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A Survey of Pigs at an Abattoir to Determine the Prevalence of *Mycoplasma suis*

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1. Acknowledgements

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I would like to express my gratitude to the CSIRO – Australian Animal Health Laboratory for allowing me to undertake this work.

2. Executive Summary

Mycoplasma suis is the causative agent of the disease known as porcine eperythrozoonosis (PE) which is also known as porcine infectious anaemia. It causes febrile acute anaemia and jaundice in feeder pigs resulting in low morbidity and high mortality. It is also known that *M. suis* causes a variety of low grade infections producing a variety of syndromes including anaemia, mild icterus, and general unthriftiness in newborns, growth retardation in feeder pigs and decreased reproductive efficiency in sows. Porcine infectious aneamia occurs worldwide and caused by the uncultivable hemotrophic bacterium *M. suis*. The actual prevalence and impact of *M. suis* infections is uncertain and there are no published reports of the organism being detected in Australian commercial pigs.
The aim of this project was to determine if *M. suis* was present in a sample of commercial feeder pigs in Australia. Diagnosis was based on a specifically designed TaqMan PCR test. Additional testing was conducted with a conventional PCR, Giemsa stained blood smears and confocal microscopy.

A total of 322 pigs were sampled, from 24 farms. *M. suis* was detected in 19 animals from 6 farms. All of these were positive by TaqMan PCR and 8 of these were also positive by conventional PCR. PCR products from five samples that were positive by conventional PCR were sequenced and a BLAST search of the data bases with the sequences gave highest identity for *M. suis* for all 5. *M. suis* was not observed in any of the Giemsa stained blood smears and could not be seen in a small number of samples tested by confocal microscopy.

A crude prevalence of *M. suis* was determined for each farm and this calculation showed that *M. suis* was present with a prevalence ranging from 0.64 - 5.4 %. A Bayesian analysis has been performed on the results in order to adjust the crude prevalence for the presumed imperfect sensitivities (Se) and specificities (Sp) of the two PCR tests (Table 5). These adjustments gave an estimate for the prevalence of *M. suis* in the farms supplying the sampled abattoir to be 4.29% with lower and upper confidence limits of 2.6% and 6.45% respectively.

In summary, based on molecular detection techniques, *M. suis* has been confirmed to be present within Australia, although the extent of its distribution is yet to be determined. The results suggest that *M. suis* may not be ubiquitous across Victorian pig farms. Based on the absence of clinical signs in the tested herds, and the lack of reporting of acute disease in commercial pig herds, it is likely that infections are sub-clinical or manifest as chronic, rather than acute disease.

3. Background to Research

It is claimed in the literature that porcine eperythrozoonosis can be a serious disease in feeder pigs and also it is often a chronic disease where it causes significant problems with unthriftiness in new born pigs and reproductive efficiency in sows. I realized that there was no evidence that work had been undertaken to determine if *M. suis* was present in Australian commercial pigs.

It is important to know if. *M. suis* is present in Australia and what effect it may be having on production in commercial piggeries.

If *M. suis* is present in Australia we need to know what the prevalence of the organism is in herds and how widespread infection with *M. suis* is in these herds. Is it worthwhile to consider trying to eradicate *M. suis* from Australian pigs?

4. Objectives of the Research Project

This project has the following aims,

1) To determine if *Mycoplasma suis* is present in a sample of feeder pigs and at what prevalence.
2) Confirm that the *M. suis* TaqMan test performs satisfactorily as a diagnostic test for the detection of *M. suis*.
3) To collect positive samples of *M. suis* for use as controls in diagnostic tests.
4) To obtain preliminary data on the impact of *M. suis* on growth rates.

5. Introductory Technical Information

Clinical Manifestations and Epidemiology

M. suis is an extracellular bacterial organism that attaches to and causes deformity and damage to porcine red blood cells. The acute form of the disease caused by *M. suis* was first described in 1932 (Hoelzle 2008). The resulting disease which is known by several names including, porcine eperythrozoonosis (PE) and porcine infectious anaemia. *M. suis* was traditionally considered a problem of feeder pigs in which it causes a febrile acute anaemia and jaundice with low morbidity and high mortality. Since the implementation of serological screening tests for *M. suis*, chronic low grade infections have been implicated in the aetiology of a variety of clinical syndromes comprising (a) anaemia, mild icterus, and general unthriftiness in newborns (b) growth retardation in feeder pigs and (c) decreased reproductive efficiency in sows. Adverse environmental conditions, nutritional mismanagement, and co-infections with other microbial agents have been suggested as important factors predisposing to the development of clinical PE (Henry, 1979, Hoelzle, 2003).

Nearly all clinically infected pigs will become carriers as antibiotic treatment will cure clinical symptoms of a PE attack however it is impossible to eliminate *M. suis* from infected pigs. Persistently and clinically unapparent infected pigs remain carrier animals and transmitters of *M. suis* within their herd or between different herds. *M. suis* can also spread to other pigs through the use of contaminated needles and syringes, ingestion of contaminated blood (cannibalism), by arthropod vectors such as mosquitos and pig louse and transplacental infection (Hoelzle, 2008, Messick,2004, Gresham,1994).

Global Distribution

Porcine infectious anaemia is a well known disease that occurs worldwide and is caused by the uncultivable haemotrophic bacterium *M suis*. The actual prevalence and impact of *M. suis* infections, however, remains uncertain. Although *M. suis* infections have been continuously reported worldwide over the past 75 years with reports from Europe, the United States, South America (Brazil), Africa (Nigeria) and Asia (China) the actual prevalence of *M. suis* in pig populations was not evaluated.

Selected Previous Prevalence Studies

A study in Germany was undertaken to determine the prevalence of *M. suis* in healthy post–weaning pigs (20-30 kg) from slaughterhouses because feeder pigs are known to be most affected. Results from this study showed that 13.9% of all pigs and 40.8% of pig farms were infected with *M. suis*. This study concluded that undetected *M. suis* infections can, in part, lead to significant economic losses in pig production. Extended feeding periods, higher feed costs, greater susceptibility to contact infectious viruses of the respiratory and intestinal tract as well as the premature slaughtering of animals can lead to considerable profit losses (Ritzmann et al 2009).

In a small study in southern Brazil *M. suis* was detected on four farms. Blood samples were taken from 121 sows in different reproductive phases, 61 piglets (9 to30 days of age) and 4 boars. Of 186 animals tested 18.2% were positive by PCR and 33.1% were positive by Southern blot (Guimaraes et al 2007).

Diagnosis of Porcine Infectious Anaemia

To date *M. suis* has not been cultivated. Alternative diagnostic tools are few and have many limitations. Laboratory confirmation of porcine infectious anaemia still relies on microscopic examination of peripheral blood smears to directly visualise *M. suis* cells attached to erythrocytes. The drawbacks of microscopy, include problems with both specificity and sensitivity, because the identifiable but short term bacteraemia linked with the onset of acute disease is lacking in chronic infections. Given the low cost and high throughput possibility, serological assays are very important in pig husbandry. No standardised assay for routine application is available for this approach. The literature currently describes three different assays (i) a complement fixation assay, (ii) an indirect haemaglutination assay and (iii) an enzyme linked immune assay (ELISA) These serodiagnostic assays share the intrinsic disadvantage of employing complex and undefined *M. suis* antigens from the peripheral blood of experimentally infected pigs. Antigens from the blood of experimentally infected pigs had to be purified in laborious and time consuming procedures. Therefore pig derived constituents and extreme variability among different batches characterise antigen preparation and this obstructs the accurate adoption and standardisation of diagnostic serological procedures (Hoelzle, 2008). Recently ELISA's using two *M. suis* recombinant antigens have been reported. It was claimed to have equal to or higher than those of the *M. suis* whole-cell ELISA and these tests may prove more reliable and reproducible for the diagnosis of *M. suis* infections (Hoelzle, 2007).

As a result of the limitations of smears and serological assays, molecular techniques, in particular PCR amplification based on the 16S rRNA gene (Messick et al 1999) and the rpoB gene (Gwaltney et al 1993) were the most effective methods for the identification of *M. suis* in animal hosts. These PCR assays were confirmed as sensitive and specific tools for the diagnosis of *M. suis*, e.g. in the blood of either acutely infected or asymptomatic carrier animals and are a better alternative than direct microscopy of blood smears (Hoelzle, 2008).

Recently further improvement of molecular diagnostic options was achieved by establishing a Light Cycler real-time PCR for the quantitative detection of *M. suis* (Hoelzle et al 2007). The conserved msg 1 gene which encodes a surface localised GAPDH analogous protein with adhesion properties was chosen for the primer and probe design.

In addition to the known advantages of real – time PCR approaches, i.e. low contamination risk, high throughput and standardisation, this *M. suis* real-time protocol is specific, sensitive, reproducible and reliable as well as working well in different matrices (e.g. blood and organs) (Hoelzle, 2007).

Furthermore, the epidemiology of the *M. suis* is largely unclear and whether *M. suis* strains of different virulence exist is not known. In addition, early accurate recognition of chronic infections is essential to develop control measures.

At AAHL I have used the msg gene sequence to design primers and probe so that they are suitable for a TaqMan PCR assay. I have obtained DNA from Dr Hoelzle in Switzerland and tested this in my assay and have confirmed that the test works. I have also used 16S primer sequences that are specific for *M. suis,* as published by Messick *et al*. I have tested these and they work with *M. suis* DNA that I obtained from Dr Hoelzle.

6. Research Methodology

Study Design

Calculations were made to determine a sample size required to be able to determine the prevalence in the source population given certain assumptions. The diagnostic sensitivity was assumed to be 80% (Hoelzle 2007 tested the Light Cycler real time assay on infected pigs the assay had 100% sensitivity) Sp 99% (Although the TaqMan specificity is only 95%, when combined with follow up 16S sequencing it will approach 100%.) Prevalence 10% (This is based on overseas findings in the literature (Ritzmann,2009; Guimaraes, 2007). Precision 4% (Based on an expected Prevalence of 10% ± 4%). Using the assumptions above we calculated that to determine the prevalence in the sample population we need to sample 312 pigs. If *M. suis* was not present in farms supplying the abattoir we needed to be able to state with confidence that the failure to find any positives was not due to insufficient sample size. Assuming a detection prevalence of 1%, this will show us that if none are detected, a sample size of 332 will ensure that this disease is truly not present in the population or at a minimum level of <1%. However it was not expected that *M. suis* would not be detected.

The Diamond Valley Abattoir in Laverton, Victoria was chosen to collect samples because it received pigs from numerous different farms in the surrounding district. Blood samples were collected from pigs at slaughter. The samples were collected following a Systematic Random Sampling technique where-in the first pig to be sampled in the chain is determined randomly and the remainder follow at a defined interval. 322 pigs were sampled at three separate collections. At each collection whole blood in an EDTA blood tube and clotted blood was collected in a plain blood tube. The clotted blood was used to collect serum for the performance of an ELISA assay which will be done by Dr Hoelzle in Germany. The whole blood was used for the preparation of blood smears and DNA extraction for the performance of a conventional PCR assay and a TaqMan PCR.

Diagnostic Testing

DNA Extraction

DNA was extracted from blood using the MagMax -96 Multisample Kit. 50 ul of blood was extracted on the Applied Biosystems MagMax Express -96 robot. DNA was eluted in 100ul of buffer.

TaqMan PCR

At AAHL, I used the msg gene sequence to design primers and probe so that they were suitable for a TaqMan PCR assay. I have obtained DNA from Dr Hoelzle and tested this in my assay to confirm that the test worked. The primer and probe sequences are listed in Table I

Table 1:

Primer/Probe Name	Sequence
M. suis – msg Fwd	TTCATGACAACAGTTCACGCTTT
M. suis – msg Rev	CTTAGGTCAGAGTGAGGAGAGTCTTG
M. suis – msg	6FAMACTTCTGACCAAAGACMGBNFQ

Experiments were performed to optimise the primer and probe concentrations. From these experiments the optimal primer concentrations were 900 nm for the forward primer and 300 nM for the reverse primer. The optimal concentration for the probe was 250 nM. PCR was performed using Applied Biosystems TaqMan 2X Universal PCR Master Mix in a 25 ul reaction containing 2 ul of template DNA. Standard PCR conditions for the ABI 7500 PCR machine were used.

Conventional PCR

Primers targeting the 16S rRNA of *M. suis* were published by Messick (1999). Primers E. suis f1 and E. suis r2 that produce a 1394 base pair fragment were used in this work. The sequences are listed in Table 2. 2 ul of template DNA was added to a reaction mix containing 12.5 ul HotStar Taq MasterMix (Qiagen) and 0.5 ul 18 uM forward and reverse primers in a 25 ul PCR reaction. PCR conditions consisted of an initial denaturation step of 95^0C for 15 minutes followed by 50 cycles of 94^0C for 1 minute, 50^0C for 1 minute and 72^0C for 2 minutes and a final extension of 72^0C for 7 minutes. The PCR was tested using *M. suis* DNA that was obtained from Dr Hoelzle and produced PCR products of the expected size.

Table 2:

Primer Name	Primer sequence
E. suis f1	AAAAAGGCCCTCGGGTCTTT
E. suis r2	CTTAACTCCAATCAAAATTACC

Sequencing and Analysis

Products that resulted from PCR using primers E. suis f1 and E. suis f2 were then gene cleaned and sequenced. Sequencing was performed in an ABI 3130XL Genetic Analyzer using a BigDyeTerminator V3.1 cycle sequencing kit. Each of sequences determined were analysed and aligned using the SeqMan Pro module of the Lasergene ver. 8.0.2 software package. The sequence was used as a query in a BLAST 2.2.23 search to obtain an identification.

Giemsa Stain

A thin blood film was prepared on a microscope slide by adding a drop of blood to a slide and then dragging the blood down the slide using another slide. The blood film was air dried and fixed in methanol. The blood film was then Giemsa stained by dipping the slide 12 times in Rapid Diff No1 (Aust. Biostain P/L) followed by 6 dips in Rapid Diff Stain No 2 (Aust Biostain P/L). The slide was rinsed by dipping twice in tap water. The slide was allowed to air dry. The slide was mounted by dipping Xylene and a cover slip was mounted using DPX (Sigma).

Confocal Microscopy

Six blood smears that were positive by TaqMan PCR and conventional PCR were prepared on microscope slides, air dried and fixed in 4% paraformaldehyde in PBS. The slides were labelled with a 1:4000 dilution of DAPI (Sigma, 5 mins diluted in water), which binds to DNA, and then washed with water. The slides were cover slipped using an aqueous mounting medium and the coverslips sealed with nail varnish. They were imaged with a Leica SP5 confocal microscope with a 405nm laser. No

fluorescent signal that would have suggested the presence of mycoplasma-like organisms was detected.

7. Discussion of Results

Samples from a total of 322 pigs were collected at slaughter and tested by TaqMan PCR, conventional PCR and Giemsa stained blood films. *M. suis* was detected by TaqMan PCR and conventional PCR. No evidence of *M. suis* was detected in blood smears stained by Giemsa stain. The results are shown in Table 3.

Table 3:

No of samples	TaqMan PCR	Conventional PCR	Giemsa stained Blood smears
322	19	8	0

A cut-off point for the TaqMan PCR has not been determined as yet. Samples that gave a positive amplification curve in the first round of testing were retested following re-extraction of a second set of blood samples. When the TaqMan was performed a second time a positive control was not included and these results were compared with the first run results. Samples that gave a typical sigmoidal amplification curve in both runs were considered to be positive.

The conventional PCR was initially performed at 32 cycles as per the Messick publication. However at 32 cycles none of the samples were positive. When the number of cycles was increased to 50 cycles, 8 positive samples were detected. 5 of the PCR products were sequenced and a BLAST search of the sequences from all 5 products showed highest identity for *M. suis*.

M. suis was detected in animals from 6 of 24 farms sampled. Animals from one farm were sampled twice. The results are shown in Table 4.

Table 4:

Farm	No. positive	No. tested	Crude Prevalence (%)
1	1	156	0.64
2	2	119	1.68
3	2	50	4.0
4	2	37	5.4
5	5	115	4.34
6	5	112	4.36
7	2	50	4.0

Farm 2 and 5 are the same farm. Animals from this farm were sampled twice.

It is important to note that there was complete correlation between the TaqMan test and the conventional PCR in that a sample that was positive in the conventional PCR was also positive in the TaqMan PCR. The TaqMan is considerably more sensitive than the conventional PCR. The results of this survey show that the TaqMan assay is a more sensitive than the conventional PCR assay and Giemsa stained blood films. An experiment to test the specificity of the *M. suis* TaqMan assay was performed with DNA of other bacteria including mycoplasmas (Table 5). None of the organisms tested showed reaction in the TaqMan assay.

Table 5:

Number	Organism
1	Mycoplasma hyopneumoniae
2	Mycoplasma agalactiae
3	Bovine Group 7 mycoplasma
4	Mycoplasmma capricolum
5	Mycoplasma bovis
6	Pseudomonas aeruginosa
7	Ornithobacterium rhinotraceale
8	Brucella abortus
9	Enterobacter cloacae
10	Mycobacterium tuberculosis
11	Pasteurella multocida
12	Salmonella typhimurium
13	Staphylococcus aureus
14	Escherichia coli

The survey has shown that the TaqMan test is a suitable test for detecting *M. suis* carriers in clinically normal animals that are not showing signs of clinical PE. It is expected that this test would detect *M. suis* in a clinically infected animal where there is likely to be much higher numbers of circulating *M. suis* The prevalence of *M. suis* infection can be estimated using the number of animals that were positive in the TaqMan test and dividing by the total number of animals sampled. This gave a crude prevalence of 5.7%.The samples were collected from 24 farms and in addition there were 14 samples collected from animals with various tattoos purchased from the Ballarat market. *M. suis* was detected in animals from 6 farms (See Table 4). Calculations of the prevalence of *M. suis* on each farm ranged from 0.64 to 5.4 percent.

A Bayesian analysis has been performed on the results in order to adjust the crude prevalence for the presumed imperfect sensitivities (Se) and specificities (Sp) of the two PCR tests (Table 6). These adjustments gave an estimate for the prevalence of *M. suis* in the farms supplying the sampled abattoir to be 4.29% with lower and upper confidence limits of 2.6% and 6.45% respectively.

Table 6:

Test system	Estimator	Estimate	5% Interval	95% Interval
Perfect RT-PCR (100% Se &Sp)	Classical	6%	3.6%	9.1%
Imperfect RT-PCR (90% Se & 95% Sp)	Classical	1.1%	0	4.9%
Perfect cPCR (80% Se &95% Sp)	Classical	2.5%	1.1%	4.9%
1 Imperfect test (RT – PCR)	Bayesian	4.9%	2.6%	7.9%
2 imperfect tests (RT – PCR & cPCR)	Bayesian	4.3%	2.6%	6.5%

Note: "intervals" are confidence intervals for classical statistics and credible or probability intervals for Bayesian statistics.

Other Evidence for the Presence of M. suis in Australia

On presentation of this work at AAVLD 2011, Stephen Davis from Berrimah Veterinary Laboratory, NT Department of Resources mentioned that a case of clinical disease in a commercial piggery in Darwin had been confirmed as M. Suis. This outbreak occurred in 2001 and subsequent work on these samples was reported by Kylie Deece. This report can be obtained on request.

Conclusions

The following conclusions can be drawn from this study

- *M. suis* has been confirmed to be present within Australia, although its exact geographical extent within the pig population is to be determined.
- Infection does not seem to be ubiquitous across all commercial piggeries, although larger studies would be needed to confirm this.
- For those herds in Victoria which are infected, the disease is likely to be sub-clinical, although whether it affects weight gain is to be determined.

Samples of blood were collected from each animal. This has provided a small amount of positive material from the animals that were positive. However all the animals were clinically normal and positive material from a clinically infected animal would provide far more significant amounts of reference material.

The data collected from this survey has not been sufficient to comment on the impact of *M. suis* infection on growth rates. To assess this, comparison of growth rates between infected and non-infected herds (or subsets of herds) in a longitudinal study would be necessary.

8. Implications and Recommendations

This project has been able to demonstrate that *M. suis* is present in Australian commercial pigs. It has been shown in other countries that *M. suis* can be a serious pathogen in pigs and has economic significance in the pig industry in many parts of the world (Hoelzle, 2007). *M. suis* affects mainly feeder pigs where it causes a febrile acute icteroanemia with low morbidity and high mortality. Moreover, under adverse environmental conditions, chronic low-grade infections may cause a variety of clinical syndromes comprising anemia, mild icterus, and general unthriftiness in newborns, growth retardation in feeder pigs, and decreased reproductive efficiency in sows.

As a result of this project a TaqMan PCR test has been developed and tested and shown to be more sensitive than a conventional PCR used as a comparison test.

Thus the TaqMan is a valuable diagnostic tool that can be utilised in further studies on the impact of *M. suis* on the Australian pig industry.

It is very important that further work be undertaken to purchase and splenectomise at least 2 pigs that are shown to be infected with *M. suis*. Splenectomising the pigs allows *M. suis* to grow to very high titres in the pig and this will enable the collection of large amounts of *M. suis* from the blood of the animals. This will provide material for positive controls for use in diagnostic tests and in further research such as whole genome sequencing. It is important to undertake whole genome sequencing of a *M. suis* isolate. This would provide valuable information as to the origin of the *M. suis* isolates in Australia and also provide comparison of the genome with two others in North America and Europe which are available in sequence data bases.

The development of a serological test such as an ELISA would provide a cheap effective test to screen for the presence of *M. suis* in a herd. An ELISA that uses a recombinant antigen has been developed in Germany.

It is recommended that further work be carried out to gain a greater understanding of the economic impact of *M. suis*. This could be done by undertaking studies of farms that have *M. suis* present in their pigs compared to farms where *M. suis* is not present.

9. Intellectual Property

No commercially valuable intellectual property has been developed from this project.

10. Technical Summary

A sensitive and specific test has been developed for the detection of *M. suis* in pigs. It has been shown that the disease is present in commercial pigs in Australia.

11. References

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12. Publications Arising

The results of this work were presented at the AAVLD conference in Sydney in December 2011. The talk was titled "A survey to detect the presence of Mycoplasma suis in pigs." Authors Trevor Taylor, John White and Peter Durr.

A manuscript for publication in a journal is being prepared.