Comparison of sampling sites and detection methods for *Haemophilus parasuis*

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Abstract

Objective To improve the isolation rate and identification procedures for *Haemophilus parasuis* from pig tissues.

Design Thirteen sampling sites and up to three methods were used to confirm the presence of *H*. *parasuis* in pigs after experimental challenge.

Procedure Colostrum-deprived, naturally farrowed pigs were challenged intratracheally with *H parasuis* serovar 12 or 4. Samples taken during necropsy were either inoculated onto culture plates, processed directly for PCR or enriched prior to being processed for PCR. The recovery of *H parasuis* from different sampling sites and via different sampling methods was compared for each serovar.

Results *H parasuis* was recovered from several sample sites for all serovar 12 challenged pigs, while the trachea was the only positive site for all pigs following serovar 4 challenge. The method of solid medium culture of swabs, and confirmation of the identity of cultured bacteria by PCR, resulted in 38% and 14% more positive results on a site basis for serovars 12 and 4, retrospectively, than direct PCR on the swabs. This difference was significant in the serovar 12 challenge.

Conclusion Conventional culture proved to be more effective in detecting *H parasuis* than direct PCR or PCR on enrichment broths. For subacute (serovar 4) infections, the most successful sites for culture or direct PCR were pleural fluid, peritoneal fibrin and fluid, lung and pericardial fluid. For acute (serovar 12) infections, the best sites were lung, heart blood, affected joints and brain. The methodologies and key sampling sites identified in this study will enable improved isolation of *H parasuis* and aid the diagnosis of Glässer’s disease.

Introduction

Haemophilus parasuis is the causative agent of Glässer's disease of pigs, a disease associated with fibrinous polyserositis, polyarthritis and meningitis. Glässer's disease is recognized as a significant disease in the pork industry worldwide.[1,2] *H parasuis* can cause infection rates of 50-70% and mortality rates above 10%.[3] Production losses due to mortality and unthrifty pigs may be considerable.[4] *H parasuis* is commonly found in the nasal cavity of apparently healthy conventionally-reared pigs. Stress factors, such as transport, unfavourable environment and adverse management practices are often associated with *H parasuis* infections.[1] Maternal and natural immunity is important in prevention of *H parasuis* infection. [2,5,6] The recent changes towards an increased frequency of high health status pigs or pig herds kept in isolation has produced a higher risk that some commercial pig populations will have reduced exposure to *H parasuis* and hence a lowered acquisition of natural immunity.[5] This reduced exposure may explain increased frequency and severity of Glässer's disease outbreaks in high health production systems and systems with particular management strategies, such as segregated early weaning and the mixing of young pigs from different sources.[2,7]

While the virulence factors involved in *H parasuis* infection are not defined[8], there seems to be a link between the causal serovar and disease severity.[9] Of the 15 known serovars, serovars 1, 5, 10, 12, 13 and 14 have been shown to be highly virulent with the ability to cause death or moribundity within four days. Serovars 2, 4 and 15 have been found to be moderately virulent, and serovar 8 mildly virulent, while serovars 3, 6, 7, 9 and 11 have been shown to be avirulent.[10]

Many attempts at isolation and identification of *H parasuis* have been unsuccessful, due to the fastidious nature of these bacteria, their fragility and their complex nutritional requirements for growth.[2] To improve the diagnostic success rate for *H parasuis*, this project was designed to evaluate which tissues are the best sources for *H parasuis* isolation. The project also sought to investigate the utility of PCR directly on clinical material for rapid confirmation of *H parasuis* infection.

Materials and methods

Pigs

Two experimental challenge trials were undertaken 5 months apart. The first used seven pigs in a serovar 12 challenge and the second used nine pigs in a serovar 4 challenge. All animal experimental work was performed with the approval of the ARI Animal Ethics Review Committee. Colostrum-deprived, naturally farrowed pigs were obtained from five sows using a previously described protocol.[11] Piglets were reared with colostrum supplement (BIOCOL, Intervet, Boxmeer, the Netherlands), a hyperimmune colostrum replacement containing anti-*E. coli* immunoglobin (Re-Sus, Nufarm Animal Health, Laverton, North VIC Australia) and a milk replacement (Wombaroo, Food Products, Adelaide, SA Australia) according to manufacturer's guidelines in an isolation-shed at the Animal Research Institute. In the serovar 4 challenge experiment, but not in the serovar 12 challenge experiment, gentamycin sulphate (50 mg/mL; Gentam, Troy Laboratories, Smithfield, NSW Australia) was given intramuscular for the first 3 days of life. By three weeks of age, the pigs were on a customized antibiotic free pig creep/weaner meal (21.24% protein) (Country Heritage Feeds Pty Ltd Highfields Queensland Australia).

Housing

The piglets were initially housed in a heated crib box, which was taken away in the third week. Facilities were cleaned daily. The temperature was decreased over three weeks starting with 29.4 –32.2°C in the first week. In the second week the temperature was 26.7 – 29.5°C, followed by 24 – 26.8°C in the third week. Piglets were monitored regularly and antibiotics administered (Gentam) as soon as bacterial disease symptoms were detected during the first five weeks of age. No antibiotics were administered after five weeks of age including after challenge with *H parasuis*.

Challenge

At six weeks of age the pigs were challenged with *H parasuis* strain H425, the reference strain for serovar 12, or strain HS1387, an Australian field isolate previously identified as serovar 4.[12] Twenty hours before challenge *H parasuis* was plated onto chocolate agar 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), On the day of the challenge the bacteria were harvested into phosphate buffered saline and the concentration of the suspension was adjusted to approximately 1×10^7 cfu/mL and the suspension kept on ice until challenge. The concentration of the final suspension used for challenge was confirmed by viable counts performed on chocolate blood agar and cfu counted the following day. The serovar 12 challenge was given as 9.43×10^6 cfu per pig while the serovar 4 challenge was 1.3×10^7 cfu per pig.

The pigs were anaesthetised using tiletamine/zolazepam (Zoletil, Virbac, Peakhurst, NSW Australia) at 6.6 mg/kg given intramuscularly and thiopental (Bomathal, BOMAC Laboratories, Auckland New Zealand) at 10 - 18 mg/kg into the ear vein. Once fully anaesthetized, the pigs were inoculated into the trachea with 1 mL of either H425 (serovar 12) or the HS1387 (serovar 4) strain of *Haemophilus parasuis*. Before challenge, the rectal temperature was measured and nasal swabs (Amies transport swabs, cotton tipped, plastic shaft) were taken from each pig.

Observations

Pigs were observed at regular intervals (between 2-6 hours depending upon disease progression) for clinical signs. As required by the Animal Ethics permit, all pigs displaying lateral recumbency and/or laboured breathing and/or cyanosis were euthanased by lethal injection, given as an overdose of pentobarbitone by intracardiac injection after anaesthesia. The experiments were terminated on days six or seven after challenge and a necropsy was performed on all pigs.

Necropsy samples

After euthanasia of pigs a necropsy was performed immediately or the pigs were stored in a cold room prior to necropsy. The maximum period between euthanasia and necropsy was between 2 to 6 hours. Using cotton swabs in Amies transport medium without charcoal, up to 15 sites were sampled for bacterial culture. These sites included: nasal cavity, tonsil, trachea, peritoneal fluid, fibrin in the peritoneum, pericardial fluid, heart blood, joint fluid from arthritic joints, joint, mandibular lymph node, liver, lung, pleural fluid, brain and cerebrospinal fluid (the latter for serovar 12 only). Samples for processing directly for PCR were taken as dry swabs, tissue blocks or fluid samples. In both trials, dry swabs were taken from the tonsil, trachea, peritoneal fluid, lung, pericardial fluid, heart blood, arthritic joint, mandibular lymph node, liver, brain and pleural fluid. In addition, dry swabs of nasal cavity, fibrin in peritoneal cavity and cerebral fluid were taken in the serovar 12 challenged pigs. Tissues collected for PCR analysis included tonsil, trachea, lung, heart, mandibular lymph node, liver and brain. Fluids collected for PCR analysis were peritoneal, pericardial, pleural, articular and cerebrospinal fluids. In the serovar 12 challenge trial, all PCRs were performed directly on the relevant tissue, swabs, or fluid. In the serovar 4 challenge study, tissues, swabs and fluids were also enriched in TM broth[13] and then examined by PCR.

Necropsy examination

Gross pathology was recorded at necropsy of all animals. In the first trial the percent of the area of affected lung was recorded. In the second trial a lung scoring system, that was modified from one originally developed for enzootic pneumonia,[14, 15] was applied to measure the severity of the lung lesions. Under this system, the highest total score awarded is 55. This indicates the proportion of consolidated lung tissue in the following sites: a maximum score of 10 is assigned to each of the apical and cardiac lobes, 5 for each diaphragmatic lobe and 5 for the intermediate lobe.

Processing of samples for bacterial culture

Swabs in Amies transport medium from tissues and fluids were inoculated onto TM/SN agar, prepared as previously described[13] and onto blood agar, the latter being cross-streaked with a nurse colony of *Staphylococcus hyicus*. The plates were then incubated aerobically for up-to 48 hours at 37ºC. All suspect colonies of *H parasuis* were single colony passaged twice before identification by PCR. For identification a 1 μL loopful of growth was thoroughly suspended in 100 μL of MilliQ filtered water. If plates were heavily overgrown with other bacteria, then a swab of the bacterial growth was taken and suspended in 1 mL of PBS and 100 μL of this suspension removed for use (colony sweep). The suspension was heated at 98°C for 5 min, followed by cooling on ice for 5 min. After centrifugation for 5 min at 30,230 x *g*, the supernatant was collected and stored at -20°C. A 1 μL aliquot of the supernatant was used for PCR analysis.

Processing of samples for PCR

Direct examination of swabs, tissues and fluid

For dry swabs, the swab tip was cut off and placed into a 1.5 mL tube to which 500 μL of PBS was added. For tissues, a 0.1 g sample was macerated and mixed with 1 mL PBS in a 1.5 mL tube. For both swab and tissue samples, the suspensions were then vortexed for 15 sec and incubated for 10 min at room temperature before revortexing again. Swab tips were removed from the tubes, releasing as much liquid as possible from the tip of the swab. For tissues 500 μL of tissue suspension was removed and added to a new 1.5 mL tube. For sampled fluids, 1 mL of the fluid was added to a 1.5 mL tube and spun at 1000 x *g* for 3 min. The resultant supernatant was collected for further processing. The swab and tissue suspensions and the fluid supernatants were centrifuged at 30,230 x *g* for 5 min and the pellets resuspended in 200 μL of a proprietary PCR buffer (PrepMan Ultra, Applied Biosystems, Foster City CA) and processed according to the PrepMan Ultra protocol provided by the manufacturer. The resultant product was stored at -20°C for PCR.

Enrichment of swabs, tissue and fluid samples for direct PCR

In the serovar 4 challenge study, each swab or about 1 cm cube of tissue sample was added to 5 mL of TM broth, prepared as previously described[13], while 1 mL of fluid was added to 9 mL of TM broth. After overnight incubation at 37ºC and vortexing, the swab and tissue were removed from the broth. The suspensions were centrifuged (1,000 x *g*, 3 min) and the pellets resuspended in 500 µL PBS. These suspensions were then centrifuged and processed using the PrepMan Ultra system as described above.

PCR

The PCR of Oliveira *et al.* [16] was used with reaction conditions modified depending on the source of the template DNA. For crude colony preparations, colony sweeps and enrichment broths the template volume was 1 µL. For all other templates, a 0.5 µL was used and the reaction mix also contained bovine serum albumin (0.002 µg/mL). The reaction was run on a Hybaid PCR Express machine with the following conditions: 1 cycle at 94°C for 1 min , 30 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 2 min and one cycle of 72°C for 10 min. The 821 bp PCR product was separated on a 1% agarose gel together with a Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) and photographed under UV illumination.

Statistical analysis

Chi-square tests were used to compare the proportion of positives between methods within the two different challenges, and between the two different challenges for the culture method. Analyses were performed using Statistix for Windows (Analytical Software, Tallahassee, FL).

Results ~~ESULTS~~

Clinical signs

Serovar 12

Disease onset and progression was rapid with pigs surviving only 3.1 days after challenge. Coughing was observed in four out of seven pigs, but only at one or two observations. Clinical signs were marked and included dyspnoea, apathy, anorexia, cyanosis, severe lameness, swollen and painful joints, prostration, tremor, muscle twitching, lateral recumbency and frothing at the mouth.

Serovar 4

Only two of the nine pigs (pig 4 with cyanosis and pig 10 with severe arthritis) required euthanasia before all remaining pigs were euthanized on day 6 or 7. The first clinical sign was coughing on day 1 post challenge. Subsequently, other signs observed included depression, reduced responsiveness to external stimuli, reluctance to move, ataxia, pallor, erythema, cyanosis, muscle twitching and tremors.

Necropsy findings

Serovar 12

Six of the seven pigs exhibited peritonitis of varying severity that increased with survival time. Overall, the gross pathological findings were not marked with two pigs showing no gross abnormalities. Other gross pathological findings included fibrin attached to the liver, perihepatitis, lesions of the lung (haemorrhagic, oedematous, discoloured, dark or mottled parenchyma), fibrin attachments to the lung, hydrothorax, an excessive amount of fluid in the pericardial sac, swelling and oedema of hock joints, and acute fibrinopurulent arthritis in the hock joints and knees.

Serovar 4

With the exception of two pigs all pigs displayed peritonitis. A much more severe form of peritonitis was observed in some pigs (as compared with serovar 12 challenge) and pigs that displayed severe peritonitis also had a high lung score. In some pigs, coils of the gastrointestinal tract were adhered together with fibrin. The pleural cavity displayed classic pathology of Glässer's disease and the lungs of all pigs were affected, ranging from mild to very severe lesions. Pathological changes of varying severity were seen in the hearts of all pigs. Other pathological findings included fibrin adherent to the liver and/or kidney, excess pericardial fluid, numerous fibrinous attachments to the heart and pericardial adhesions to the epicardium, lungs and pleura.

Culture and PCR results

Serovar 12

Though few gross pathological changes apart from lung changes were noted in the serovar 12 challenged pigs, it was possible to recover *H parasuis* from a number of organs. The trachea, lung, heart blood, affected joints and brain of all animals sampled were culture positive (Table 1). The best method to detect *H.parasuis* was culture of swabs taken from tissues and fluids. Significantly more positive results were obtained when swabs were cultured (82% or 68% without colony sweep) compared to swabs examined directly by PCR (44%) ($P < 0.01$ and $P < 0.05$, respectively). When swabs from the nasal cavity were cultured the plates were all overgrown by other bacteria. However, a PCR performed on the mixture (sweep) did yield positive results in 5 of 6 pigs. When DNA was extracted directly from swabs, only the nasal cavity and the trachea tested positive in all animals tested (Table 1).

Table 2 compares the results of culture and the matching direct PCR test. Apart from contaminated sites such as the nasal cavity and tonsils, all swab samples from other (internal body) sites with culture scores of 0 or 1 for *H. parasuis* (0 to 10 colonies) had concurrent negative direct PCR results, with the exception of the peritoneal fluid in one pig (no.13). Some samples that resulted in a growth of 11 to 100 colonies on culture plates (culture score 2) also produced negative PCR results when the swab was processed directly. With one exception (the pericardial fluid of pig 13), all samples with culture scores of 3 or 4 (more than 100 colonies) were positive by direct PCR of swabs.

Serovar 4

The culture-based detection of *H parasuis* from all sites was less successful for serovar 4 (47% positive) than for serovar 12 (82% positive) ($P < 0.01$). In contrast to the serovar 12 challenge strain which was recovered from multiple tissues from all animals, after serovar 4 challenge only samples from the trachea of all animals were positive by culture (including colony sweep PCR) (Table 3). Other sites with high culture success rates were pleural fluid and fibrin from the peritoneum (5/6 animals). The nasal cavity was the next most successful location to detect the bacteria.

In comparing culture (47% positive results) and direct PCR of swabs (33% positive) there were no significant differences between the methods (Table 3). All plates that were inoculated with tonsillar swabs were contaminated with Gram-negative bacilli. As experienced with serovar 12, if the plates were contaminated with other bacteria and *H parasuis* could not be isolated, then direct PCR improved the results.

In two sampling sites (pericardial fluid and heart), the culture method did not give better results than PCR (Table 3). For the pericardial fluid the results of direct PCR on the swabs (5/9) or fluid (4/7) with and without an enrichment step were better than culture (4/9). PCR on heart also yielded more positive results (3/9) than culturing swabs from the heart blood (1/9). The small sample size for joint fluid and pleural fluid with and without enrichment did not allow for a comparison between direct PCR examination and culture of fluid.

Direct PCR on enrichment swabs and tissue blocks taken from internal organs and fluids (but not from the upper respiratory tract, such as nasal cavity, tonsil or trachea) yielded overall more positive results (but not significantly different) than direct PCR on swabs (Table 3). The exception for this overall higher yield of direct PCR on enrichment swabs and tissue were samples from the peritoneal fluid and brain (Table 3). The trend of better results for direct PCR on tissue (except upper respiratory tract samples) compared to direct PCR on tissue swabs was also seen in serovar 12 challenge. Enrichment did not improve the PCR results for tissue and fluid except for the liver and pericardial fluid (Table 3).

The detection of *H parasuis* serovar 4 was dependent on the severity of clinical signs and gross pathological findings. Pig 4 had to be euthanized due to cyanosis and was the first pig to display severe signs of the disease. *H parasuis* was cultured from all sites sampled from this pig except the nasal cavity (Table 4). Despite the joints and mandibular lymph node of this pig appearing grossly normal at necropsy, these sites were also positive for *H parasuis*. From pig 3, which showed few clinical signs and few lesions at necropsy, *H parasuis* could only be recovered from the tonsil and trachea. Pigs 7 and 8 also only exhibited mild clinical sign and were still very active at the end of the experiment. Apart from the recovery of *H* *parasuis* from these two pigs in the upper respiratory tract (nasal cavity, tonsils and trachea), only one other sampling site (pleural fluid for pig 7; lung for pig 8) yielded *H parasuis* (Table 4). Pig 9 did not have marked changes at necropsy, and was affected with an enlarged and inflamed hock joint with associated lameness.. In this pig, *H parasuis* was only recovered from two other sites outside the upper respiratory tract, being the inflamed hock joint and pleural fluid. All five pigs with lung scores of 55 yielded *H parasuis* from more samples (range 6 – 12) than pigs without such a high score (range 2 – 5)(Table 4). These five pigs all had fibrin present in the peritoneal cavity from which *H parasuis* was recovered. Four out of these five pigs with a high score yielded *H. parasuis* on culture of lung and pericardial fluid, three were culture positive in the liver or pleural fluid and two in joint cultures (Table 4).

As with serovar 12 challenge, nearly all sites (with the exception of nasal cavity, tonsil and trachea) that yielded no or limited growth (culture score $\leq$1) resulted in negative PCR results when the swabs were processed directly for PCR. The only exceptions were the pericardial fluid of pig 6 and the brain of pig 2 (Table 4). Only samples from two sites with a culture growth score of 2 or greater resulted in negative PCR results when the swab was processed directly (heart pig 4, trachea pig 7, Table 4).

Discussion

The duration and severity of the disease caused by serovar 12 is comparable to that found in overseas studies with serovar 12 or with other highly pathogenic serovars, e.g. serovar 1 and 5.[4,6,17] In the current experiment all seven pigs died within 3.5 days of challenge. Using intraperitoneal injections in SPF pigs, Kielstein and Rapp-Gabrielson[9] classified serovars 1, 5, 10, 12, 13 and 14 as very pathogenic causing death and morbidity within four days, while serovar 4 together with serovars 2 and 15 were classed as moderately pathogenic, not causing death but producing a milder or less acute form of Glässer's disease. As reported by Kielstein and Rapp-Gabrielson[10] we found that most pigs survived a serovar 4 challenge, with only one of the pigs requiring euthanasia due to lateral recumbency, cyanosis and laboured breathing. However, using the serovar as an indicator or predictor of virulence for a particular strain is problematic, since exceptions do occur. Thus Takahashi *et al.* [17] reported all eight pigs died when challenged with a serovar 2 strain. Angen *et al.*[18] have concluded that due to the known genetic variability and the know strain variation in virulence that the serovar of an isolate cannot be considered as a stable marker for virulence.

Virulence may also depend on the degree of host immunocompetence, which can differ according to several parameters between and within breeds.[19,20,21] Animal differences and time differences are likely confounding factors between the current two experiments and genetic differences and environmental factors could have influenced the difference in virulence observed in the current to a certain degree.

For both serovars the culture plates of nasal cavity samples were overgrown with other bacteria species and therefore, *H parasuis* was only isolated from one of 14 pigs. Oliveira *et al.* [22] also reported difficulties in recovering *H parasuis* from the nasal cavity due to contamination of samples by bacterial flora of the upper respiratory tract. However, PCR tests on the colony sweeps yielded positive results for 11 out of 14 pigs. Sampling the nasal cavity and tonsils will not provide a conclusive diagnosis for Glässer's disease, as *H parasuis* is considered a commensal in the upper respiratory tract and both pathogenic and non-pathogenic strains can be isolated from these sites.[7] Thus, *H parasuis* present in nasal cavity or tonsils might not necessarily be the causal serovar for the disease,.[23] and sampling the internal organs is recommended to determine the disease-causing serovar of *H parasuis*. Another problem with sampling from these upper respiratory tract sites is the presence of *Actinobacillus indolicus*, which can be part of the natural flora[8] and interferes with the *H parasuis* PCR, giving false positive results.[16]

When attempting culture for *H parasuis* from pigs with a milder form of Glässer's disease such as that caused by serovar 4, the current study results suggest sampling of pleural fluid, fibrin in the peritoneum, lung, peritoneal fluid and pericardial fluid. If a pig suffered from an apparent acute septicaemic Glässer's disease form such as caused by serovar 12, sites such as lung, heart blood, affected joints and brain represent the preferred samples. Since pigs can be infected by more than one genotype of *H parasuis* simultaneously, which can be isolated from different body sites in the same pig, several sites should be sampled.[23] However, as only two serovars of *H. parasuis* were used in this study, further studies are needed to examine whether other serovars of similar pathogenicity to those tested here will result in the same recommendation for sampling sites. There could also be differences among strains of the same serovar, even though the duration and severity of the disease found in this study were comparable to overseas studies with the same serovars.

Oliveira[23] noted that isolation of *H parasuis* from chronically infected animals is usually unsuccessful. This observation corresponds to the current findings with serovar 4 challenged pigs, where the recovery depended on disease severity. Thus the pig that had to be euthanased due to cyanosis yielded *H parasuis* from nearly all tissues and fluids sampled, whereas animals that showed only mild clinical signs had few culture positive sites.

All pigs in this study were euthanased and then stored in a cold room until the necropsy (which was within two to six hours after death). The samples were processed in the laboratory straight after necropsy. As this is not always possible in the field, several measures can be undertaken to enhance the survival of *H parasuis*. Oliveira[23] suggested euthanasing and sampling pigs with clinical signs characteristic of acute infection rather than sampling dead pigs. It is recommended that samples are kept on ice, as this prolongs the survival of *H parasuis*. According to Morozumi and Hiramune,[24] *H parasuis* survives in physiological saline at 42°C for only one hour, at 37°C for two hours and at 24°C for eight hours, while at 5°C the amount of surviving cells after eight hours only decreases slightly. The use of transport swabs with Amies medium without charcoal is recommended as it assists survival of *H*. *parasuis*.[25]

The most successful method to detect *H parasuis* in the current study, regardless of the challenge serovar, was based on inoculation of swabs of tissue or fluids onto solid media. This approach also allows the organism to be characterised by serotyping and genotyping, and enables storage for vaccine development if required. However, swabs from the nasal cavity and tonsils of challenged pigs were often overgrown by contaminants. A colony sweep was able to provide a positive PCR result for a number of these bacterial mixtures. However, some caution has to be taken with colony sweeps, as the PCR used here has only been validated on closely related species of bacteria and other pig pathogens. As the PCR test has not been validated on all bacteria found in the nasal cavity of a pig it is not known if false positives occur.

This study had ideal culture conditions, good quality samples, rapid laboratory processing, a suitable growth medium and experienced laboratory diagnosticians. It is possible that in circumstances where laboratories are handling samples that are suboptimal or suitable media is unavailable or laboratory staff lack relevant experience, that PCR could outperform culture. This is the situation with the PCR for *Avibacterium paragallinarum* where under optimal conditions in Australia culture and PCR perform equally[26] but PCR outperformed culture under suboptimal conditions in China.[27] A direct PCR from a relevant swab (without plate inoculation), while not providing the option to do serotyping and/or genotyping, might thus be useful when sampling conditions for *H parasuis* are suboptimal, the viability of *H parasuis* may have been compromised, or when the laboratory lacks expertise and/or suitable media.

The results in this study indicate the direct PCR failed to perform as well as culture when low numbers of *H parasuis* were present in the samples. This is consistent with the findings of Oliveira *et al.*, [16] who reported that the PCR required a minimum of 100 cfu mL^{-1} for detection. In the current study, sites showing $\leq$10 cfu *H parasuis* per plate resulted in negative results in the direct PCR, except for one sample of peritoneal fluid (serovar 12 challenge), pericardial fluid and brain (serovar 4 challenge). Some samples with moderate growth (11 – 100 cfu/plate) also resulted in negative results for the direct PCR. Similar, following serovar 12 challenge, pericardial fluid was the only sample where a culture yielding over 100 cfu/plate resulted in a negative PCR results. This may be due to uneven bacterial distribution in pericardial (or peritoneal) fluids such that sampling fails to reach a treshold of 100 cfu/mL.

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References

1. Rapp-Gabrielson VJ. *Haemophilus parasuis*. In *Diseases of swine* (Ed, B.E. Straw, D'Allaire, S. Mengeling, W.L., Taylor, D.J.) Iowa State University Press, Ames Iowa, 1999: 475 - 481.

2. Ferri EFR, Gutierrez CB, de la Puente VA, Garcia del Blanco N, Navas J, Paniagua ML, del Rio ML, Monter JL, Garcia de la Fuente YJN. Bacterial meningitis in pigs: Glasser's disease. *Porci* 2000; 59: 43 - 60.

3. Baumann G, Bilkei G. Effect of vaccinating sows and their piglets on the development of Glässer's disease induced by a virulent strain of *Haemophilus parasuis* serovar 5. *Vet Rec* 2002; 151: 18 -21.

4. Bak H, Riising HJ. Protection of vaccinated pigs against experimental infections with homologous and heterologous *Haemophilus parasuis*. *Vet Rec* 2002; 151: 502 - 505.

5. Solano-Aguilar GI, Pijoan C, Rapp-Gabrielson V, Collins J, Carvalho LF, Winkelman N. Protective role of maternal antibodies against *Haemophilus parasuis* infection. *Am J Vet Res* 1999; 60: 81 -87.

6. Blanco I, Galina-Pantoja L, Oliveira S, Pijoan C, Sanchez C, Canals A. Comparison between *Haemophilus parasuis* infection in colostrum-deprived and sow-reared piglets. *Vet Microbiol* 2004; 103: 21 - 27.

7. Vahle JL, Haunes JS, Andrews JJ. Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of Cesarean derived colostrum deprived (CDCD) swine. *Can J V Res* 1997; 61: 200 - 206.

8. Oliveira S, Pijoan C. *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Vet Microbiol* 2004; 99: 1 - 12.

9. Rapp-Gabrielson VJ, Gabrielson DA. Prevalence of *Haemophilus parasuis* serovars among isolates from swine. *Am J Vet Res* 1992; 53: 659 - 664. 7.

10. Kielstein P, Rapp-Gabrielson VJ. Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *J Clin Microbiol* 1992; 30: 862 - 865.

11. Oliveira S, Galina L, Blanco I, Canals A, Pijoan C. Naturally-farrowed, artificially-reared pigs as an alternative model for experimental infection by *Haemophilus parasuis*. *Can J Vet Res* 2003; 67: 146 - 150.

12. Turni C, Blackall PJ. Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis*. *Vet Microbiol* 2005; 106: 145 – 151.

13. Lancashire, JF, Tamsin, DT, Blackall PJ, Jennings PJ. Plasmid-encoded Tet B tetracycline resistance in *Haemophilus parasuis*. *Antimicrob Agents Chemother*.2005; 49: 1927 – 1931.

14. Pointon AM, Davies PR, Bahnson PB. Disease Surveillance at Slaughter. In: Straw BE, D'Allaire DS, Mengeling WL, Taylor DJ (Eds.), Diseases of Swine, Iowa State Press, Iowa, 1999: 1111 – 1132.

15. Goodwin RFW, Hodgson RG, Whittlestone P, Woodhams RI. Some experiments relating to artificial immunity in enzootic pneumonia in pigs. J Hyg 1969: 193 – 208.

16. Oliveira S, Galina L, Pijoan C. Development of a PCR test to diagnose *Haemophilus parasuis* infections. *J Vet Diag Invest* 2001; 13: 495 - 501.

17. Takahashi K, Nagai S, Yagihashi T, Ikehata T, Nakano Y, Senna K, Maruyama T, Murofushi J. A cross-protection experiment in pigs vaccinated with *Haemophilus parasuis*

serovars 2 and 5 bacterins, and evaluation of a bivalent vaccine under laboratory and field conditions. *J Vet Med Sci* 2001; 63: 487 - 491.

18. Angen O, Svensmark B, Mittal, KR. Serological characterisation of Danish *Haemophilus parasuis* isolates. *Vet Microbiol* 2004; 103: 255 - 258.

10. Edfors-Lilja I, Wattrang E, Magnusson U, Fossum C. Genetic variation in parameters reflecting immune competence of swine. *Vet Immunol Immunopathol* 1994; 40: 1-16.

20. Joling P, Mok KS, de Vries Reilingh G, Wever PJ, Cornelis RS, Oskam JP, Henken AM. An evaluation of immune competence in different swine breeds. *Ve. Q* 1993; 15: 9 - 15.

21. Nguyen VP, Wong CW, Hinch GN, Singh D, Colditz IG. Variation in the immune status of two Australian pig breeds. *Aust. Vet. J.* 1998; 76: 613 - 617.

22. Oliveira S, Batista L, Torremorell M, Pijoan C. Experimental colonization of piglets and gilts with systemic strains of *Haemophilus parasuis* and *Streptococcus suis* to prevent disease. *Can J Vet Res* 2001; 65: 161 -167.

23. Oliveira S. Improving rate of success in isolationg *Haemophilus parasuis* from clinical samples. *J Swine Health Prod* 2004; 12: 308 - 309.

24. Morozumi T, Hiramune T. Effect of temperature on the survival of *Haemophilus parasuis* in physiological saline. *Natl Inst Anim Health Q (Tokyo)* 1982; 22: 90-1.

25. del Rio M L, Gutierrez B, Gutierrez CB, Monter JL, Ferri EFR. Evaluation of survival of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in four liquid media and two swab specimen transport systems. *Am J Vet Res* 2003; 64: 1176-1180.

26. Chen X, Miflin JK, Zhang P, Blackall PJ. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. *Avian Dis* 1996; 40: 398 – 407.

27. Chen X, Chen Q, Zhang P, Feng W, Blackall PJ. Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. *Avian Pathol* 1998; 27: 296 – 300.

Table 1 Results of culture and PCR analysis for samples from seven pigs challenged with serovar 12, presented as the number of pigs positive/pigs sampled.

Site or tissue	*H parasuis* culture positive [a]	Direct PCR positive on	
		swab	tissue or fluid
Brain	7/7	1/7	3/5
Cerebrospinal fluid	-	-	3/4
Mandibular lymph node	5/6	0/6	1/3
Nasal cavity	0(5)[b]/6	6/6	-
Tonsil	0(2)/6	3/6	3/7
Trachea	7/7	7/7	3/5
Lung	7/7	2/7	3/7
Pleural fluid	3/4	1/4	2/4
Pericardial fluid	2(4)/7	0/7	1/6
Heart blood/ heart	6/6	3/5	0/1
Liver	3/3	0/3	0/2
Peritoneal fluid	3(4)/6	3/6	5/7
Fibrin in peritoneum	3(4)/6	2/6	-
Joint or synovial fluid	7/7	6/7	5/7
Total sites	53(64)/78	34/77	29/58
% positives	$68(82)^{A}$	44^{B}	50^{B}

[a] If *H parasuis* was not identifiable by culture due to contaminated overgrowth, presence of *H parasuis* was checked by PCR on a colony sweep.

[b] Results in brackets include positives obtained by colony sweep of contaminated culture plates.

[A,B] Values with a different superscript (within a row) are significantly different $P < 0.05$.

Table 2 Growth of *H parasuis* (on a scale of 0 - 4) from serovar 12 challenge pigs compared to direct PCR results from swabs.

Site or tissue	Pig						
	2	6	7	8	11	13	14
Brain	2[a]/N[b]	1/N	1/N	2/N	2/N	2/P	1/N
Mandibular lymph node	1/N	-	1/N	2/N	0/N	2/N	1/N
Nasal cavity	0 (+)[c]/P		0(+)/P	0(+)/P	0(+)/P	0(-)/P	0(+)/P
Tonsil	0(+)/P	0(-)/P	0(-)/P	0(-)/N	-	0(-)/N	0(+)/N
Trachea	2/P	2/P	3/P	3/P	3/P	3/P	3/P
Lung	2/N	1/N	2/N	2/P	2/N	2/N	2/P
Pleural fluid	2/P	-	0/N	-	-	1/N	1/N
Pericardial fluid	0/N	0(+)/N	0/N	0(+)/N	2/N	3/N	0/N
Heart blood/ heart	2/N	2	2/N	2/P	-	3/P	2/P
Liver	2/N	-	-	-	2/N	2/N	-
Peritoneal fluid	1/N	0(+)/N	-	0/N	3/P	0/P	2/P
Fibrin in peritoneum	2/N	0(+)/N	0/N	0/N	-	2/P	2/P
Joint or synovial fluid	3/P	2/P	2/P	2/N	3/P	4/P	2/P

[a] *H parasuis* growth was measured on the following scale: 0 = no growth or *H parasuis* growth could not be identified due to contamination; 1 = 1 to 10 cfu; 2 =11 to 100 cfu; 3 = > 100 cfu; 4 = lawn growth.

[b] P and N refer indicate positive and negative PCR results on DNA extracted directly from swabs.

[c] (+) and (-) indicates positive and negative colony sweep PCR results from contaminated culture plates

Table 3 Results of culture and PCR analysis for nine pigs challenged with serovar 4, presented as the number of pigs positive/pigs sampled.

Site or tissue	*H parasuis* culture [a]	Direct PCR swab	Direct PCR tissue or fluid	PCR enrichment swab	PCR enrichment tissue or fluid
Brain	1/9	1/9	1/9	0/9	0/9
Mandibular lymph node	1/9	0/9	0/9	1/9	0/9
Nasal cavity	1(6)[b]/8	-	-	-	-
Tonsil	0(4)/9	7/9	-	0/9	-
Trachea	8(9)/9	8/9	4/8	5/9	4/8
Lung	4(5)/9	2/9	3/9	2/9	2/9
Pleural fluid	5/6	-	1/1	-	1/2
Pericardial fluid	4/9	5/9	4/7	5/9	5/8
Heart blood/ heart	1/9	0/9	3/9	1/9	1/9
Liver	3/9	0/9	1/8	3/9	4/8
Peritoneal fluid	5/9	5/9	4/8	4/9	3/8
Fibrin in peritoneum	5/6	-	-	-	-
Joint or synovial fluid	3/9	2/9	1/1	3/9	2/4
Total sites	41(52)/110	30/90	22/69	24/90	22/74
% positives	37 (47)	33	32	27	30

[a] If *H parasuis* was not identifiable by culture due to contaminated overgrowth, presence of *H parasuis* was checked by PCR test on a colony sweep.

[b] Results in brackets include positive obtained by colony sweep of grossly contaminated culture plates.

Table 4 Growth of *H parasuis* (on a scale of 0 - 4) in samples from serovar 4-challenged pigs compared to direct PCR results from swabs. Lung scores and number of positive sample sites per pig (according to culture and PCR colony sweeps) are also indicated.

Site or tissue	Pig								
	1	2	3	4	5	6	7	8	9
brain	0[a]/N[b]	0/P	0/N	1/N	0/N	0/N	0/N	0/N	0/N
Mandibular lymph node	0/N	0/N	0/N	1/N	0/N	0/N	0/N	0/N	0/N
Nasal cavity	0(+)[c]	0(+)	-	0(-)	0(-)	0(+)	0(+)	3	0(+)
Tonsil	0(-)/P	0(-)/P	0(+)/P	0(+)/P	0(-)/N	0(-)/P	0(-)/P	0(+)/N	0(+)/P
Trachea	2/P	0(+)/P	4/P	3/P	4/P	2/P	4/N	3/P	3/P
Lung	1/N	0/N	0/N	0(+)/N	3/P	2/P	0/N	1/N	0/N
Pleural fluid	-	1	-	3	-	2	2	0	2
Pericardial fluid	2/P	1/P	0/N	3/P	2/P	0/P	0/N	0/N	0/N
Heart blood/ heart	0/N	0/N	0/N	2/N	0/N	0/N	0/N	0/N	0/N
Liver	0/N	0/N	0/N	1/N	1/N	1/N	0/N	0/N	0/N
Peritoneal fluid	2/P	3/P	0/N	3/P	2/P	2/P	0/N	0/N	0/N
Fibrin in peritoneum	3	3	-	4	2	2	-	-	0
Joint or synovial fluid	0/N	0/N	0/N	1/N	0/N	4/P	0/N	0/N	3/P
Lung score	55	55	2	55	55	55	11	13	13
Positive results	6	6	2	12	6	8	3	4	5

[a] H. parasuis growth was measured on the following scale: 0 = no growth or *H parasuis* growth could not be identified due to contamination; 1 = 1 to 10 cfu; 2 =11 to 100 cfu; 3 = > 100 cfu; 4 = lawn growth.

[b] P and N indicate positive and negative PCR results on DNA extracted directly from swabs. No direct PCR was performed on samples from the nasal cavity.

[c] (+) and (-) indicate positive and negative colony sweep PCR result from contaminated culture plates