of sampling more than one batch of piglets to avoid low frequency serovars being missed. It is possible that some of the failures of autogenous Glässer's vaccines that have been observed on farms could be due to the fact that not all serovars present on the farm were incorporated in the vaccine.

Even though the Farm 2 experiment did not prove that the controlled exposure method worked, it certainly gave an indication that the method could work. None of the sick pigs from the treatment group yielded serovar 15, which is a disease-causing serovar on the farm, while some of the sick pigs of the control group in both experiments yielded this serovar.

Another important point that emerged from this experiment is that the farm to be used in further experiments has to have an acute problem with *H. parasuis* to allow a demonstration of vaccine efficacy.

The preliminary experiment on Farm 4 highlights several points. One point is that the ability of a serovar to spread among pigs (essentially, its frequency of detection) is a very important point in the control of the organism. A strain that is easily spread, a good colonizer, and has a high pathogenicity, as seen on this farm, is likely to be difficult to control. In contrast, Farm 1 had a non-pathogenic strain which spread rapidly, while the pathogenic strain could only be found when sampling was increased. A strain that is non-pathogenic and spreads easy would be an ideal strain for a vaccine, as it would give protection, and would easily spread amongst pigs, particularly if it is the first strain encountered by the naïve pig. It is possible that such "super colonizers" might exclude or prevent other strains from colonizing.

The dosage trial on Farm 4 showed that the colonization in the nasal cavity did not seem to depend on the dosage level. However, on Farm 2, a colonization of 32 % was achieved in the first trial, even though a dose of only 7×10^5 cfu was used, compared to the low colonization level of 2.4% in the dosage trial on Farm 4. The Farm 2 and Farm 4 trails were performed with different serovars (6 and 9 respectively). It is not clear whether colonization in the nasal cavity depends on the strain or other factors. Indications seen throughout these experiments suggest

that the detectable presence of the vaccine strain in the nasal cavity might not be essential for the protective immunity.

Serovar profiling method

H. parasuis is part of the natural flora of the upper respiratory tract of pigs, so it is not surprising that *H. parasuis* was found in the nasal cavity of weaned pigs on two farms with an extensive history of freedom from Glässer's disease. Strains that spread rapidly will make it hard to find strains with low prevalence. Therefore, a large sample size is needed to find low prevalence strains indicating that multiple serovars may not easily be detected if one strain is more prevalent than the others. Clearly, the question arises of how many colonies have to be sampled to find strains circulating at a low prevalence. The result of the current study, that many isolates of *H. parasuis* can be obtained from a given herd, but in most cases only one or two strains predominate, coincides with the finding of Smart and Miniats (1993). Except for a few limited studies, such as on the colonisation rate of weaned pigs (Kirkwood *et al*. 2001; Cegielski *et al*. 1999), and attempts to determine the prevalent serovars in pigs (Kirkwood *et al*. 2001; Smart *et al*. 1988; Olvera *et al*. 2006), there is no actual study looking at the diversity within individual pigs across several farms with more than two serovars; nor are there any studies of the prevalence of strains over time. Therefore, there is no guidance as to how many samples have to be taken to establish the serovars present in a pig, especially if one strain is dominant.

Sampling sick pigs which showed clinical signs suggestive of Glässer's disease yielded only strains of serovars regarded as pathogenic and serovar NT when the first 13 farms were evaluated. Despite the dominant serovar NT strain on Farm 1, the pathogenic serovar 4 strain on this farm was recovered effortlessly in the nasal cavity of a sick pig (only one colony was collected). These results, combined with the fact that pathogenic strains of *H. parasuis* given in artificial challenges can be found in the nasal cavity (Oliveira *et al*. 2001; Segales *et al*. 1997), suggest that there is definitely a correlation between sick pigs and the presence of pathogenic serovars of *H. parasuis* in the nasal cavity. Overall, it seems that strains of *H. parasuis* of known pathogenic serovars can be found in the nasal cavity of sick pigs and are most likely disease-causing strains.

The multiplicity of serovars found on Australian pig farms was higher than other researchers have observed. Smart *et al*. (1988) observed that most farms harboured two to four strains. Most of the farms in the current studies conform to the observation of Smart *et al*. (1988). Oliveira *et al*. (2003) looked at 10 herds in America and recovered 1 to 3 serovars per herd.

Our results suggest that the serovar can change in time if certain strains are used for vaccination. These changes in serovar prevalence might have to do with the normally low prevalence of pathogenic strains in healthy animals (Oliveira and Pijoan 2002) while diseased animals might have a higher prevalence of pathogenic strains. A study by Olvera *et al*. (2007) found that the diversity of strains isolated on a farm was affected by antimicrobial treatment. Immediately following antibiotic treatment, they could only recover one of the three strains present on the farm before antimicrobial treatment and this strain was resistant to the antibiotic used. However, one year after treatment the diversity of the strains was back to that seen before the treatment. This would suggest that the susceptible strains were still there at a low prevalence and it stresses the importance of serovar profiling to find all the strains that are potentially disease causing organisms. Overall, the results from farms where sick pigs were sampled

indicate that both methods (swabbing healthy weaned pigs and sick pigs) should be combined to enhance results of the profiling.

In summary, *H. parasuis* was found on farms where no outbreak of Glässer's disease had occurred. Isolates of serovars recognized as being pathogenic can be easily missed if another strain is more prevalent. Detection of isolates occurring at a low prevalence requires examining multiple colonies per nasal swab. Sampling healthy weaned pigs does not necessarily reveal all pathogenic serovars. The sample size of pigs is important in the detection of strains on a farm. A bigger sample size of sick pigs on some of the farms might have revealed all pathogenic serovars on the farms.

Real Time PCR

The isolation of *H. parasuis* is difficult, as the bacterium is very sensitive to pH changes and heat (Morozumi and Hiramune 1982). *H. parasuis* is also a slow growing, fastidious organism with specific nutritional requirements (Ferri *et al.* 2000; Oliveira and Pijoan 2004). This makes recovery of the organism after sample collection and transport to the laboratory very difficult. Once in the laboratory the isolation is very difficult as *H. parasuis* is easily overgrown by other faster growing bacteria. Therefore, the method of identification by culture is not always optimal and PCR-based methods are an attractive alternative. The available conventional PCR assays display problems with non-specific bands (Oliveira *et al.* 2001) or sensitivity (Angen *et al.* 2007). The real time PCR method described here is more specific and does not give a positive reaction with any of the 24 non-target species (including all close relatives) tested. Oliveira *et al*. (2001) reported a sensitivity of 0.5 cfu per reaction, while Angen *et al*. (2007) reported a sensitivity of 5.3 cfu per reaction for their respective PCR tests. According to our sensitivity assays, the real time PCR showed a sensitivity of 9.5 to 0.83 cfu per reaction for the boiling method of DNA extraction and 47.5 CFU per reaction to 0.42 cfu per reaction for the PrepMan Ultra method. However, when looking at the data from the challenged animals, where the Oliveira *et al*. (2001) PCR was used as well, the real time PCR gave significantly higher numbers of positive results than the Oliveira *et al*. (2001) PCR. Overall, our data indicate that the real time PCR is more sensitive than the conventional PCR, even though our minimum detectable cfus per reaction were higher than the reported minimum detectable cfus of the conventional PCR (Oliveira *et al*. 2001).

In a study by Turni and Blackall (2007), the culture method was deemed more successful than the conventional PCR method, when detecting *H. parasuis* in sick animals. The results of the current study indicate that this does not hold true for the real time PCR. The real time PCR performed better than culture, although not at a statistically significantly level for swab samples. In laboratories where the culture methods are not as optimized as in our laboratory, the real time PCR should perform even better compared to culture. Therefore, we believe that real time PCR will enhance the diagnosis of *H. parasuis*, especially for laboratories that are not experienced with the culture of *H. parasuis* or, in situations where culture is not possible.

The sensitivity assay revealed that the real time PCR detected low numbers of *H. parasuis* even in the presence of large numbers of non target cells. The ability of the real time PCR to perform well in the presence of large number of other bacteria, was seen in the results obtained from the samples processed from the tonsils of the challenged animals. The real time PCR significantly outperformed the conventional PCR-culture method.

When using this real time PCR assay as a diagnostic tool it has to be pointed out that the presence of *H. parasuis* in the upper respiratory tract does not mean that there is a problem with *H. parasuis*, as non-pathogenic serovars can be found in the upper respiratory tract. Therefore, if the real time PCR is used as the diagnostic tool to determine the cause of disease, the assay should be used from samples from internal organs and tissues.

The *infB* gene targeted real time PCR for *H. parasuis* performed well for pure culture, swabs from tissue and fluid or tissue and fluid processed directly regardless of contamination by other bacterial species. The high sensitivity and specificity of the real time PCR make it an ideal diagnostic tool for *H. parasuis*. The real time PCR has distinct advantages over conventional PCR, such as less risk of non-specific reactions and contamination, less handling time (no post-reaction analysis) and the potential of high throughput automation.

Conclusion

This project has aimed at improving the diagnostic capability to detect *H. parasuis* and to implement a new method to control *H. parasuis* on Australian farms. During the period of the project the aim of better diagnosis has been fulfilled. Tools like the Real Time PCR, serovar profiling and genotyping of *H. parasuis*, have given the Australian pig industry the tools to diagnose *H. parasuis* and to implement vaccine strategies to control the problem. The knowledge gained during the project has helped to implement all these tools and make them already available to the pig industry well before the end of the project.

The implementation of the controlled exposure as a vaccination method has had its difficulties. The project has been able to generate data that indicates that this method will work. It is certainly a safe method and all the tools are in place to determine the serovars on the farm and pick a suitable serovar for controlled exposure.

6. Limitations/Risks

Controlled exposure

So far it has become clear that the method of controlled exposure is extremely safe and all the tools are in place to establish the method on a farm. The difficulties in establishing that the method gives statistically significant protection are due to the difficulties encountered on the farm with disease outbreaks associated with *H. parasuis*. Other factors influence the picture *H. parasuis* represents on a farm. The clinical signs displayed due to *H. parasuis* infection are not very disease specific, but could be associated with other diseases as well.

The project has shown that for *H. parasuis* vaccine research on farms, a large farm that has an acute outbreak with a large sample size is needed to get statistically significant results.

Diagnostic methods

The two diagnostic tools that have been developed (Real Time PCR and Serovar Profiling) are the tools that were previously missing, and limited the capacity to diagnose the disease on farms. It is now possible for Australian farmers to use these tools to aid in the diagnosis of Glässer's disease. These tools have to be applied with the constraints identified in this project. As an example, the simple presence of *H. parasuis* in the nasal cavity, as detected by the real time PCR, does

not mean that a pig is suffering from Glässer's disease. *H. parasuis* is part of the natural flora of the upper respiratory tract of pigs. Therefore, strains in the nasal cavity could be non-pathogenic and not being involved in disease at all. It is also important to think about sample size to make sure all strains on the farm are being sampled.

7. Recommendations

Controlled exposure

The concept of controlled exposure needs to be tested on a large farm that has an acute outbreak and were no other obvious diseases interfere with the outcome. It needs to be a farm where the clinical signs can be attributed to Glässer's disease and where there is still a death rate due to Glässer's disease.

For implementation of the method a freeze drying method for *H. parasuis* needs to be developed, as shipping this fastidious organism in a live state is not an easy task and has major limits.

Serovar profiling and Real Time PCR

Both these methods are available to the pig industry and have already helped pig farmers to control their *H. parasuis* problem.

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Appendix 1 - Preliminary experiment to test safety of controlled exposure method – Farm 1

Glässer's disease is still a major problem in the pork industry. Autogenous vaccines, besides being expensive, only give protection if all the strains present on the farm are included in the vaccine. The objective of the project is to reduce production costs and improving herd feed conversion efficiency by offering a solution to farmers for the control of Glässer's disease. The solution is controlled exposure of piglets while they are still under the protection of maternal antibodies. Controlled exposure will give the piglets protection against the serovars on the farm but will also give cross-protection to other serovars. Therefore, if another serovar is introduced onto the farm the results will not be catastrophic. This would mean that the high cost of antibiotics to control Glässer's is not necessary any more and mortalities plus growth retardation due to Glässer's would be avoided.

This report covers a preliminary field trial that was performed chiefly as a safety trial. At six days of age, pigs were exposed – by nasal spray - to the moderately pathogenic serovar 4 present on the farm. The sows had been immunized against this serovar prior to farrowing with a killed vaccine that contained the serovar 4 strain from this farm. The pigs were then regularly sampled to establish the colonization of the challenge strain.

Results of sampling

Date	Procedure	Days post exposure	*H. parasuis* (Hps) recovered
31/7/06	Sampled the nasal cavity of 52 piglets from 10 sows (4-5 piglets per sow) to establish serovars present on the farm.	14 days before exposure	Found Hps in the nasal cavity of piglets from two sows. This was a serovar NT (confirmed by gel diffusion (GD) and Indirect Haemagglutination assay (IHA)). The strain that was recovered from this farm as a disease causing strain was serovar 4
14/8/06	Exposed 29 piglets at six days of age to 1.04 x 10^4 cfu (delivered in 1 ml dose to nostril) of the serovar 4 strain recovered from the farm. Piglets were ear marked with purple tags. All pigs were in the same nursery room. Came from 4 sows (0180, W213, 0212, 073). 25 piglets were ear tagged with yellow tags as control group. These piglets were in a different room of the nursery. Came from 5 sows (0177, 0357, TP1507, Y339, W355). First to third parity sows were used.	0	
22/8/06	18/8/06 control pig died due to scours. 20/8/06 control pig died due to scours. 6 control pigs were treated with Amoxycillin. Sampled the nasal cavity of 18 treatment and 23 control piglets. Found *Actinobacillus indolicus* in the nasal cavity of one treatment piglet. Found *Actinobacillus rossii* in one control piglet.	8	none
28/8/06	Sampled the nasal cavity of 11 treatment and 23 control piglets Sampled nasal cavity of a sick pig in weaner room, which was not part of the experiment. Clinical signs: held head at strange angle, laboured breathing and had been in weaner room for longer than normal. Recovered *H. parasuis* serovar 4 from the nasal cavity of this pig.	14	none
4/9/06	Sampled the nasal cavity of 18 treatment and 23 control piglets. 2 control pigs (16, 13) and 1 treatment pig (1) have *A. indolicus* in the nasal cavity.	21	none

Date	Procedure	Days post exposure	*H. parasuis* (Hps) recovered
11/9/06	Sampled the nasal cavity of 29 treatment and 23 control pigs. Prior to sampling control pigs (except pig 3 and 23, which had a light weight at birth) have been moved to weaner room	28	13 of 23 control pigs had serovar NT 8 of 29 treatment pigs had serovar NT
20/9/06	Sampled the nasal cavity of 29 treatment and 23 control pigs. Prior to sampling treatment pigs have been moved to weaner room. Pig 3 and 23 are still in nursery.	37	21 of 23 control pigs had serovar NT Pigs 3 and 23 of control group were free of Hps 17 of 29 treatment pigs had serovar NT
26/9/06	Sampled the nasal cavity of 16 treatment pigs (ones not positive for Hps last sampling) and 2 control pigs that were still in nursery last time. Both control animals were still in nursery. Rest of control pigs were sent to grow out shed, while we were there.	43	14 out of 16 treatment pigs had serovar NT 2 control pigs did not have Hps
2/10/06	Sampled 29 treatment pigs. Treatment group was still in the weaner room. From each plate collected 6 colonies, which were processed and genotyped to find serovar 4 amongst them. Also sampled 2 control pigs.	51	All treatment pigs had Hps serovar NT 11 of 29 treatment pigs had Hps serovar 4 2 control pigs had Hps
17/10/06	Sampled 23 control pigs and 21 treatment pigs From each plate collected up to 6 colonies, which were processed and genotyped to find serovar 4 amongst them.	66	All Pig had Hps

Results of genotyping

Date	Procedure	Genotype	*H. parasuis* (Hps) serovar
28/08/06	Compared the genetic profile of the exposure strain and the strain found in the sick pig (that was not part of the experiment) with the strain found in the pre-exposure sampling with an ERIC PCR	The serovar 4 of the challenge strain has a distinctly different genetic profile than the serovar NT that was found in the pre-exposure sampling. The genetic profile of the strain from the sick pig was the same as the exposure strain.	
11/9/06	Compared the genetic profile of Hps recovered from the nasal cavity from 8 treatment pigs and 13 control pigs All ERIC PCR were run with two controls – the serovar 4 (exposure strain) and the serovar NT	All except one pig from the treatment group had the same genetic profile	The 20 isolates showing the common genetic profile were non-typable serovar (confirmed by GD) The single isolate with the different genetic profile was also confirmed as serovar NT by GD and IHA.
20/9/06	Compared the genetic profile of Hps recovered from the nasal cavity from 17 treatment pigs and 21 control pigs. Had more than one sample (more than one colony collected of the original culture plate) for some of the pigs. Compared a total of 61 isolates.	All of the animals had Hps with the same genetic profile as the NT serovar recovered in the pre-trial sampling and in the previous sampling.	
26/9/06	Compared the genetic profile of Hps recovered from the nasal cavity from 14 treatment pigs. Compared a total of 51 isolates	All of the animals had Hps with the same genetic profile as the NT serovar recovered in the pre-trial sampling and in the previous sampling.	
2/10/06	Compared the genetic profile of Hps recovered from the nasal cavity from 29 treatment pigs and 2 control pigs Sampled about 6 colonies from each original culture plate, resulting in a comparison of 139 isolates.	18 isolates displayed a genetic profile identical to profile of the serovar 4 strain, while the rest of the isolates (121) displayed a profile identical to the profile of the serovar NT strain. The 18 isolates displaying the serovar 4 profile came from 11 pigs.	

Date	Procedure	Genotype	*H. parasuis* (Hps) serovar
17/10/06	Compared the genetic profile of Hps recovered from the nasal cavity from 21 treatment pigs and 23 control pigs. Sampled up to 6 colonies from each original culture plate, resulting in a comparison of 193 isolates.	3/21 treatment animals yielded Hps that displayed a serovar 4 profile as well as Hps with the NT serovar profile (comparison of 89 isolates). 6/23 control animals yielded Hps that displayed a serovar 4 profile as well as Hps with the NT serovar profile (comparison of 104 isolates). For all the rest of the animals (18 treatment and 17 controls) only Hps with the serovar NT profile was found.	

The different genetic profile discovered on the 11/9/06 from one animal might have been a mixed culture, as it was the only time this profile appeared and in further sampling the genotype recovered from this animal was the normal genotype of the NT serovar recovered from all animals during the trial.

None of the challenged pigs showed any clinical signs associated with Glässer's disease, indicating that the controlled exposure method appears to be safe with the moderately pathogenic serovar 4 strain used in this study.

The spread of the presumably non-pathogenic strain of the NT serovar (none of the control group showed any signs of disease) among all pigs of both treatment and control groups prevented a differentiation between the groups. The results suggest that this non-typable strain is an ideal candidate for controlled exposure due to its rapid spread and safety.

Conclusion:

Due to the rapid spread of the non-typable *H. parasuis* interfering with the differentiation of the control and treatment groups and the lack of any sign of Glässer's disease in the control group this farm is unsuitable for the evaluation of the controlled exposure method.

Appendix 2 - Controlled exposure experiments on Farm 2

Introduction

Glässer's disease has in recent times emerged again as one of the major disease problems in the pork industry (Nedbalcova *et al.* 2006). The disease is associated with poly-serositis, poly-arthritis and meningitis. The causative agent of this disease is the bacterium *Haemophilus parasuis* (Nedbalcova *et al.* 2006). Major losses occur due to mortalities and poor growing pigs. The problem is mainly observed shortly after weaning (Oliveira and Pijoan 2004). At this stage pigs colonised by *H. parasuis*, which have developed their own immunity while under the protection of maternal antibodies, spread the bacterium to susceptible pigs that were not colonised (Oliveira *et al.* 2001).

There are 15 serovars of *H. parasuis*, being either very, moderately, mildly or none pathogenic (Kielstein and Rapp-Gabrrielson 1992). The commercial and autogenous vaccines available in Australia are made with killed bacteria and give generally protection only to the serovar/s present in the vaccine. However, vaccination with live bacteria is supposed to give cross-protection. This concept that live organisms provide more cross-protection than killed organisms has been noted with other bacteria within the family *Pasteurellaceae*. Studies with *Actinobacillus pleuropneumoniae* have induced cross-immunity between a variety of serovars of this organism after natural respiratory infection (Furesz *et al*. 1997; Prideauz *et al*. 1999). Similar results have been reported outside the family *Pasteurellaceae*. As an example, studies with *Neisseria meningitidis*, have confirmed that infection with live bacteria (mutant virulant strain) gives cross-protection against a range of strains with different serogroup, serotype and serosubtyping antigens (Newcombe *et al*. 2004). It was also suggested that the cross-protection is long-term. A study done in 1993 by Nielsen supported this concept of cross-protection for live *H. parasuis* strains. *H. parasuis* serovars 2, 3, 4 and 7 given as a single aerosol gave heterologous protection to serovar 5 challenge (Nielsen 1993). It is proposed that intranasal inoculation with a live strain induces mucosal antibodies to a spectrum of antigens, including common antigens, which result in cross-protection[8]. Live intranasal inoculation is practiced on some commercial farms in America, where they control expose young piglets to all the strains of *H. parasuis* on the farm. This concept of controlled exposure has been developed in America (Oliveira *et al*. 2001), it relies on maternal antibodies to protect against systemic invasion while the piglet is developing its own immunity to the colonising strain of *H. parasuis* that has been introduced via nasal inoculation at 5 days of age to insure all pigs are colonised. The current project has taken this concept of controlled exposure further by only using one of the strains on the farm and relying on cross-protection.

Method

Preliminary survey

Serovar profiling was done on a 400 sow farm in Queensland, Australia, where pigs displayed symptoms of Glässer's disease. Symptoms displayed were coughing, anorexia and death in pigs shortly after weaning. Nasal swabs were taken from 40 pigs at weaning (three weeks of age) from 10 different sows. Swabs from the nasal cavity were also taken from 7 sick pigs displaying signs of Glässer's disease. A necropsy was also performed on a sick pig. Amies transport swabs were used to swab the nasal cavity. The collected swabs were kept on ice until they were

inoculated on to BA/SN agar.[9] Blood agar plates cross-streaked with a nurse colony of *Staphylococcus hyicus* were used as purity control. Colonies of *H. parasuis* were identified with PCR (Oliveira et al. 2001). All *H. parasuis* isolates were genotyped with the Enterobacterial repetitive intergenic consensus-based (ERIC) PCR (Oliveira *et al*. 2003) and at least one representative of each genotype was serotyped. Serotyping was done via the gel diffusion and indirect haemagglutination assay (Turni and Blackall 2005). A serovar 6 was chosen as the challenge strain.

Pigs

In the first trial the control and treatment group consisted of 19 and 21 sows, retrospectively. In the second trial the control and treatment group consisted of 20 and 22 sows, retrospectively. All sows were vaccinated twice prior (3 and 5 weeks prior) to farrowing with an autogenous killed vaccine from the serovar 6 obtained from the farm in the preliminary survey.

Bacterial strain

The serovar 6 *H. parasuis* strain from the farm serotyped as serovar 6 in the gel diffusion but was a serovar NT in the IHA. The strain was revived from -70°C storage three days prior to preparation onto BA/SN agar (Turni and Blackall 2007). It was plated onto chocolate agar plates, that consisted of BBL™ Blood Agar Base (Becton Dickinson, Sparks, MD USA), 5 % defibrinated sheep blood (Bio-Lab, Melbourne VIC) and 0.0025% reduced nicotinamide adenine dinucleotide (NADH) (Roche Diagnostics, Mannheim Germany), twice before harvesting after 18 hours incubation at 37°C. *H. parasuis* was harvested into 2 ml of phosphate buffered saline and then diluted to a concentration of 7×10^5 cfu/ml. A sample of the prepared inoculum, as well as samples of the left over inoculum was plated onto chocolate agar plates and a count performed after one day incubation. The inoculum was kept on ice until being used for inoculation four hours after preparation. Survival counts after 7 hours showed that the prepared inoculum was at the same concentration for this period if kept on ice. 0.5 ml of the inoculum was sprayed into each nostril of the pigs.

Experimental set-up

The nursery consisted of sow stalls that did not allow for contact between the piglets of different sows, as each stall was partitioned off. In the first experiment the control group of sows was in a different shed to the treatment group sows. In the second experiment both sows were in the same shed with the control group grouped together and the treatment group in two groups.

The farm was set up with weaning sheds that consisted of three sections; each week one of the sections would be filled with weaned pigs. For the purpose of the experiment the experimental groups where one week apart with he control group being the first weaned, followed by the treatment group the following week leaving a section between the two groups. This was done to prevent nose to nose contact between the two groups. The experiment was then repeated.

Protocol

First experiment: The groups consisted of 193 piglets for the control group and 207 pigs for the treatment group. 207 pigs were controlled exposed at four to eight days of age. Of these piglets 179 pigs for the control and 186 for the treatment group were weaned at 21-25 days of age for the control and 19-21 days of age for the treatment group. The freshly prepare inoculum had a concentration

of 1.47 x 10^4 cfu/ml *H. parasuis*, while the inoculum counted 10 hours after preparation had a concentration of 6.7 – 7.4 x 10^3 cfu/ml *H. parasuis*.

Second experiment: The groups consisted of 199 piglets for the control group and 217 pigs for the treatment group. 217 pigs were controlled exposed at four to eight days of age. Of these piglets 176 pigs for the control and 193 for the treatment group were weaned on two separate days at 21-22 days of age and 21 – 24 days of age for the control and 21-22 days of age and 21 – 24 days of age for the treatment group. The freshly prepare inoculum had a concentration of 2.85 x 10^4 cfu/ml *H. parasuis*, while the inoculum counted 10 hours after preparation had a concentration of 0.7 – 1.47 x 10^4 cfu/ml *H. parasuis*.

Both experiments: At weaning between 47 and 63 pigs were nasal swabbed to serovar profile the groups. In the first experiment 54 pigs for the control and 47 pigs for the treatment group were swabbed. In the second experiment 63 pigs for the control and 48 pigs for the treatment group were nasal swabbed at weaning. Sick pigs in all groups were sampled twice a week to establish the serovars causing disease. Number of mortalities, number of antibiotic treatments and symptoms of disease were recorded throughout the experiment. Each pig was ear-marked with the sow number. Once the pig was treated it received an ear tag. At the end of the experiment the number of ear tags was recorded. Some pigs were sampled more than once during the experiment.

Results

Preliminary survey

The healthy pigs yielded serovars: 5, 6, 9, 9/13, 10, 15 and NT, while the sick pigs yielded serovars: 12, 15 and NT. Serovar 15 was recovered from the lung tissue of the necropsied pig. From 12% (5) of the 40 healthy pigs sampled at weaning *H. parasuis* was not recovered.

Experiments

The data of the experiments is summarised in Tables 1 and 2. Table 3 represents a summary of the sick and dead pigs during the experiments. Table 4 summarises all the serovars found on the farm with the number of genetic profiles for each serovar

Table 1 Data for experiment 1 is represented in this table. Sampling of sick pigs is recorded with the symptoms and serovar recovered from these pigs. The pigs were ear- marked and could be traced back to their mother. The highlighted fields represent serovars which are potentially pathogenic according to literature[4] or our experience.

group	sow	piglets tested/sow	serovars	maximum serovars/piglet	sick at weaning 2/4/07 and 5/4/07	symptoms	serovars	5/4/07 Thur (4 days post weaning) not ear tagged	symptoms	serovars	6/4/07 no treated /sow - symptom - antibiotic	13/4/07 Fri sampling sick eartag pigs	symptoms/ treatment	serovar	13/4/07 sampled without ear tag	symptoms	serovar	13/4/07 no treated /sow - symptom - antibiotic	17/04/07 ear tagged and not ear tagged	symptoms	serovar	18/4/07 no treated /sow - symptom - antibiotic	25/4/07 no treated /sow - symptom - antibiotic	26/4/07 Thurs sampled without ear tag (got Penicillin after swabbing)	symptoms (all got Bomox)	serovars	26/4/07 already ear tagged no treated /sow - symptom - antibiotic
c	1	3	NT + 9	2	1	cough	9	1	cough, diarrh																		
c	2	3	9 + 9/13	1				1	cough	9/13												1 - dopey Bomox	1 - skinny Bomox				
c	3	2	9 + 2	1																			1 - crook leg Propen	1	swollen hock joint	10	
c	4	3	NT + 2	1	1	skinny	NT	1	sick	NT	2 - skinny Bomox	1	backbone showing - Bomox	15	1	skinny	15										
c	5	3	9	1																							
c	6	3	9 + NT	1	1	cough	9															1 small skinny Bomox	1 - skinny Bomox				1 - Bomox
c	7	2	NT	1				1 1	skinny skinny skinny	Protease	1 - skinny Bomox	1	skinny - Bomox	NT	1	skinny - Bomox	NT		1 1	swollen leg skinny							
c	8	3	9 + NT	1	1	skinny	9				2 - skinny, scour - Bomox + Trisoprim	1	skinny - Bomox	9	1	skinny - Bomox	9					1- bad leg Banacillin Bomox					
c	9	3	9	1																		1 - skinny ear swollen Bomox					
c	10	2	NT	1																1	skinny	6			heavy breathing	6	
c	11	3	15	1				1	cough	15	1 - skinny Bomox	2	skinny - Bomox	15+10 15+10									1 - skinny Bomox				
c	12	3	15	1							1 - skinny Bomox	1 1	skinny cough - Bomox	15 5or12						1	cough	13+10	1 - skinny Bomox				1 - Bomox
c	13	3	NT + 10	1																1	skinny	10					
c	14	3	NT	1				1	diarrh	2/1																	
c	16	3	NT	1				1	cough	NT																	
c	17	3	NT	1								1	crook leg - Propen	15 and 9													
c	18	3	NT	1							1 - skinny Bomox																
c	19	3	10	1																		1 - skinny backbone showing Bomox					
c	45	3	NT + 11	1	1 1	skinny(sick) discoord.	NT NT					1 1	skinny, scour - Trisoprim, Bomox backbone - Bomox	15 no Hps									1 - skinny Bomox 1 - skinny, scour Bomox Trisoprim				1 - Bomox

group	sow	piglets tested/sow	serovars	maximum serovars/piglet	at weaning 2/4/07 and 5/4/07	symptoms	serovars	5/4/07 Thur (4 days post weaning) not ear tagged	symptoms	serovars	6/4/07 no treated /sow - symptom - antibiotic	13/4/07 Fri sampling sick eartag pigs	symptoms/ treatment	serovar	13/4/07 sampled without ear tag	symptoms	serovar	13/4/07 no treated /sow - symptom - antibiotic	17/04/07 ear tagged and not ear tagged	symptoms	serovar	18/4/07 no treated /sow - symptom - antibiotic	25/4/07 no treated /sow - symptom - antibiotic	26/4/07 Thurs sampled without ear tag (got Penicillin after swabbing)	symptoms (all got Bomox)	serovars	26/4/07 already ear tagged no treated /sow - symptom - antibiotic
t	20 (wrong)	1	1/11	1	1	sick	9				1 - skinny Bomox	1	small, skinny, scour, died 12/4														1 - Bomox
t	21	2	9 + 6	1																		1 - skinny, scour Bomox Trisoprim	1 - skinny Bomox 1 - skinny + crook leg Bomox	3	skinny	10 6 9 + 6	
t	22	2	NT	1															1	backbone showing	5or12						
t	23	2	9	1														1 - cough Bomox									
t	24	2	10	1								1	cough - Bomox died?						1	backbone showing		1 - skinny Bomox	1 - skinny Bomox 1 - coughing Bomox 1 - backbone showing Bomox				1 - Bomox
t	25	2	9	1											1	skinny	9	1 - skinny (small) Bomox									
t	26	3	6 + 9	1																							
t	27	3	10 + 9	1								1		9	1		9	1 - cough Bomox				2 - skinny Bomox	1 - backbone showing Bomox	1	skinny	6	
t	28	3	6 + 9 + 10	1								1	skinny - Bomox	9+6					1	skinny backbone showing		1 - skinny little Bomox					
t	29	3	6 + 9/13	1								1 1	cough - Bomox skinny- Bomox	no Hps 9													2 - Bomox
t	30	3	6 + 9	1														1 - cough Bomox	1	diarrhoea	9	2 - skinny Bomox					
t	31	2	10	1																				1	coughing	10	
t	32	3	9 + 11	1							1 skinny - Bomox																
t	33	2	6 + 9	1																							
t	34	2	6 + 9/13	1																			1 - backbone showing Bomox	1	skinny	NT	1 - Bomox
t	35	2	6 + 10	1																		1 - skinny Bomox					
t	36	2	9 + ?	1	1	sick	10					1	scour - Trisoprim	no ?Hps									1 - backbone showing Bomox	2	skinny	9/10	
t	37	2	9	1																							
t	38	3	6 + 9/13	1											1	skinny	5or12	1 - skinny Bomox	1 (tagged) 1	skinny backbone showing		1 - dopey Bomox	1 - skinny Bomox 1 - backbone showing Bomox	1	skinny	9/10	1 - Bomox
t	39	2	6	1																							
t	40	3	6	1								1	scour - Trisoprim	no Hps	1	skinny	?	1 - skinny Bomox				2 - skinny Bomox	1 - backbone showing Bomox				

Table 2 Data for experiment 2 is represented in this table. Sampling of sick pigs is recorded with the symptoms and serovar recovered from these pigs. The pigs were ear- marked and could be traced back to their mother. The highlighted fields represent serovars which are potentially pathogenic according to literature[4] or our experience.

group	sow	weaned/ not weaned (17/5/07)	piglets tested/sow	serovars	maximum serovars/piglet	at weaning 10/5/07 and 17/5/07	symptom	serovar	22/5/07 Tues no treated(Fri)/sow - symptom - antibiotic// all treated today	symptom	serovar	29/5/07 Tues no treated(Fri)/sow - symptom - antibiotic/ all treated today	symptom	serovar	29/5/07 no ear tag no treated/sow - symptom - antibiotic/ all treated today	symptom	serovar	5/6/07 Tues no treated(Fri)/sow - symptom - antibiotic/ all treated today	symptom	serovar	5/6/07 healthy pigs sampled	serovar	8/06/2007 all already ear tagged pigs treated with Bomox and Trisoprim
C	1	yes	3	9																			
C	2	yes	3	9(13)																			
C	3	yes	3	9(13)																			
C	4	yes	3	10																			
C	6	yes	3	9																			
C	7	yes	3	9																			
C	8	yes	3	9								1 Bomox + Trisoprim 1 Bomox + Trisoprim	skinny skinny + diarrhoea	6 15+6									
C	9	yes	3	9																			
C	10	yes	1	9																			
C	11	yes	3	9					1 Bomox + Trisoprim	skinny	no Hps												
C	12	yes	3	9																			
C	13	yes	3	9								1 Bomox + Trisoprim	skinny	no Hps									
C	14	yes	3	9					1	swollen shoulder joint	no Hps												
C	15	yes	1	11																			
C	17	yes	1	9(13)					1 Bomox + Trisoprim	skinny	no Hps	1 Bomox + Trisoprim	skinny	15									
C	18	yes	1																				
C	19	yes	3	10																			
C	20	yes	1	9/10																			
C	19	no	3	9(13)																			
C	20	no	3	15																			
C	22								1 Bomox + Trisoprim	very skinnyski	no Hps												
C	9	no	3	9(13)																			
C	5	no	3	9(13)								1 Bomox + Trisoprim	skinny	no Hps									
C	15	no	3	9																			
C	16	no	3	9								1 Bomox + Trisoprim	skinny	9/10	1 Bomox + Trisoprim	skinny	6 + 15						
C	17	no	3	9(13)								1 Bomox + Trisoprim 1	skinny	6									
C	18	no	3	9								1 Bomox + Trisoprim 1 Bomox + Trisoprim	skinny skinny	NT 6+ 10									
C	10	no	1	9(13)																			

group	sow	weaned/ not weaned	piglets tested/sow	serovars	maximum serovars/piglet	at weaning 10/5/07 and 17/5/07	symptom	serovar	22/5/07 Tues no treated(Fri)/sow - symptom - antibiotic/ all treated today	symptom	serovar	29/5/07 Tues no treated(Fri)/sow - symptom - antibiotic/ all treated today	symptom	serovar	29/5/07 no ear tag no treated/sow - symptom - antibiotic// all treated today	symptom	serovar	5/6/07 Tues no treated(Fri)/sow - symptom - antibiotic/ all treated today	symptom	serovar	5/6/07 healthy pigs sampled	serovar	8/06/2007 all already ear tagged pigs treated with Bomox and Trisoprim
T	12	yes	1	9(13)														1 Bomox + Trisoprim	slow	no Hps			
T	19	yes	1	6																			
T	20	yes	1	9								1 Bomox + Trisoprim	skinny	6									
T	21	yes	1	6																	healthy	5 + 6	
T	22	yes	2	9								1 Bomox + Trisoprim	(only slightly) skinny	no Hps	1	skinny	no Hps				healthy		
T	23	yes	2	9(13)																			
T	24	yes	2	9(13)								1 Bomox + Trisoprim	not really skinny	no Hps							healthy	6	
T	25	yes	2	10											1	skinny	10 + NT	1 Bomox + Trisoprim 1 Bomox + Trisoprim	skinny skinny + slow	10 no Hps	healthy	6	
T	26	yes	2	9											1	skinny	no Hps				healthy healthy healthy	6 6 6	
T	27	yes	2	10																	healthy	10 + 6 + 6	
T	29	yes	2	10																	healthy healthy	no Hps 6	
T	30	yes	2	NT																	healthy		
T	31	yes	2	9																	healthy healthy healthy	9 no Hps 9	
T	32	yes	2	NT					1 Bomox + Trisoprim	coughed Fri	no Hps	1 Bomox + Trisoprim	skinny	no Hps							healthy	no Hps 6	
T	33	yes	2	6																			
T	34	yes	2	NT																			
T	35	yes	3	6		1	cough	13															
T	36	yes	3	6																			
T	37	yes	2	13																			
T	40	yes	2	9																			
T	38	no	2	9(13)																			
T	39	no	2	9(13)																	healthy	no Hps	
T	40	no	2	9(13)																			
T	41	no	2	6														1 Bomox + Trisoprim	treated due to skinny today diarrhoea		healthy	no Hps	
T	42	no	2	9(13)																			
T	43	no	2	9(13)																			

Table 3 This is a summary of the numbers of pigs born, death during farrowing, total numbers weaned, numbers tested at weaning and total numbers treated for both experiments.

				Death in farrowing shed due to:						
	Age (days) at weaning	Total number weaned	Total no born	overlay	broken leg	non viables	malnutrition	Total pigs tested at weaning	N of tested with Hps	Total no treated
first experiment	weaned on one day									
Control group (c)	21-25	179	193	10	1		3	54	53	15
Treatment gorup (t)	19-21	186	207	18			3	48	47	26
second experiment	weaned on two days (Thurs+Mo)									
Control group (c)	21-22, 21-24	176	199	13		7	3	69	63	16
Treatment gorup (t)	21-22, 21-24	193	217	13		10	1	50	48	10

Table 4 All serovars present on the farm together with the numbers of genotype per serovar are recorded in this table.

Serovars	Genotypes
1/11	1
2	1
2/1	
6	2
9	4
9/10	1
9/13	1
10	1
11	1
15	2
5	1
12	
13	1
NT	2
14	18

Discussion

This farm is certainly exceptional in regards to the number of serovars of *H. parasuis* found on the farm. When serovar profiling farms over the years we have encountered one farm that had six serovars, but most had from one to five serovars with an average of three serovars per farm. The preliminary sampling on this farm only yielded 8 serovars, while further sampling in the experiments yielded a total of 14 serovars.

In the first trial we did not recover *H. parasuis* from 1.9% of control group and 2.1% of treatment group sampled. In the second experiment *H. parasuis* was not recovered from 8.7% of control and 4% of the treatment group. These percentages are lower than was seen in the preliminary sampling where 12% of the samples did not yield *H. parasuis*.

If the principle of cross-protection of a live strain of *H. parasuis* works, this would mean that the total number of pigs getting sick with *H. parasuis* should reflect the percentage of pigs that are naïve (not colonized) at weaning. This is certainly not the case for the first experiment. Antibiotic treatment was given to 8.4% of the control group pigs and 14% of the treatment group pigs in the first experiment (that is assuming that they had to be treated due to Glässer's disease). In the second

experiment the percent of pigs getting sick (9.1% for control group and 5.2% for the treatment group) was close to the number of naïve pigs in the two groups. The problem with executing experiments on a farm is that other factors will affect the experiment and such a factor might be co-infection and environmental factors. As no necropsies were done on any of the pigs, the symptoms these sick pigs displayed can not be linked definitely to *H. parasuis* infection or other diseases.

When sampling at weaning in both experiments, it is noticeable that serovar 6 was found only in the treatment group. It is not obtained from all treatment animals sampled, but pigs can have more than one serovar of *H. parasuis* in their nasal cavity. This finding of serovar 6 only being retrieved from samples of the treatment group suggest that the controlled challenge has established colonization of the strain.

When comparing the serovar profile of the two experiments it can be seen that the profile changed. In the second experiment the serovar 9 and 9(13) became much more dominant, especially in the control group. Serovar 2 and 1/11 from trial one were not seen in trial two. The serovar 13 in the second trial was not encountered in the first trial. This would suggest that these serovars are not very dominant and are only present in a small number of sows.

H. parasuis is a problem on most farms about 7 - 14 days after weaning. This farm seems no exception to this. In the preliminary sampling 12, 15 and NT were sampled from sick picks, with the necropsy yielding serovar 15. Experience with serovar profiling farms has indicated that if there is a problem with *H. parasuis* on a farm then sampling sick pigs seems to yield pathogenic strains as is seen in the preliminary sampling of this farm (12 and 15 are pathogenic strains, pathogenicity for NT is not known). Seeing that serovar 15 was obtained from a necropsy sample it can be assumed that this serovar is causing problems on the farm. The interesting observation in both experiments is that sampling sick pigs from the control group yields serovar 15, while none of the sick pigs from the treatment group yields this serovar. Especially in the second trial, it becomes apparent that the sick pigs in the treatment group do not yield *H. parasuis* after weaning and if then it is the serovar distributed by the sow. In the first trial a lot more pigs of the treatment group were treated with antibiotics than in the control group (26 verses 15). However, 11 days after weaning, the time when serovar 15 became apparent in the control group, most of the sick pigs of the treatment group had serovars that were non-pathogenic. The pathogenic serovars were only displayed 20 days past weaning, but except of the serovar 9/10, the other serovars were seen at weaning. It might be that these pigs at 20 days past weaning had other problems than *H. parasuis*.

This trial gives some indication that controlled exposure has the potential to work, but the concept needs to be proven on a farm that has an acute problem with *H. parasuis* and were most of the pigs are not colonized with a strain of *H. parasuis*.

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Appendix 3 - Preliminary Trial – Farm 4

Method

A total of 13 sows were vaccinated 5 and 3 weeks before farrowing with a *Haemophilus parasuis* serovar 9 strain from the farm . This is a non-pathogenic serovar based on literature reports. The outbreaks of Glässer's disease on this farm in the past have been associated with a *H. parasuis* isolate that shows a serovar 1/11 cross-reaction. The sows in this trial were split into two groups at farrowing. Seven sows were allocated to the treatment group and six sows allocated to the control group. The piglets of the sows from the treatment group were given a controlled exposure of the serovar 9 strain ($2.4 - 2.8 \times 10^5$ colony forming units per pig) at 5 days of age. There were a total of 71 piglets in the treatment group (given the controlled exposure) and a total of 63 pigs in the control group (not given a controlled exposure).

Two days before weaning 31 pigs of the treatment group and 29 pigs of the control group were nasal swabbed.

At weaning (21 days of age) both groups were sent into the same igloo but in opposite pens. The treatment group pigs were in the pen with all the male pigs and the control group pigs were in the pen with the female pigs. A total of 425 pigs were weaned into that igloo (across both pens) on the day. The other pigs in the igloo were given Draxxin and an inactivated Glässer's disease vaccine that contained the pathogenic strains from the farm but not the serovar 9 strain, at weaning.

Eight days after weaning, one pig from the treatment group and three from the control group were sampled via nasal swabbing. These pigs were selected due to "hollow" looking appearance. On that day, the second phase food was introduced which contained Amoxicillin. Ten days after weaning only second phase food was fed.

Eleven days after weaning, nasal swabs were taken from 39 treatment pigs and 52 control pigs. On that day, Amoxicillin was added to the water, with the Amoxicillin being added daily for 5 days.

Thirteen days after weaning, nasal swabs of another 6 treatment pigs were collected.

At 14 days after weaning, one treatment pig was shifted to the sick pen.

At 18 days post weaning, Amoxicillin was again added to the water (it had not been present for the previous two days). A nasal swab from one treatment pig was collected on that day.

At 25 days after weaning, the pigs were shifted into another igloo.

Results

Table 1. Detailed results and observations

Date	Groups/ observations	Result of swabbing
19 days of age	nasal swabbed 29 control group pigs and 31 treatment group pigs	retrieved serovar 9 from one pig from control group
5 days after weaning	all pigs looked fine	
8 days after weaning	sampled 4 pigs: Treatment group - one hollow looking Control group: two hollow looking and one hollow and skin a bit scruffy looking	Control group: one scruffy pig and one hollow pig - serovar 1/11 third pig - serovar 9
11 days after weaning	all pigs had fallen back, looked tugged in, no coughing swabbed 52 control group pigs and 39 treatment group pigs. Classed pigs according to good, average and poor condition Control group: 28 good, 13 average and 11 poor (54, 25 and 21%) Treatment group: 20 good, 14 average and 5 poor (51, 36 and 13%) took 4 worst control group pigs (6.3%) into the nursery	Control group: 25 pigs (48%) had serovar 1/11, 24 pigs (46%) no Hps, 2 pigs had serovar 9 and 1 pig ? Challenge group: 27 pigs (69%) had serovar 1/11 and 12 pigs (31%) no Hps
13 days after weaning	pigs looked much better and even the four in the nursery, which did not get extra antibiotics except Amoxicillin in feed, looked much better sampled 6 treatment group pigs, two coughed, two looked a bit scruffy and two healthy	Hps serovar 1/11 from two coughing pigs and from one scruffy pig
14 days past weaning	one treatment group pig taken to sick pen - too skinny	
15 days after weaning	pigs looked same as two days before pig in sick pen did get milk and Amoxicillin and had improved	
18 days after weaning	all pigs looked fine, even the ones in the nursery and sick pen one treatment group pig was very small and was sampled	Hps serovar 1/11 from small pig
21 days after weaning	pigs from nursery had been returned to igloo 19 days past weaning, small pig, which was sampled at 18 days, was taken to sick pen	

No trends were observed between sickness of piglets and sow number.

The control pigs were as sick as the rest of the pigs in the igloo. One observation was that of all the other pigs in the igloo, the male pigs looked more affected by the disease than the female pigs.

Out of 425 pigs in the igloo one pig died and 15 pigs (3.5%) were pulled out.

Summary

As these two groups of pigs were in a large igloo with other pigs, all pigs had to be treated when pigs were falling back in health. The strain that causes disease on the farm is very pathogenic and treatment has to be given early, otherwise pigs do not recover. Despite being on antibiotics and having being given a conventional killed vaccine, the pigs not in the control or treatment groups were also falling back, and needed treatment on day 11.

The aim of this experiment was to establish whether the number of sick pigs and the mortality rate in the control group would not exceed the death rate observed previously on farm. It was also meant to establish the safety of the controlled exposure method.

None of the pigs in the experiment died. The four pigs taken out into the nursery where taken out to avoid the treatment of antibiotics which had to be given to all pigs in the igloo via the water. These pigs did not get the antibiotic in the feed. Therefore, the number of sick pigs taken out was only slightly higher due to our artificial intervention (6.3% compared to 3.5%).

In summary, the experiment established that the death rate and the number of sick pigs would not increase in pigs that are not given the vaccine used for the farm and the antibiotics used at weaning.

The management of the farm has agreed to allow further experiments on the farm.

The treatment group had 13% poor pigs when pigs were classed compared to 21% in the control group. However, there were more average looking pigs in the treatment group.

One observation that would indicate that the controlled challenge dose is too low is the fact that *H. parasuis* was not recovered from the nasal cavity of the treatment pigs two days before weaning. Therefore, the next step is to establish a challenge dose at which all treated pigs get colonised.

Appendix 4 - Dosage Trial – Farm 4

Background

During the preliminary trial to test the safety of controlled exposure of *Haemophilus parasuis*, it was discovered that, at weaning, the particular strain used for the controlled exposure –a serovar 9 strain– –given as a controlled exposure (at 5 days of age) in the nostrils of the exposed pigs could not be retrieved from the nasal cavity at weaning. As this could suggest that the dosage rate for the controlled exposure was too low, a titration of higher dosage levels was used to establish whether higher concentration of the controlled challenge inoculum could be retrieved back when sampled just before weaning.

Method

Experiment

Eight sows were vaccinated with an autogenous, killed vaccine twice before farrowing (three and five weeks prior to farrowing). The autogenous vaccine contained the serovar 9 strain, which was used for the controlled challenge of the sows' piglets. There were four dosages used (expressed as organisms given per piglet) were: - 1,000,000 organisms, 10,000,000 organisms, 100,000,000 organisms and 1,000,000,000. The four groups of sows and their litters were well separated by space and had no opportunity to have contact with each other. The sows used in the experiment were low parity sows, as young sows are most likely not to be colonised with *Haemophilus parasuis*.

The piglets were controlled exposed at 6 – 7 days after birth and ear tagged on the day. No antibiotics were administered for a week after challenge. One day before weaning (20 days of age) all the pigs were nasal swabbed to check for colonisation. The pigs were then weaned into two pens in the same eco-shelter. They were separated by sex into these two pens. The eco-shelter holds a total of 400 pigs. At 10 days after weaning a total of 64 pigs from the groups were nasal swabbed. An additional 40 pigs from the other animals (not tagged) in the pens were nasal swabbed (20 female and 20 male).

Table 1. Details of pigs and sampling regime

Ear tag (dosage)	Sow number	Males	Females	Total
Controlled exposed				
Red (10^6 cfu/ml)	U46672			11
Red (10^6 cfu/ml)	U4672			11
Purple (10^7 cfu/ml)	U4689			10
Purple (10^7 cfu/ml)	U4687			10
Blue (10^8 cfu/ml)	U4654			10
Blue (10^8 cfu/ml)	U46683			10
White (10^9 cfu/ml)	U4671			11
White (10^9 cfu/ml)	U4703			12
Sampled one day before weaning				
Red (10^6 cfu/ml)	U46672			10
Red (10^6 cfu/ml)	U4672			11
Purple (10^7 cfu/ml)	U4689			10
Purple (10^7 cfu/ml)	U4687			10
Blue (10^8 cfu/ml)	U4654			10
Blue (10^8 cfu/ml)	U46683			10
White (10^9 cfu/ml)	U4671			11
White (10^9 cfu/ml)	U4703			11
Sampled 10 days after weaning				
Red (10^6 cfu/ml)		8	9	17
Purple (10^7 cfu/ml)		5	10	15
Blue (10^8 cfu/ml)		13	3	16
White (10^9 cfu/ml)		5	11	16
Not tagged		20	20	40

Dosage of controlled exposure

H. parasuis HS2991 (serovar 9) was revived from storage four days prior to the controlled exposure. The strain was plated onto BA/SN agar and blood agar as purity control. After 24 hours at 37°C, the strain was plated onto BA/SN plates. After another 24 hours, the strain was plated onto 50 BA/SN plates. After overnight incubation, the BA/SN plates were used to inoculate 100 chocolate agar plates plus 50 purity control plates which were then incubated for 18 hours. The plates were then harvested to make the four dosages of controlled challenge.

10^6 -Two chocolate agar plates were harvested into 6 ml PBS. A 1,000 µl aliquot of this dilution was added to 4 ml of PBS. From this another 1,000 µl was diluted into 4 ml PBS and that was then added to 46 ml PBS. Prior experiments have shown that this should have yielded 10^6 cfu/ml.

10^7 - Two chocolate agar plates were harvested into 12 ml PBS. Ten ml of this dilution were added to 40 ml PBS. This should have yielded 10^7 cfu/ml.

10^8 - Four plates were harvested into 24 ml of PBS. This was repeated and both dilutions combined. This should have yielded 10^8 cfu/ml.

10^9 - Ten chocolate agar plates were harvested into 12 ml of PBS. This step was repeated twice more and all dilutions combined into one bottle. This should have yielded 10^9 cfu/ml.

All dosages were done in duplicate.

The final dilution for each dosage level was counted by transferring 1,000 µl of the final dilution into 9 ml PBS and doing serial dilutions. A 100 µl aliquot of the relevant dilutions expected to contain 10^4, 10^3 and 10^2 cfu/ml from each dosage level were spread onto BA/SN plates and incubated overnight for counting the next

day. Upon arrival back from the farm the remaining control challenge inocula, which had been kept on ice were counted.

Inoculation

Each pig was given 0.5 ml of the relevant inoculum into each nostril. Piglets with the lowest dosage were done first followed by piglets with progressively higher dosages.

Results

Table 2. Actual doses (expressed as cfu/ml) at preparation and after vaccination

	Predicted Dose			
Preparation	10^6	10^7	10^8	10^9
Count before vaccination	~3.6×10^8	4.3×10^7	3.7×10^8	1.08×10^9
Count after vaccination (9 hours after harvest)	5.1×10^6	3.2×10^7	0.9×10^8	0.6×10^9

Sampling at one day before weaning

H. parasuis was recovered from two piglets, which were both from sow U4654. They had been given a dosage of 10^8 cfu/ml. This means that just two animals out of the 83 animals sampled yielded *H. parasuis*. The serovar recovered from both animals was serovar 9 (the same as given in the controlled exposure and which is also present on the farm).

Sampling 10 days after weaning

Out of the 64 controlled exposed animals sampled, *H. parasuis* was recovered from one pig, which had been challenged with 10^6 cfu/ml. The serovar recovered was serovar 9.

Out of the control animals three animals yielded *H. parasuis*. Two of them had serovar 9, while the third had serovar 1/11.

Discussion

The dosage level did not seem to make a difference in the recovery of *H. parasuis* from the nasal cavity. The low levels at which *H. parasuis* was recovered in the controlled exposured pigs matched the normal low level of serovar 9 which has been found previously on the farm. As an example, in the preliminary experiment two pigs out of 52 control pigs were colonised with serovar 9, which is a prevalence of 3.8%.

As the inoculum of *H. parasuis* was still viable upon return to the laboratory, the assumption can be made that there was nothing wrong with the inoculum. Every precaution was taken not to negatively affect colonisation, including preventing antibiotic treatment for 7 days after the controlled challenge.

The technique of nasal swabbing only samples the bacteria in the upper part of the nasal cavity. It is possible that the bacteria sprayed into the nasal cavity do not colonise in this part of the nasal cavity. It might establish in the lower part of the nasal cavity or in the tonsils and even trachea.

The low prevalence of serovar 1/11 in the control pigs, which should, but did not, at day 10 after weaning come down with Glässer's disease is noteworthy. In the preliminary trial 25 pigs out of 52 control pigs had serovar 1/11, which is a prevalence of 48%.

On the farm there are now sows coming through that have been vaccinated twice again since the strain was left out of the vaccine and caused the outbreak of Glässer's disease on the farm. According to the veterinarian of the farm the disease might now be under control. The animals did not look like they had fallen back, as seen in the preliminary trial.

Appendix 5 - Serovar profiling of *Haemophilus parasuis* on Australian farms by sampling live pigs

Introduction

Haemophilus parasuis, the causative agent of Glässer's disease, is one of the early colonizers of the nasal mucosa of piglets. [1,2] It can be isolated from the nasal cavity, tonsils and trachea of pigs via swabs from H. parasuis challenged pigs (Oliveira and Pijoan 2004; Kirkwood *et al*. 2001; Vahle *et al.* 1997). For isolation of disease causing strains on farms it is usually not recommended to swab from the nasal cavity, as the strains of *H. parasuis* found in the nasal cavity are not necessarily the strains that cause disease (Oliveira and Pijoan 2002). Apparently non-pathogenic isolates of H. parasuis have a high prevalence in the nasal cavity, while pathogenic isolates have a low prevalence(Oliveira and Pijoan 2002). The usual recommendation for isolation of disease causing organisms is to obtain samples from tissues (heart, brain) or fluids (peritoneal, pleural, pericardial, joint) collected at necropsy (Oliveira and Pijoan 2002; Oliveira 2004; Turni and Blackall 2007). This is an expensive and not always successful exercise and multiple isolates still have to be serotyped in case there are multiple serovars present (Oliveira and Pijoan 2002).

A study by Kirkwood *et al*. (2001) found that litters from multiparous sows had a higher level of colonisation with *H. parasuis* than litters from young sows (primiparous). When comparing weaning at two and four weeks it became apparent that the longer period with their mother increased the colonisation levels of the litter (Cegielski *et al*. 1999). These findings suggest that swabbing weaned pigs from multiporous sows (with weaning occurring at 3 weeks of age) should optimize the recovery rate. As pigs suffering from Glässer's disease have the disease causing strain in their nasal cavity (Turni and Blackall 2007) sampling sick pigs might enhance the detection of pathogenic strains on the farm. This paper reports on our evaluation of the ability of nasal swabbing to create a profile of the *H. parasuis* serovars present on farms.

Rafiee *at al*. (2000) evaluated the enterobacterial repetitive intergenic consensus-based (ERIC) PCR as a finger-printing tool for *H. parasuis* strains and concluded that each of the 15 reference strains gave a unique ERIC PCR finger-print, which was reproducible and stable on repeated tests. This reproducibility was also noted for field isolates. A study by Oliveira *et al.* (2003) in which 98 *H. parasuis* isolates from 15 North American herds and multi-farm systems were genotyped by the ERIC PCR and then serotyped via gel precipitation concluded that the genotype of an isolate is a good predictor of the serovar group with a few exceptions where some strains with a similar genomic fingerprint belonged to different serovars. It was not clear whether these exceptions were a real effect or due to incorrect serotyping. In the current study the ERIC PCR was used to genotype isolates and the interpretation standard according to Oliveira (2007) was used.

Method

The data for this paper was collated from work done A) to find a farm free of *H. parasuis,* B) to screen farms for suitability for Glässer's disease vaccine trials and C) to establish the serovars present on farms for diagnostic and disease control purposes. A total of 13 farms (seven in Queensland, one in New South Wales and five in South Australia) were initially sampled over a period of a year. The data was then assessed and a further seven farms were sampled. Except for two farms that never had an outbreak of Glässer's disease (farm 1 and 2), the remaining farms involved in this study either had previous Glässer's disease outbreaks or were

suspected of having Glässer's disease on their farm. Two farms had a previous diagnosed history of Glässer's disease associated with a known serovar (Farms 3 and 7 (Table 1)). The objective of sampling on these farms was to profile the serovars of *H. parasuis* on the farms.

Three farms were sampled more than once (Farm 3, 10 and 13). This occurred as these farms were involved in either experimental vaccine trials (Farm 3) or investigations to monitor the efficacy of conventional autogenous vaccination programs. On Farm 3 twenty-three pigs (from 5 sows) were sampled over the period of 14 days to 72 days of age.

Nasal swabs were either taken from pigs from multiparous sows at weaning time or from sick pigs displaying symptoms typically seen in Glässer's disease (e.g. coughing, anorexia) (Turni and Blackall 2007). The sample sizes varied as it depended on the number of multiparous sows available on the day and on the number of sick pigs that had signs that were possible associated with Glässer's disease (Table 1). There was also an upper limit of 50 swabs that could be handled in the laboratory. From Farm 12, a high health farm, we received only 4 swabs yet 12 pigs were sampled (Table 1). This farm has a connection with Farms 10 and 11 and samples were sent in to determine whether the prevalent serovar on the other farms was also present on this farm.

Necropsies were performed on some farms at the same time as sampling was done. Specifically, necropsies were performed on one pig (Farms 9, 10 and 11), two pigs (Farm 20), three pigs (Farm 13), five pigs (Farm 14 and 17), eight pigs (Farm 16) and ten pigs (Farm 15). The pigs necropsied were extremely sick pigs displaying symptoms typically seen in Glässer's disease. Swabs of tissue or tissue blocks were taken and sent to the laboratory. Swabs of the tissue samples were taken at the laboratory and all swabs were then processed as detailed below.

Sick animals for sampling were selected on the basis of showing clinical signs and there was no attempt made to definitely establish the cause of the clinical signs. The necropsy samples came from different animals than the swabs from sick pigs, except on Farms 15 and 16. Nasal swabs were taken from three or 10 pigs (Farms 16 and 15 respectively) from which lung samples were also taken at necropsy.

Swabs were placed in Amies transport media and kept on ice until inoculated on to BA/SN agar, prepared as previously described (Turni and Blackall 2007), and on to blood agar, the latter being cross-streaked with a nurse colony of Staphylococcus hyicus. The plates were then incubated aerobically for 18 - 24 hours at 37ºC. Suspect colonies of *H. parasuis* were selected and sub-cultured on BA/SN for DNA processing, storage and serotyping.

On most farms only one to two typical *H. parasuis*-like colonies were examined per pig. On Farm 3 at age 43 days up to 4 colonies and at age 72 days up to six colonies were prepared per pig with a total of 34 and 103 isolates, respectively, genotyped. As well, additional colonies were examined on Farms 9 (up to four colonies), 10 (up to three colonies) and 11 (two colonies). Colonies were prepared for PCR as previously described (Turni and Blackall 2007) Isolates were confirmed as *H. parasuis* by the PCR of Oliveira *et al*. (2001) ERIC PCR, as described by Oliveira *et al*. (2003) was used to group the strains within one farm according to genotype profile. A total of 556 isolates were genotyped. The genotype profile was compared only within each farm and the interpretation standard according to Oliveira (2003) was used. If two isolates had the same genomic fingerprint, i.e. an identical band pattern including location and intensity, they were assumed to be the same strain. For multiple sampling on a farm the known strains were run as controls in the ERIC PCR. If only small numbers of *H. parasuis* were being

compared and the differences were quite obvious, then gels were analysed by eye. Otherwise the Bionumercis software (Bionumeric version 4.50, Applied Maths Inc, Sint-Martens-Latem, Belgium) was used to analyse the gels. Within each genotype representative isolate/s was/were serotyped by gel diffusion (GD) testing (Turni and Blackall 2005). If a clear cut answer could not be obtained, the isolates were then examined by the indirect haemagglutination (IHA) test (Turni and Blackall 2005). Isolates which did not react in any of the tests were regarded as non-typable and were termed serovar NT.

The pathogenicity of the serovars was according to the classification of Kielstein and Rapp-Gabrielson (1992) with serovars 1, 5, 12, 13 and 14 being highly pathogenic, serovars 2, 4 and 15 being moderately pathogenic, serovar 8 being slightly pathogenic and serovars 3, 6, 7, 9 and 11 being non-pathogenic.

Results

Overall, a total of 556 isolates of *H. parasuis* were genotyped – with 150 isolates being serotyped.

Of the 20 farms studied, only one farm (Farm 20) failed to yield any isolates of *H. parasuis*. On this farm, nasal sampling was performed on 15 sick pigs. Necropsy of two pigs on Farm 20 also failed to yield *H. parasuis* – although *Actinobacillus pleuropneumoniae* was recovered from the lung of one pig.

On the two farms with an extensive history of freedom from Glässer's disease (Farms 1 and 2), *H. parasuis* of serovars 6 and NT were found in the nasal cavity of weaned pigs (serovar NT on Farm 1 and serovars 6 and NT on Farm 2). However, the colonisation rate was very low (6.7 and 12.5%).

The initial sampling from Farm 3 of 52 pigs yielded serovar NT but not serovar 4, which had caused an outbreak in the past on this farm. Sampling one sick pig in the weaning shed at a later stage yielded a serovar 4 isolate with the same genetic profile as the disease causing strain collected when the outbreak occurred (Table 1). Subsequent sequential sampling (14, 20, 27, 34, 43 and 72 days of age) of a group of pigs over time revealed that this naïve group of pigs remained apparently free of *H. parasuis* until shortly after entering the weaner room (at 34 days of age 13 out of 23 pigs yielded *H. parasuis*). A week after being sent to the weaner room, all sampled pigs in the weaner room (two pigs had been held back) were colonised by *H. parasuis* (21 pigs in the weaner room sampled). At this sampling the NT serovar was the only serovar recovered. At 72 days of age up to six colonies of suspect H. parasuis were picked from each of the inoculated BA/SN plate of all 23 pigs. A total of 103 isolates of H. parasuis were compared using the genetic profile as generated by ERIC PCR. All 23 pigs were shown to harbour serovar NT while six of the pigs had a serovar 4 with these isolates having the same ERIC PCR profile as the strain associated with disease on this farm.

Sampling on Farms 7 and 13 established that sampling healthy weaned pigs does not necessarily reveal all serovars associated with disease on the farm (Table 2). The serovar 12 found during necropsy at an earlier time than the sampling was not found in any of the healthy pigs sampled on Farm 7. The serovar 4 found at necropsy was not found in any healthy pigs sampled from farm 13. In contrast, sampling sick pigs seems to increase the chance of obtaining pathogenic serovars, as no sick pig from the first 13 farms in this study yielded an isolate of *H. parasuis* of a serovar regarded as non-pathogenic. This is demonstrated on Farms 10 and 11 where only sick pigs were sampled and known non-pathogenic strains (like serovars 3, 6, 7, 9 and 11) were not found. However, the farms sampled following evaluation of the method on the first 13 farms yielded 2 to 9 sick pigs with a non-pathogenic serovar (Table 2). On Farm 14 two pigs yielded serovar 6, on Farm 16

eight pigs yielded serovar 7 and one pig serovar 9, on Farm 17 two pigs yielded serovar 9 and on Farm 19 three pigs yielded serovar 9.

The result on Farm 9 where two potentially pathogenic serovars (serovars 5 and 10) were found only in the healthy pigs and not in the sick pigs suggests that sampling healthy pigs is still capable of revealing the presence of potentially pathogenic serovars on a farm (Table 2).

The multiplicity of serovars especially on Farm 9, where a total of eight serovars were detected, is noteworthy. This multiplicity was also seen on Farm 15 with six serovars. Most of the other farms had at least two serovars. The number of serovars recovered varied from one (Farms 1 and 12), two (Farms 2, 3 and 11), three (Farms 4, 5, 7, 8, 10 and 17), four (Farms 6 and 18), five (Farms 13, 14, 16 and 19), six (Farm 15) and eight (Farm 9).

Farm 12, a high health farm, has a connection with Farms 10 and 11 (Table 1). The farm management wanted to know whether the serovar NT found on Farms 10 and 11 was also on Farm 12. A total of 12 pigs, ranging in age from two to four weeks, were sampled with four swabs being used across the 12 pigs. One of the four swabs yielded H. parasuis of serovar NT, with this isolate having the same genotype as the NT strain on the two other farms (Table 1).

Farm 13 again showed sampling both healthy and sick pigs gives a better chance of finding more serovars on a farm. The genetic profile of the serovar found in the lung samples on this farm was different than the serovar 4 found in the nasal cavity of sick pigs at the initial sampling (Table 2). Therefore not all the genotypes present on the farm were found in the initial sampling. The vaccine strain was found in one sick pig only. A second sampling on Farm 10, after the vaccination program with the NT serovar was started, revealed only serovar 4 for one group. This was not a dominant strain previously.

Table 3 shows that occasionally more than one genotype is present for one serovar (Farms 9, 10, 11, 13, 15, 16 and 19). The serovars are not distributed evenly, even on farms where only sick pigs were sampled, as on Farms 10, 11, 16 and 19.

The success rate of isolation of *H. parasuis* from the nasal cavity was higher than from lungs on Farms 15 and 16. On Farm 15 *H. parasuis* was recovered from the nasal swabs of six out of 10 pigs, while only three lung samples yielded *H. parasuis*. On Farm 16 two out of three nasal swabs yielded *H. parasuis* while the lungs did not yield any *H. parasuis*. It is worth noting that the serovars of isolates from the nasal cavity and lung of the same pig did not always match up. On Farm 15, the lung sample of pig 1 yielded serovar 4 , while the nasal swab yielded two genotypes of serovar 4 (one the same genotype as the lung sample). In other examples, pig 6 yielded serovars 4 and 5 from the lung, but serovar 4/6 from the nasal swab, while pig 10 had serovar 4 in the lung but no *H. parasuis* could be retrieved from the nasal swab. The other four animals from Farm 15 yielded H. parasuis from their nasal cavity but not their lung.

Discussion

H. parasuis is part of the natural flora of the upper respiratory tract of pigs (Nedbalcova *et al*. 2006) so it is not surprising that *H. parasuis* was found in the nasal cavity of weaned pigs on two farms with an extensive history of freedom from Glässer's disease. According to Kielstein and Rapp-Gabrielson (1992) serovar 6, the serovar found on these two farms, is a non-pathogenic serovar. It would appear that the non-typable (NT) serovars found on these two farms without outbreaks were also non-pathogenic as the prevalence of these strains was very low and these farms have been free of Glässer's disease outbreaks. Further sampling of both

healthy and sick pigs would be required before any conclusions can be reached on whether Farm 20 is truly free of *H. parasuis*, as only sick pigs were sampled on this farm.

The NT strain found on Farm 3 was assumed to be non-pathogenic, as it spread so rapidly without causing any symptoms of disease in the naïve pigs. The low prevalence of the pathogenic serovar 4 strain on Farm 3 matches the finding of other researchers of the low prevalence of pathogenic strains (Oliveira and Pijoan 2002). The large sample size needed to find this low prevalence strain indicates that multiple serovars may not easily be detected if one strain is more prevalent than the others. Clearly, the question arises of how many colonies have to be sampled to find strains circulating at a low prevalence. The result of the current study, that many isolates of *H. parasuis* can be obtained from a given herd, but in most cases only one or two strains predominate, coincides with the finding of Smart *et al*. (1988). Except for a few limited studies, such as on the colonisation rate of weaned pigs (Kirkwood *et al*. 2001; Cegielski *et al.* 1999), and attempts to determining the prevalent serovars in pigs (Kirkwood *et al*. 2001; Smart *et al*. 1988; Olvera *et al*. 2006), there is no actual study looking at the diversity within the single pig on several farms (on farms with more than two serovars) and the prevalence of strains over time. Therefore, there is no guidance as to how many samples have to be taken to establish the serovars present in a pig, especially if one strain is dominant.

Not all pathogenic serovars on a farm were detected when only healthy weaned pigs were sampled. On Farm 7 serovar 12, a highly pathogenic serovar, was found from an earlier necropsy sample but not detected in any of the 30 nasal swab samples. This serovar 12 strain was particularly hard to grow and might have easily been missed in nasal swabs due to its poor growth. The difference in growth characteristics of various strains has been observed by others (Smart *et al*. 1988) and it has been suggested that some strains could be missed due to this heterogeneity(Smart *et al*. 1988).

Sampling sick pigs which showed clinical signs suggestive of Glässer's disease yielded only strains of serovars regarded as pathogenic and serovar NT when the first 13 farms were evaluated. Despite the dominant serovar NT strain on Farm 3, the pathogenic serovar 4 strain on this farm was recovered effortlessly in the nasal cavity of a sick pig (only one colony was collected). These results, combined with the fact that pathogenic strains of *H. parasuis* given in artificial challenges can be found in the nasal cavity (Oliveira and Pijoan 2004; Segales et al. 1997) suggest that there is definitely a correlation between sick pigs and the presence of pathogenic serovars of *H. parasuis* in the nasal cavity. Overall, it seems that strains of *H. parasuis* of known pathogenic serovars can be found in the nasal cavity of sick pigs and are most likely disease-causing strains.

The finding of strains of non-pathogenic serovars of *H. parasuis* in sick pigs on Farms 14, 16, 17 and 19 complicates the correlation between sick pigs and the presence of the disease-associated serovars of *H. parasuis* in the nasal cavity noted on the first 13 farms. However, there might be other explanations for these findings of non-pathogenic serovars in sick pigs. One explanation could be that the "sick" pigs with non-pathogenic serovars were not suffering from Glässer's disease. On Farm 17 the non-pathogenic serovar was isolated from a pig out of a shed that had no sick pen and pigs were quickly chosen based on poor condition or coughing. On Farm 20 it certainly seems that none of the sick pigs suffered from Glässer's disease as the necropsy samples revealed *Actinobacillus pleuropneumoniae,* but not *H. parasuis*. The serovar 9 isolate on Farm 16 came from a sick pig that also had two pathogenic serovars. It is most likely that the pig was naïve before

entering the weaner facility, picked up the serovar 9 together with the pathogenic strains and hence became sick due to lack of protection against systemic invasion.

The other explanation could be that these isolates of so-called "non-pathogenic" serovars differ from the formal reference strains that were used in the pathogenicity studies described in the literature. The serovar 7 isolate on Farm 16 was atypical in terms of the serotyping reaction. This isolate was identified as serovar 7 by the IHA methodology but could not be serotyped by the GD method. Similar unusual reactions occurred with the isolate of non-pathogenic serovar 6 on Farm 14 – with no reaction in the IHA but a reaction in the GD method. It is possible that these "atypical" isolates are not truly non-pathogenic.

The multiplicity of serovars on Farm 9 and 15 were higher than other researchers have observed. Smart et al. (1988) observed that most farms harboured two to four strains. The other farms in the current studies conform with the observation of Smart *et al*. (1988). Oliveira *et al*. (2003) looked at 10 herds in America and recovered 1 to 3 serovars per herd.

Our results suggest that the serovar can change in time if certain strains are used for vaccination. As an example on Farm 13, the vaccine strain on farm 13 was found in one of nine sick pigs and none of the weaned pigs. In similar light, after vaccination with the NT serovar on Farm 10 one group of seven sick pigs had serovar 4 only, which was not the dominant serovar before vaccination. These changes in serovar prevalence might have to do with the normally low prevalence of pathogenic strains in healthy animals (Oliveira and Pijoan 2002), while diseased animals might have a higher prevalence of pathogenic strains and cause a higher spread of these strains. A study by Olvera *et al*. (2007) found that the diversity of strains isolated on a farm was affected by antimicrobial treatment. Immediately following antibiotic treatment, they could only recover one of the three strains present on the farm before antimicrobial treatment and this strain was resistant to the antibiotic used. However, one year after treatment the diversity of the strains was back to the diversity before the treatment. This would suggest that the susceptible strains were still there at low prevalence and it stresses the importance of serovar profiling to find all the strains that are potentially disease causing organisms. Overall, the results from farms where sick pigs were sampled indicate that both methods (swabbing healthy weaned pigs and sick pigs) should be combined to enhance results of the profiling.

On Farm 13 the genotype of serovar 4 isolate found in the lungs was a different genotype from the serovar 4 isolate found in the nasal cavity of diseased pigs. However, nasal sampling the pigs at a later stage did yield an isolate of the same genotype as the lung samples. This suggests again that sample size is important, especially if more than one serovar is causing Glässer's disease on the farm. Sampling lung samples and nasal cavity at the same time on Farm 15 revealed that the serovar might not necessarily be the same from both sampling sites. The explanation for this might be that several serovars cause the disease. Hence, multiple nasal samples might have yielded the pathogenic isolates found in the lung. The finding that multiple strains can be found in the lung indicating that more than one strain can be involved in a clinical outbreak as has been reported previously by Olvera *et al*. (2006).

This study found only two different genotypes for some serovars, while other authors have discovered a much higher genetic diversity within serovars (Oliveira *et al*. 2003)

In summary, *H. parasuis* was found on farms where no outbreak occurred. Isolates of serovars recognised as being pathogenic can be easily missed if another strain is

more prevalent. Detection of isolates occurring at a low prevalence requires examining multiple colonies per nasal swab. Sampling healthy weaned pigs does not necessarily reveal all pathogenic serovars. The sample size of pigs is important in the detection of strains on a farm. A bigger sample size of sick pigs on some of the farms might have revealed all pathogenic serovars on the farms.

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Table 1. Results of swabbing the nasal cavity of pigs from multiparous sows at weaning or swabbing pigs with symptoms of disease possibly associated with Glässer's disease from 20 farms across Queensland, New South Wales and South Australia. Pathogenicity was assigned to the serovars according to Kielstein and Rapp-Gabrielson.[14] Farms with multiple entries were sampled at different times. Necropsy samples came from different pigs to the sampled pigs, except on Farms 15 and 16.

Farm	Sample size	Age of pigs	sick/ healthy	No. with Hps	no of serovars recovered			No with Hps/no necropsied	autogenous/commercial-sow/piglet vaccine used
					potentially pathogenic	non-pathogenic	non-typable or pathogenicity not referenced		
1	30	21-22 days	healthy	2			1		
2	24	24 days	healthy	3		1	1		
3	52	3 weeks	healthy	5			1	?/?	autogenous sow
3	1	over 5 weeks	sick	1	1*				
3	23 23 23	34 days[a] 43 days 72 days	healthy	13 21 23	 1*		1 1 1		
4	30	3 weeks	healthy	12	2	1			
5	30	3 weeks	healthy	11	2	1			
6	30	3 weeks	healthy	17	2	1	1		
7	30	3 weeks	healthy	16	1	1	1	?/?	
8	30	3 weeks	healthy	17	1	1	1		
9	40 7	3 weeks 3 - 5 weeks	healthy sick	35 6	3* 2*	2	2 1	1/1	
10	7 8 2 (very sick)	31 days 49 days 28-49 days	sick	4 7 ?[b]	2*		1	1/1	
10	6 6 7	4 weeks 5 weeks 6 weeks	sick	1 4 4	 2* 1		1 1 -		autogenous sow
11	11	3 weeks onwards	sick	8	1		1	1/1	
12	12	2 - 4 weeks	healthy	1 swab			1		
13	31 9	at weaning ?	healthy sick	27 4	 1	1	1 2	 2/3	autogenous sow/commerial piglets
13	6	?	sick	2	1*				autog. sow/com. piglet
14	25 5	3 weeks 5-6 weeks	healthy sick	23 3	2* 1	1 1	1 -	3/5	
15	10 20	19 days ?	healthy sick	7 13	4* 4*		1	4/10	
16	38	4 weeks	sick	16	3	2*		0/8	
17	40	3 - 6 weeks	sick	21	1	1	1*	1/5	
18	10	?	sick	9	2		2		
19	15	6 - 7 weeks	sick	12	2	1	2		
20	15	?	sick	0				0/2	

? number with Hps / number necropsied not known. Necropsis done proir to the study period.

***** one of the serovars has the same genotype as the strain found at necropsy on the farm.

[a] sampling was done prior to this at 14, 20 and 27 days of age, which yielded no *H. parasuis*

[b] isolation of Hps impossible due to proteus contamination

Table 2 Detailed results of farms where a) sampling healthy pigs did not necessary reveal all pathogenic strains (Farms 7 and 13), b) sampling sick pigs revealed only pathogenic strains or possible pathogenic strains (Farms 3, 9, 10, 11, 13, 15 and 18) and c) sampling sick pigs revealed non-pathogenic strains (Farms 14, 16, 17 and 19)

Farm	Sample size	Sick/healthy pigs	No. with Hps	Serovars recovered			Serovars of necropsied sick pigs
				potentially pathogenic	non-pathogenic	non-typable or pathogenicity not referenced	
7	30	healthy	16	4	9	NT	12
13	31	healthy	27		9	NT	
	9	sick	4	4		4/7,10/13/4	4
13	6	sick	2	4 *****			
3	1	sick	1	4*****			4
9	40	healthy	35	5, 10, 15*****	6, 9	NT, 9/13	15
	7	sick	6	12, 15*****		NT	
10	15	sick	11	4*****, 15		NT	4
10	19	sick	9	4*****, 15		NT	
11	11	sick	8	15		NT	4
15	10	healthy	7	4*, 10, 12, 13			4, 5
	20	sick	13	4*, 5*, 10, 12		4/6	
18	10	sick	9	10, 10/6, 10/15, 15			
14	25	healthy	23	15*, 5	6	9/13	15, NT
	5	sick	3	5	6		
16	38	sick	16	4, 10, 12	7, 9		
17	40	sick	21	15	9	NT*	NT
19	15	sick	12	4, 15	9	NT, 4/6/7	

Table 3 Details of the serovars and genotypes found on four farms.

Farm	Serovars (no of pigs with serovar)	Genotypes (for serovar)
3	NT (23)[A], 4 (6)	1 (NT)[B], 1(4)
9	6 (10), 10 (9), 9 (5), 9/13 (5), 15 (4), 5 (2), NT (2)	1 (5), 1 (6), 1 (9), 1 (9/13), 1 (10), 2 (15), 1 (NT)
10	NT (9), 4 (2), 15 (2),	2 (15), 1 (4), 1 (NT)
11	NT (6), 15 (3)	2 (15), 1 (NT)
13	NT (19) , 9 (8), 4 (3), 4/7 (1), 10/13/4 (1)	2 (9), 1 (4), 1 (4/7), 1 (10/13/4), 1 (NT)
15	4 (10), 10 (9), 12 (4), 4/6 (1), 5 (1), 13 (1)	2 (4), 1 (4/6), 1 (5), 1 (10), 1 (12), 1 (13)
16	7 (8), 4 (3), 12 (3), 9 (1), 10 (1)	2 (4), 1 (7), 1 (9), 1 (10), 1 (12)
19	NT (5), 9 (3), 4 (2), 4/6/7 (2), 15 (1),	2 (NT)1 (4), 1 (4/6/7), 1 (9), 1 (15)

[A] The serovar is presented and then the number of pigs with that serovar is given in brackets. The serovars are ordered according to their prevalence

[B] The various genotypes are listed with the serovar being presented in brackets.

Appendix 6 - Validation of a Real Time PCR for *Haemophilus parasuis*

Introduction

In 1906, a disease of young pigs associated with polyserositis and arthritis was described by Karl Glässer (Sutherland and Simmons 1947). This disease is now known as Glässer's disease and is caused by the bacterium *Haemophilus parasuis* (Oliveira and Pijoan 2004). *Haemophilus parasuis* is a fastidious and delicate organism, characteristics which cause problems in the diagnosis of the disease (Ferri *et al.* 2000). Recommendations on the best sampling sites and on transport media for swabs and temperatures are available in the literature and from laboratories involved in the processing of samples (Oliveira 2004; Turni and Blackall 2007a). However, the conditions in the field are not always optimal, which makes culturing samples difficult and creates a dependence on a sensitive and specific PCR to use on DNA templates extracted from tissues, fluids and swabs directly. The PCR developed by Oliveira *et al*. (2001) has problems in specificity giving a weak positive with *Actinobacillus indolicus*, lowering the value of the test when it is applied to samples from upper respiratory sites where both *H. parasuis* and *A. indolicus* can be present (Oliveira *et al.* 2001). The PCR developed by Angen *et al*. (2007) does not report problems with specificity, but seems not as sensitive as the one of Oliveira *et al.* (2001) (Angen *et al*. 2007). The PCR of Oliveira *et al*. (2001) can detect a minimum concentration of 1 x 10^2 cfu ml^{-1} of *H. parasuis* (Oliveira *et al*. 2001). When the conventional PCR of Oliveira *et al*. (2001) was compared to the culture method for swabs taken from pigs challenged with *H. parasuis*, the culture method was more sensitive than the conventional PCR (Turni and Blackall 2007a). According to Espy *et al*. (2006) the sensitivity of the real time PCR is in some cases greater than the conventional methods used for bacteriological diagnostics (culture and conventional PCR). A real time PCR has the potential to be highly sensitive and specific (Espy *et al*. 2006; Mackay 2004; Valasek and Repa 2005).

The primers developed for the two conventional PCRs for *H. parasuis* target the 16S ribosomal RNA gene, which is conventionally used as a taxonomic and phylogenetic marker (Wilson et al. 1990). However, a preliminary study in our laboratory could not differentiate *Pasteurella mairii* from *H. parasuis* for the short amplification product of the real time PCR technology selected, when using the 16S rRNA as the target. A possible alternate gene to the 16S rRNA gene is the *inf*B gene. Hedegaard *et al*. (2000) concluded that the *inf*B gene might be useful as a genetic marker for phylogenetic studies. The *inf*B gene codes for the two forms of the translation initiation factor IF2 – IF2 alpha and IF2 beta (Hedegaard *et al*. 2000).

This paper describes a real time PCR method developed at the Animal Research Institute targeting the *inf*B gene of *H. parasuis*. The validation of this method includes the testing of DNA from *H. parasuis* reference and field strains, DNA from closely related bacterial species and DNA extracted from swabs of tissue and fluids and from fluid and tissue samples from seven serovar 12 and nine serovar 4 experimentally challenged pigs.

Material and Methods

Bacteria

To determine the specificity of the real time PCR, a total of 68 *H. parasuis* strains covering all 15 recognised serovars were tested. This selection included 15 reference strains, the taxonomic type strain, 42 Australian field isolates collected

from 13 farms representing 10 identified serovars and 10 isolates from Denmark representing 9 serovars. All isolates/strains were identified by the PCR of Oliveira *et al*. (2001) and serotyped according to the method by Turni and Blackall (2005). A total of 36 non-*H. parasuis* isolates covering 24 species were used. Tables 1 and 2 provide the details of the isolates used in this study.

A conventional PCR targeting the nuclear ribosomal RNA 16S rRNA gene with universal bacteria primers (5' GAGTTTGATCCTGGCTCAG 3' and 5' AAGGAGGTGWTCCARCC 3') was performed on the template of all bacterial species other than *H. parasuis* to confirm that the template was suitable for use in PCR. The 16S PCR was run as described by Miflin and Blackall (2001) with the exception that the mix was briefly UV irradiated before the primers and template were added to the mix to inactivate any contaminating DNA.

Clinical samples

The clinical samples examined were generated in previously described experiments that used 16 naturally farrowed, colostrum deprived pigs subjected to intra-tracheal challenge with either *H. parasuis* serovar 4 (HS 1387) or serovar 12 (H425) (Turni and Blackall 2007a).

DNA preparation from bacterial isolates and samples from challenged animals

Bacteria species were grown on BA/SN (Turni and Blackall 2007b) overnight and a 1 μl loopful of growth was suspended in 100 μl of water. The suspension was heated at 98°C for 5 min, followed by cooling on ice for 5 min. After centrifugation for 5 min at 17,380 x *g*, the supernatant was collected and stored at -20°C. A 1 μl aliquot of the supernatant was used for PCR analysis. Alternatively, 1 μl loop of growth was suspended in 200 μl of PrepMan Ultra (Applied Biosystems, Foster City CA) and boiled for 10 min suspended in boiling water. After cooling the suspension for 3 min, the suspension was spun at 17,380 x *g* for 3 min and the supernatant collected and stored at -20°C until use.

DNA from the swab, tissue and fluid samples of the *H. parasuis* challenged animals were prepared with the PrepMan Ultra method as previously described by Turni and Blackall (2007a).

Real time PCR

Available sequences of the *infB* gene of *H. parasuis* (gene bank accession numbers: DQ781806, DQ781808, DQ781810 - DQ781817) and related species (accession numbers: DQ211781 - *Actinobacillus rossii*, DQ211790 - *Pasteurella mairii*, DQ211791 - *P. aerogenes*, EF059970 - *A. porcinus*, EF059971 - *A. indolicus*) on GenBank were aligned with the software Sequencher 4.5 (Gene Code Corporation, Ann Arbor, MI). Primers and probes were designed for a species-specific region of the gene with the Primer Express® Software (Applied Biosystems, CA, USA). The primers designed were the forward primer CTinfF1: 5' CGACTTACTTGAAGCCATTCTTCTT 3' and reverse primer CTinfR1: 5' CCGCTTGCCATACCCTCTT 3', which target the 392 to 466 base pair region of the gene. The FAM labelled TaqMan probe with a TAMRA quencher was supplied by Applied Biosystems (CA, USA) with the following sequence CTinfP 5' 6FAM-ATCGGAAGTATTAGAATTAAGTGC –TAMRA 3'. The 25 μl reaction mix consisted of 7 μl of H_2O, 10 μl of 5 Prime RealMasterMix Probe (Quantum Scientific, Milton, QLD, Australia), 100 nmol l^{-1} CTinfF1, 400 nmol l^{-1} CTinfR1, 100 nmol l^{-1} of CTinfP and 5 μl of template (maximum concentration of 200 ng μl^{-1}). The template was diluted one in ten if DNA was prepared from a culture plate. The reaction was run on a Corbett Rotor-Gene 3000 (Corbett, Mortlake, NSW Australia) real time thermal cycler with the following cycling conditions: first cycle at 95°C for 2 min, followed

by 45 cycles of 95˚C for 20 sec and 58˚C for 60 sec, this is then followed by one cycle at 28˚C for 1 min. Fluorescence data were acquired once every cycle at the end of the extension phase. The amplified product was 74 base pairs long.

Sensitivity of the real time PCR

Growth of *H. parasuis* CCUG3712^T was harvested from an overnight incubated NADH supplemented chocolate agar plate (Turni and Blackall 2007a) into phosphate buffered saline (PBS) and the concentration of the suspension was adjusted to approximately 1.8 x 10^8 cfu ml^{-1}. The concentration of the suspension was confirmed by viable counts performed on chocolate blood agar that were incubated for 24 hour. The suspension was then diluted in a 10 fold series in PBS to give dilutions containing 10^0 to 10^5 cfu ml^{-1}. A 1 ml aliquot of each dilution was used to extract DNA with the PrepMan Ultra method and 100 μl of each dilution was used to extract DNA with the boiling and cooling method. The extraction of DNA was done in triplicate for each method and each dilution. The sensitivity experiment was repeated for the boiling and cooling method. The sensitivity was also tested for dilutions of *H. parasuis* that also contained 1.265 x 10^8 *Escherichia coli* per dilution. Furthermore, the sensitivity was tested by diluting *H. parasuis* in pericardial fluid from healthy pigs instead of PBS.

Sensitivity was also tested for three serovar reference strains of *H. parasuis*: SW 124 (serovar 4 regarded as moderately pathogenic), Nagasaki (serovar 5 regarded as highly pathogenic) and 174 (serovar 7 regarded as non-pathogenic). The harvested growth was again adjusted to approximately 1.8 x 10^8 cfu ml^{-1} and the concentration confirmed by viable count. However, instead of diluting the cell suspension the DNA was extracted with both methods from the original suspension. After determining the DNA concentration with a spectrophotometer the DNA suspensions were then 10 fold serial diluted with sterile H_2O.

Genotyping of *H. parasuis* field isolates with ERIC PCR

All 42 Australian and the 10 Danish field isolates of *H. parasuis* were genotyped to determine the number of different genotypes present in the collection. The Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, performed as previously described by Oliveira *et al.* (2003), was used to genotype the isolates. A dendrogram was constructed with the Bionumeric software (Bionumeric version 4.50, Applied Maths Inc, Sint-Martens-Latem, Belgium). Visual comparison of the band pattern was used to determine the similarity cutoff point.

Comparison of PCR results using clinical sourced material

The real time PCR was run on DNA samples from a previous challenge experiment performed by Turni and Blackall (2007a). This challenge experiment was set up in two parts: in the first trial seven naïve animals were challenged with serovar 12, while in the second trial nine naïve animals were challenged with serovar 4. Tissue and fluid samples were taken from these animals two to seven days after challenge. These tissue and fluids were processed in two different ways: one sample was cultured, while the other sample was processed for PCR. Two different samples were taken for PCR: one was a swab from tissue or fluid and the other was the tissue and fluid samples. In the current work the real time PCR was run on DNA that had been stored at -20°C from these challenge trials. The results of the real time PCR were compared to results of the conventional PCR of Oliveira *et al*. (2001) and to results of the culture method, both of which have been reported previously (Turni and Blackall 2007a).

DNA was tested from tissue and fluids swabs of the brain, mandibular lymph node, trachea, lung, pleural fluid, pericardial fluid, heart, liver, peritoneal fluid, fibrin in

the peritoneum and joint or synovial fluid for both the serovar 12 and 4 challenge. The other types of samples analysed were DNA obtained from tissue samples from tonsils (only serovar 12), trachea, lung, heart, lymph node and brain for animals challenged with either serovar 12 or 4. In addition fluid samples (peritoneal, pleural, pericardial, articular and cerebrospinal) were obtained and DNA extracted from these samples in the serovar 4 challenge experiment. In the serovar 4 challenge no culture was performed on the tissue and fluid samples that were processed directly for DNA.

A total of 71 and 90 samples of DNA from swab of tissue and fluids were analysed from serovar 12 and 4 challenge experiments, respectively. For the tissue and fluid samples a total of 26 and 52 samples of DNA were processed from serovar 12 and 4 challenge experiment, respectively.

Statistics

A generalized linear mixed model with method (real time PCR/PCR/culture), serovar, tissue and interaction as fixed effects and animal within serovar as a random effect was fitted to the data assuming a binomial error distribution with a logit link function. Pair-wise differences between means of the logits were tested using a protected least significance difference procedure (P = 0.05). All analysis was done using GenStat (Statistics. VSN International, Hemel Hempstead).

Results

Validation of Real Time PCR method

The *infB* gene gave good separation for *H. parasuis* from the closely related bacterial species tested in this study. None of the closely related species amplified in the real time PCR. All 15 reference strains, the type strain, the 42 Australian and 10 Danish field isolates of *H. parasuis* tested gave positive results with the real time PCR.

Visual examination of the dendrogram, constructed from the ERIC PCR gel profile of the 42 Australian and 10 Danish field isolates, showed that a >96 % similarity cut-off was appropriate for recognizing genotypes and 46 genotypes were recognized amongst the 52 Australian and Danish field isolates.

Sensitivity

The Ct scores and the concentrations of the reaction mix for which all three samples yielded a positive result for the CCUG3712 are shown in Table 3. The results for the three other serovar reference strains of *H. parasuis* tested are also shown. From these data the cut-off points for a positive were determined. The cut-off points for the questionable and negative Ct scores were determined from the Ct values where not all three replicates of CCUG3712 yielded uniform positive results (data not shown). The positive cut-off point was selected as the highest Ct score shown in Table 3. Hence, the selected cut-off points were as follows:

Positive - a Ct score up to 42

Questionable - a Ct score of 43 to 45

Negative - a Ct score above 45

Real Time PCR compared to conventional PCR and culture method

Overall, the real time PCR from 161 swabs of tissue and fluid gave a total of 50 (31.06%) more positive results than the conventional PCR over the two challenge trials and 14 (8.70%) more positive results than the culture method (Table 4). The difference between the real time PCR and the conventional PCR were statistically significant for both serovar challenges. In the serovar 12 challenge the real time

PCR had 37 (52.11%) more positives than the conventional PCR. In serovar 4 challenge the difference was only 13 (14.45%) more positive results (Table 4).

When the real time PCR was run with the DNA obtained from tissue and fluid samples the real time PCR obtained significantly more positive results than the conventional PCR and the culture method (Table 4).

A more detailed analysis of the instances where the real time PCR gave a positive result with samples that were negative by culture is presented in Table 5. Many of the culture negative/real time PCR positive samples involved the tonsils. The Ct scores for tonsil swab samples, which came up negative in culture, ranged from 26.95 to 36.05, while the Ct scores from tonsil samples ranged from 29.43 to 38.34. A closer look at data from Table 4 reveals that swabs from tonsils gave six positive results out of six samples for the real time PCR and three for the conventional PCR for serovar 12. For serovar 4 the real time PCR produced nine positive results out of nine tonsil swab samples and the conventional PCR produced seven positive results. When the tonsil tissue was processed directly the real time PCR produced seven positive results out of seven samples and three positive samples for the conventional PCR for the serovar 12 challenge. Cultures on the tonsils were mostly overgrown and a sweep was performed on the growth which was analysed with a conventional PCR (Turni and Blackall 2007a). If the conventional PCR came up negative the culture was called negative. From the analysis of the real time PCR it becomes apparent that this sweep method with the conventional PCR is not sensitive enough to detect low numbers of *H. parasuis* in the sweep.

Discussion

A real time PCR was developed with primers that target the *infB* gene. The commonly used 16S rRNA gene was not species-specific to separate *H. parasuis* from the other closely related species for the short amplification sequence of a real time PCR (data not shown). No region could be found within the 16S rRNA gene where the primer sequences for *H. parasuis* were sufficiently different from other species.

While studying the *infB* gene as a possible tool to study the population structure of *Streptococcus agalactiae*, Hedegaard *et al*. (2000) discovered that the *infB* gene revealed limited intra-species diversity within *S. agalactiae*. Hedegaard *et al.* (2000) concluded that the *infB* gene is useful as a genetic marker for phylogenetic studies of species. This observation that the *infB* gene can separate species has also held true for *H. parasuis* in the current study. The gene proved to be a good target for real time PCR primers for *H. parasuis*, separating *H. parasuis* from all other closely related species. The intra-species variation of the *infB* gene also seems to be very limited for *H. parasuis*. All of the 42 field samples and 10 Danish isolates, which represented 46 different genotypes, as well as all 15 serovar reference strains and the taxonomic type strain (CCUG3712), amplified with the primers targeting the *infB* gene.

The isolation of *H. parasuis* is difficult, as the bacterium is very sensitive to pH changes and heat (Morozumi and Hiramune 1982). *H. parasuis* is also a slow growing, fastidious organism with specific nutritional requirements (Ferri *et al.* 2000; Oliveira and Pijoan 2004). This makes recovery of the organism after sample collection and transport to the laboratory very difficult. Once in the laboratory the isolation is very difficult as *H. parasuis* is easily overgrown by other faster growing bacteria. Therefore, the method of identification by culture is not always optimal and PCR-based methods are an attractive alternative. The available conventional PCR assays; display problems with non-specific bands (Oliveira *et al.* 2001) or sensitivity (Angen *et al.* 2007). The real time PCR method described here is more

specific and does not give a positive reaction with any of the 24 non-target species (including all close relatives) tested. Oliveira *et al*. (2001) reported a sensitivity of 0.5 cfu per reaction, while Angen *et al*. (2007) reported a sensitivity of 5.3 cfu per reaction for their PCR. According to our sensitivity assays, the real time PCR showed a sensitivity of 9.5 to 0.83 cfu per reaction for the boiling method of DNA extraction and 47.5 CFU per reaction to 0.42 cfu per reaction for the PrepMan Ultra method. However, when looking at the data from the challenged animals, where the Oliveira *et al*. (2001) PCR was used, the real time PCR gave significantly higher numbers of positive results. Overall, our data indicate that the real time PCR is more sensitive than the conventional PCR, even though our minimum detectable cfus per reaction were higher than the reported minimum detectable cfus of the conventional PCR (Oliveira *et al*. 2001).

In a study by Turni and Blackall (2007a) the culture method was deemed more successful than the conventional PCR method when detecting *H. parasuis* in sick animals. The results of the current study indicate that this does not hold true for the real time PCR. The real time PCR performed better than culture, although not at a statistically significantly level for the DNA from swab samples. In laboratories where the culture methods are not as optimized as in our laboratory, the real time PCR should perform even better compared to culture. Therefore, we believe that real time PCR will enhance the diagnosis of *H. parasuis*, especially for laboratories that are not experienced with the culture of *H. parasuis* or in situation where culture is not possible.

Strains of *H. parasuis* can be easily found in the nasal cavity of sick pigs (Turni 2009). However, nasal swabs are not suitable for use in the conventional PCR of Oliveira and Pijoan (2004) as that PCR gives false positive reactions with *A. indolicus*, an organism known to be present in the upper respiratory tract of pigs (Oliveira and Pijoan 2004). However, as the real time PCR described in this study does not give false positives for *A. indolicus*, it is possible to use this assay on nasal swabs or other upper respiratory tract samples. From the results of the current study it is apparent that the conventional PCR also has problems in detecting the presence of *H. parasuis* in overgrown cultures and that the use of sweep method and subsequent conventional PCR analysis (as performed on overgrown culture plates in this study) is not an effective method for the detection of *H. parasuis*. The Ct scores of the real time PCR assays on the tonsil samples indicate that there were sufficient numbers of bacteria in the tissue to indicate that sufficient numbers of *H. parasuis* were present and that these organisms should have been transferred to the culture plate and should have been present in the subsequent plate sweeps used as a source for the conventional PCR. This problem of the conventional PCR (low sensitivity in the presence of other bacteria) is overcome with the real time PCR. The sensitivity assay revealed that the real time PCR detected low numbers of *H. parasuis* even in the presence of large numbers of non target cells. The ability of the real time PCR to perform well in the presence of large number of other bacteria was seen in the results obtained from the samples processed from the tonsils of the challenged animals. The real time PCR significantly outperformed the conventional PCR-culture method.

When using this real time PCR assay as a diagnostic tool it has to be pointed out that the presence of *H. parasuis* in the upper respiratory tract does not mean that there is a problem with *H. parasuis*, as non-pathogenic serovars can be found in the upper respiratory tract. Therefore, if the real time PCR is used as the diagnostic tool to determine the cause of disease, the assay should be used from samples from internal organs and tissues.

In conclusion, the *infB* gene targeted real time PCR for *H. parasuis* performed well for pure culture, swabs from tissue and fluid or tissue and fluid processed directly regardless of contamination by other bacterial species. The high sensitivity and specificity of the real time PCR make it an ideal diagnostic tool for *H. parasuis*. The real time PCR has distinct advantages over conventional PCR - less risk of non-specific binding and contamination, less handling time (no post reaction analysis) and the potential of high throughput automation.

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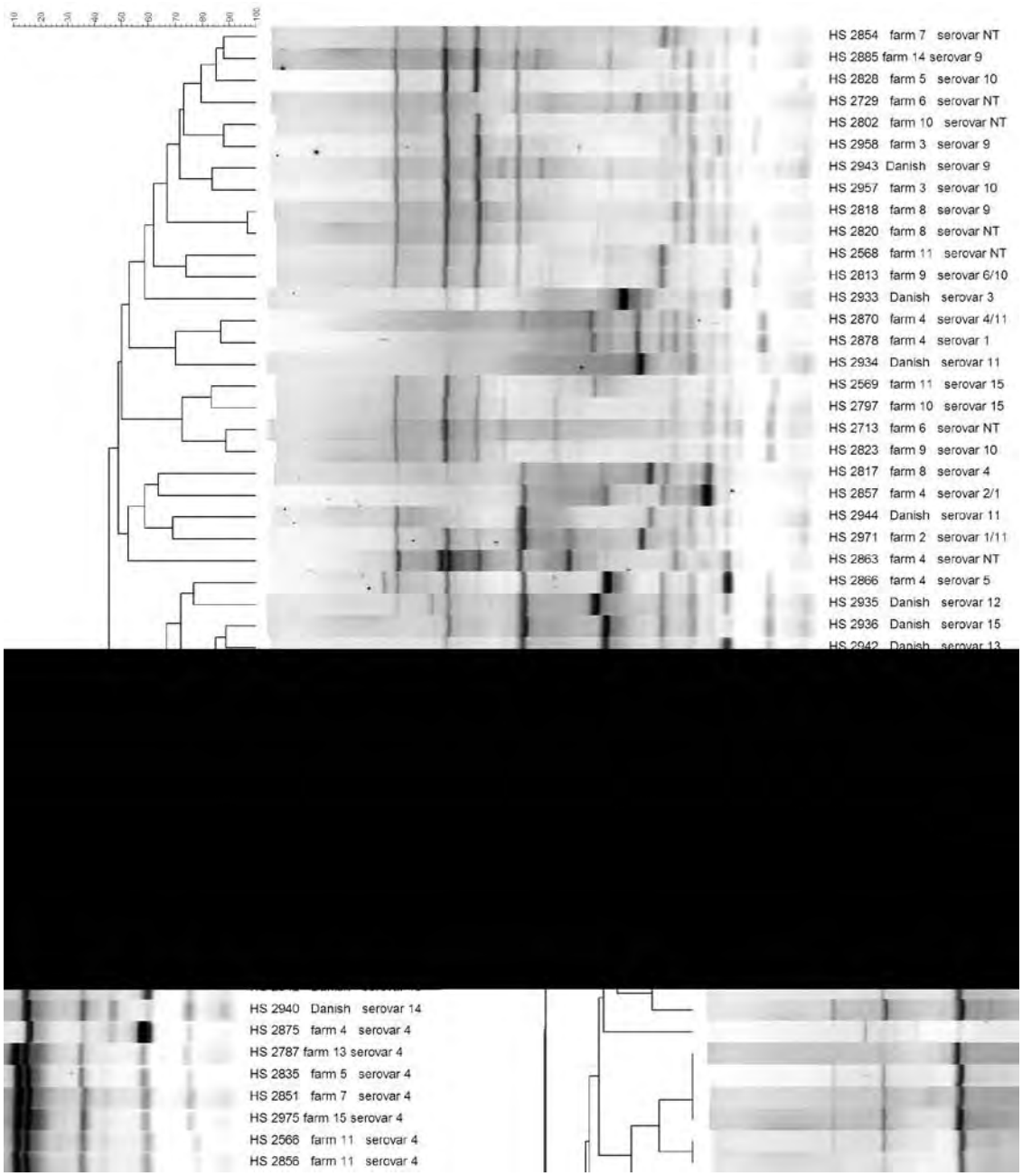

Figure 1 Dendrogram showing the clustering of ERIC PCR finger-prints of 42 Australian and 10 Danish field isolates of *H. parasuis*. Using a cut-off of 96% similarity, a total of 46 genotypes were recognised

Table 1. *H. parasuis* strains used to validate the specificity of the real time PCR

Strain*	Serovar	Description
NR4	1	Serovar Reference
SW140	2	Serovar Reference
SW114	3	Serovar Reference
SW124	4	Serovar Reference
Nagasaki	5	Serovar Reference
131	6	Serovar Reference
174	7	Serovar Reference
C5	8	Serovar Reference
D74	9	Serovar Reference
H367	10	Serovar Reference
H465	11	Serovar Reference
H425	12	Serovar Reference
IA-84-17975	13	Serovar Reference
IA-84-22113	14	Serovar Reference
IA-84-15995	15	Serovar Reference
CCUG3712		Type Strain
Field isolate (1)*	1	NC – healthy pig (D)†
Field isolate (1)	1/11	NC – sick pig (B)
Field isolate (1)	2/1	NC – healthy pig (D)
Field isolates (9)	4	NC – healthy pig (D) NC – sick pig (A, E, G, H, M) Lung (K, Q) Trachea (K)
Field isolate (1)	4/11	NC – healthy pig (D)
Field isolates (4)	5	NC – healthy pig (D, F) NC – sick pig (L) Lung (P)
Field isolate (1)	6	NC – healthy pig (N)
Field isolate (1)	6/10	NC – sick pig (I)
Field isolate (1)	7	NC – sick pig (E)
Field isolates (4)	9	NC – healthy pig (C, N) NC – sick pig (H) Lung (E)
Field isolate (3)	10	NC – healthy pig (C) NC – sick pig (E, I)
Field isolate (3)	12	NC – healthy pig (L) NC – sick pig (E) Lung (R)
Field isolate (1)	13	Lung (O)
Field isolate (4)	15	NC – healthy pig (C) NC – sick pig (J, K) Lung (F)
Field isolates (7)	NT	NC – healthy pig (D, F, G) NC – sick pig (H, J, K) Lung (F)
Danish field isolates (10)	2, 3, 9, 11, 12, 13, 14, 15, NT	Two isolates of serovar 11

* The number of field isolates in each category is given in brackets.

† NC stands for nasal cavity, Farms are labelled from A to R and is indicated in brackets

Table 2. Non-*H. parasuis* strains used to validate the specificity of the real time PCR

Species	Strain	Description
Actinobacillus capsulatus	BR515[A]	Field isolate
Actinobacillus equuli	CCUG 2401[A]	Type strain
	BR92	Field isolate
Actinobacillus indolicus	CCUG39029[A]	Type strain
	HS2394	Field isolate
Actinobacillus ligniersii	BR453[A]	Field isolate
Actinobacillus minor	CCUG38923[A]	Type strain
	HS2189	Field isolate
Actinobacillus pleuropneumoniae	Shope 4074[A]	Type and serovar 1 reference strain
	K17	Serovar 5 reference strain
	WF83	Serovar 7 reference strain
	1096	Serovar 12 reference strain
	HS143	Serovar 15 reference strain
	HS2924	Field isolate
Actinobacillus porcinus	CCUG38924[A]	Type strain
	HS2108	Field isolate
Actinobacillus rossi	CCUG12395	Type strain
	BR488[A]	Field isolate
Actinobacillus suis	CCUG11624[A]	Type strain
Bibersteinia trehalosi	BR458	Field isolate
Bordetella bronchiseptica	BR467[A]	Field isolate
Bisgaard Taxon 8 / *A. equili/A. arthritidis*	BR509	Field isolate
Escherichia coli	BR494[A]	Field isolate
Haemophilus parainfluenzae	NCTC7857[A]	Type strain
Mannheimia haemolytica	CCUG408[A]	Type strain
	BR441	Field isolate
Mannheimia varigena	CCUG38462[A]	Type strain
Pasteurella aerogenes	CCUG9995[A]	Type strain
Pasteurella canis	NCTC11621[A]	Type strain
Pasteurella langaaenis	NCTC11411[A]	Type strain
Pasteurella mairii	CCUG27189[A]	Type strain
	BR133	Field isolate
Pasteurella multocida	NCTC10322	Type strain
Pasteurella stomatis	NCTC11623[A]	Type strain
Pasteurella species B	SSIP683[A]	Reference strain
Streptococcus suis	CCUG7984[A]	Type strain

[A]Indicates that both DNA extraction methods were used with this strain/isolate

Table 3. Results of sensitivity testing of real time PCR for *H. parasuis*

Strain	DNA extraction method	Cell count in positive reaction at minimum positive concentration	Ct score of minimum positive concentration	DNA concentration (ng ul^{-1}) of original solution	Cell count (cfu ml^{-1}) of original solution
CCUG3712*	PrepMan Ultra	47.5 cfu	37.12 ± 0.63	-	1.9×10^8
CCUG3712*	Boiling/cooling	9.5 cfu	37.04 0.51	-	1.9×10^8
CCUG3712* (Repeat)	Boiling/cooling	2.45 cfu	38.20 ± 0.55	-	0.49×10^8
CCUG3712* + 1.265×10^8 *E. coli*	Boiling/cooling	2.45 cfu	38.06 ± 0.49	-	0.49×10^8
CCUG3712* + Pericardial fluid	Boiling/cooling	2.45 cfu	39.17 ± 2.35	-	0.49×10^8
SW124	Boiling/cooling	9.5 cfu	38.42	14.9	1.9×10^8
Nagasaki	Boiling/cooling	9.5 cfu	38.06	10.9	1.89×10^8
174	Boiling/cooling	0.83 cfu	42.71	11.6	1.66×10^8
SW124	PrepMan Ultra	0.48 cfu	37.90	65.7	1.9×10^8
Nagasaki	PrepMan Ultra	0.47 cfu	40.02	49.7	1.89×10^8
174	PrepMan Ultra	0.42 cfu	42.46	55.4	1.66×10^8

* For CCUG3712, reactions were performed in triplicate while single reactions were used for all other strains. For CCUG3712, the lowest dilution at which all three replicates were positive was defined as the minimum positive dilution.

Table 4. Positive and negative results from all swab samples from tissue and fluid compared by the three methods for the two experimental challenge trials. Positive and negative results from all tissue and fluid samples are compared for the three methods in the two challenges. In the serovar 4 challenge, tissue and fluid were not cultured. Different letters indicate a statistical significant difference between the data

Sample type	Challenge type	Number of positive results*			Number of samples
		Real-time PCR	PCR#	Culture#	
Swab of tissue/fluid	Serovar 12	64 (90)[a]	27 (38)[b]	57 (80)[a]	71
	Serovar 4	43 (48)[a]	30 (33)[b]	36 (40)[a,b]	90
	Total	107 (66)[a]	57 (35)[b]	93 (58)[a]	161
Tissue/fluid	Serovar 12	26 (100)[a]	11 (42)[b]	9 (35)[b]	26
	Serovar 4	32 (62)[a]	18 (35)[b]	-	52
	Total	58 (74)[a]	29 (37)[b]	-	78

* Result in brackets is percentage.
# Previously published results see Turni and Blackall (2007a)

Table 5. Samples of tissues and fluid for which the real time PCR produced a positive result yet the culture method gave a negative result. For the tonsils most of the cultures were overgrown and a conventional PCR was performed on the mixed growth, if the PCR came up negative the culture was called negative

Sample Type	Challenge Serovar	Number of tissues that were positive in real-time PCR but negative by culture (total number sampled)									
		Brain	Heart	Joint	Lung	Lymph Node	Pericardial Fluid	Peritoneal Fibrin	Peritoneal Fluid	Pleural Fluid	Tonsil
Swabs	12	0 (7)	0 (5)	0 (6)	0 (7)	1 (6)	1 (7)	2 (6)	2 (6)	1 (4)	4 (6)
	4	2 (9)	2 (9)	1 (9)	0 (9)	1 (9)	1 (9)	-	1 (9)	-	5 (9)
Tissue/Fluid	12	1 (2)	0 (1)	-	6 (7)	2 (3)	-	-	-	-	7 (7)