An investigation into the extent of occurrence of novel pathogenic *Brachyspira* species, including newly recognised agents of swine dysentery, in Australian pig herds

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Executive Summary

Swine dysentery (SD) is a bacterial disease of grower and finisher pigs, causing colitis and bloody mucoid diarrhoea. In infected herds it can have a major economic impact through reduced growth rates, poor food conversion, mortalities and costs of control.

The classical agent of SD is an anaerobic spirochaete called *Brachyspira hyodysenteriae*, although in the last decade two other species named "*Brachyspira hampsonii*" and *"Brachyspira suanatina"* have been identified as also causing SD in North America and Europe. As these species may have a different epidemiology to *B. hyodysenteriae,* the main aim of the current study was to determine whether they occurred in Australia. A subsidiary linked aim was to provide updated information on the occurrence, distribution and characteristics of *B. hyodysenteriae* strains that are currently present in Australia.

A total of 611 faecal and colonic samples from 89 herds were received and tested at Murdoch University over the 12-month study period. No isolates of the two new species were identified from herds with disease and it was concluded that it was unlikely that they are present in Australia, and so no further specific measures are needed.

B. hyodysenteriae was found commonly, being identified in 27 (30.3%) of tested herds, including all six herds that showed no signs of disease and all five herds with mild signs of disease not attributed to SD. A number of these 11 herds either had given apparently "false positive" reactions in a serological ELISA for SD, or were epidemiologically linked to such herds. One of the infected herds without disease was a breeding herd providing stock to other herds. The finding of *B. hyodysenteriae* in herds without disease is significant and poses problems for diagnosis and control of SD in Australia. The other 16 infected herds had a history of having had SD or had relevant clinical signs.

Multilocus sequence typing (MLST) applied to a subset of the *B. hyodysenteriae* isolates revealed that they were diverse and distinct from earlier Australian isolates or those from overseas. Related isolates were found in some herds with epidemiological links. MLST was shown to be a very useful tool for monitoring *B. hyodysenteriae* strains in Australia.

Resistance to four key antimicrobial agents (tiamulin, tylosin, lincomycin, oloquindox) occurred and was more common compared to Australian isolates tested in 2007. Furthermore three multi-drug resistant isolates were identified from different herds. Resistant isolates represent a serious risk for future control of SD in Australia.

Application of a test for virulence-associated genes showed a high agreement between lack of these genes and origin of the isolates from herds with no disease or only mild disease. In a number of cases where isolates lacking the genes were found in herds with disease, other isolates that had the genes also were present and were presumed to cause the disease. This test appears to be a good predictor of strains that are less able to colonise and cause disease, and can be recommended for more wide scale diagnostic use.

In view of the high prevalence of colonisation amongst Australian herds, including breeding herds, it is recommended that a protocol be developed to provide confidence about whether or not herds are colonised with *B. hyodysenteriae*. This might include regular serological ELISA testing coupled with culture and PCR of colonic samples at slaughter check. In addition, surveillance for *B. hyodysenteriae* amongst Australian herds should be ongoing to provide detailed profiling of current *B. hyodysenteriae* strains, including strain typing by MLST, antimicrobial susceptibility testing and undertaking virulence gene testing. These data will assist in the long-term control of SD in Australia.

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1. Introduction

Swine dysentery (SD) is a severe mucohaemorrhagic colitis that classically results from infection of the caecum and colon with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*. SD can severely depress feed conversion efficiency in the grower/finisher phases, and in addition control requires considerable antimicrobial use, whilst the disease represents an animal welfare issue. Multi-drug resistant strains of *B. hyodysenteriae* have been recorded in Europe, and are likely to exist elsewhere. Classical SD recently has re-emerged in the North and South America, and remains endemic in most other regions of the world. It is known to occur in farms across Australia (Hampson *et al*, 2015).

Until recently *B. hyodysenteriae* has been believed to be the only causative agent of swine dysentery; however, outbreaks of bloody diarrhoea indistinguishable from SD have been documented since 2007 in grower-finisher pigs in Canada and the USA in farms where *B. hyodysenteriae* could not be identified. Investigation of these cases led to the recognition of novel, strongly ß-haemolytic *Brachyspira* isolates, for which the name "*Brachyspira hampsonii*" has been proposed (Chandler *et al*, 2012). Experimental inoculations of pigs have established the pathogenic potential of this new species (Rubin *et al*, 2013; Costa *et al*, 2014). In addition, in 2013 cases of SD caused by *B. hampsonii* were recorded in pigs in Europe, and the species has been isolated from migratory waterbirds in Canada and Spain, and these species are thought to be reservoirs of the pathogen (Martínez-Lobo *et al*, 2013; Rubin *et al*, 2013; Mahu *et al*, 2014; Rhode *et al*, 2014).

A distinct strongly ß-haemolytic *Brachyspira* called "*Brachyspira suanatina*" that causes an SD-like disease also has been described in feral waterbirds and pigs in Scandinavia (Råsbäck *et al*, 2007).

The re-emergence of *Brachyspira* spp. including resistant strains of *B. hyodysenteriae* and novel species like "*B. hampsonii*" as pathogens has re-ignited significant concerns for pork-producers worldwide.

In Australia, a case of colitis associated with an "atypical" strongly ß-haemolytic *Brachyspira* strain also has been recorded, although it was not investigated fully. Australian pig veterinarians are aware of the importance of "*B. hampsonii"* and related species, but the prevalence of these pathogens amongst and within Australian herds is still not known. The lack of availability of diagnostic tools capable of identifying "*B. hampsonii"* is likely to be a contributing factor to this lack of data.

The main aim of this project was to determine to what extent novel pathogenic *Brachyspira* species, including the recently described "*B. hampsonii*" are present in Australian pig herds. A subsidiary aim was to investigate distribution, diversity, antimicrobial susceptibility and predicted virulence of strains of *B. hyodysenteriae* from Australian herds, including those with mild or no obvious disease.

2. Methodology

2.1 Detection of novel agents of swine dysentery in Australian herds

Sample collection

This project involved collaboration between Murdoch University, pig veterinarians from around Australia and diagnostic laboratories that submitted samples for testing. Throughout the duration of the project, laboratory facilities and staff at Murdoch University were made available for screening field samples from pig herds. Samples were requested from herds with a history of SD, whether or not they showed disease at the time of sampling, from herds with mild enteritis of uncertain aetiology, and from herds that had did not have disease but had given positive reactions ("false positives") in a serological ELISA for SD in a previous study.

Bacteriological culture

All faecal or colonic samples that were received were swabbed onto Trypticase Soy agar (TSA) plates containing 5% defibrinated ovine blood, and cultured for 5 to 7 days at 37°C in a culture jar with an anaerobic atmosphere generated by an AnaeroGen™ 2.5L Sachet (Oxoid). Zones of haemolysis around the inoculated area indicated growth, and confirmation was obtained by resuspending surface growth in phosphate buffered saline (PBS) and viewing with a phase-contrast microscope. Those samples which contained motile spirochaetes were retained for testing by Polymerase Chain Reaction (PCR).

Diagnostic PCR

The identification of the *Brachyspira* species cultured from the field samples was determined using published PCR tests for *B. hyodysenteriae* (La *et al*, 2003), *Brachyspira intermedia* (Phillips *et al*, 2006) and *Brachyspira pilosicoli* (La *et al*, 2003). New PCR tests for "*B. hampsonii*" and "*B. suanatina*" were developed using unique primers targeting the haemolysin A gene (*tlyA*) and DNA-dependent RNA polymerase gene (*rpoC*), respectively. The specificity of the new PCR assays were confirmed using genomic DNA from 107 strains of *Brachyspira* spp. obtained from the culture collection at the Reference Centre for Intestinal Spirochaetes at Murdoch University. A genus-specific PCR for *Brachyspira* spp. (Phillips *et al*, 2006) also was included.

For the field samples, PCR assays were applied to growth harvested from the primary isolation plates and resuspended in sterile water. The PCR assays were performed in 25 µl reactions consisting of 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase, 0.2 mM of each dNTP and 0.5 µM of forward and reverse primers. Cycling conditions involved an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 15 s and primer extension at 72°C for 1 min. The products were separated by gel electrophoresis and visualised over UV light after staining with ethidium bromide.

2.2 Antimicrobial Susceptibility Testing

Growth of spirochaetes

Isolates were subcultured until pure. The spirochaetes to be tested for antimicrobial susceptibility then were cultured on a fresh TSA plate at 37°C for 5 days, as previously described (section 2.1). The cells were harvested from the agar plate by resuspending the surface growth with 2 ml sterile PBS, transferred into a microfuge tube and then counted using a haemocytometer. The spirochaete cells were collected by centrifuging at 5,000 *g* and the pellet resuspended with sterile PBS at a density of 10^6 cells per ml.

Susceptibility testing

Antimicrobial susceptibility of the field isolates was assessed using the agar-dilution method. The test plates consisted of TSA containing 5% defibrinated ovine blood and the appropriate antibiotic concentration. Control plates did not include antibiotics. The plates were incubated for 5 days at 37 °C in anaerobic jars and then observed for haemolysis. The isolates were tested for susceptibility to varying concentrations of tiamulin (0.25, 0.5, 1, 4 and 8 µg/ml), tylosin (1, 5, 25, 50 and 100 µg/ml), lincomycin (2, 4, 16, 36, and 72 µg/ml) and olaquindox (0.1, 0.5, 2 and 4 µg/ml).

A total of 10^5 cells were drop-inoculated onto the control and sensitivity plates. Each isolate was tested in duplicate and *B. hyodysenteriae* control strain WA1 was included in each batch of tests. Growth of the strains on the control and sensitivity plates was checked visually after 5 days incubation. Zones of haemolysis present around the growth on the control plates were determined, and isolates were recorded as being susceptible to the antimicrobial concentration in the test plates if no such zones were observed. Any surface growth was scraped off the plate and examined under a phase contrast microscope to confirm purity and the endpoint.

Interpretation of results

The first sensitive colony zone and the last resistant colonies were checked for spirochaete growth by phase-contrast microscopy. The minimum inhibiting concentration (MIC) of the antibiotic was reported as the lowest concentration of antimicrobial that inhibited growth. MIC breakpoints used to assist interpretation of the results are presented in Table 1.

Table 1. MIC breakpoints (µg/ml) for *in-vitro* antimicrobial susceptibility tests performed on Brachyspira isolates recovered from field samples.

Antimicrobial	Sensitive	Intermediate	Resistant	Reference
Tiamulin	≤ 0.25	> 0.25 ≤ 2	> 2	Pringle *et al*, 2012
Tylosin	≤ 1	> 1 ≤ 4	> 4	Ronne and Szancer, 1990
Lincomycin	≤ 4	> 4 ≤ 36	> 36	Ronne and Szancer, 1990
Olaquindox	< 0.1	-	-	Uezato *et al*, 2004

2.3 Multi-locus Sequence Typing (MLST) of B. hyodysenteriae isolates

Growth of spirochaetes

Thirty-one *B. hyodysenteriae* field isolates and four historical Australian isolates were typed by MLST. The historical isolates were obtained from the Reference Centre for Intestinal Spirochetes at Murdoch University and originated from three different farms that were of interest to one of the submitting veterinarians because of their connections to currently infected farms. The field isolates were subcultured to purity prior to use, and the historical isolates were subculture from storage at -80°C. All isolates were cultured on a fresh TSA plate at 37°C for 5 days, as previously described (section 2.1). The cells were harvested from the agar plate by resuspending the surface growth with 2 ml sterile PBS, transferred into a microfuge tube and then counted using a haemocytometer. The cells were collected by centrifuging at 5,000 *g* and the cell pellet was resuspended with sterile PBS to a density of 10^6 cells per ml.

DNA extraction

High molecular weight DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Ten ml of a 10^8 cells per ml culture of *Brachyspira* was harvested by centrifugation at 5,000 *g*. The cell pellet was resuspended in 180 µl of lysis buffer containing 20 µl of proteinase K (10 mg/ml) and incubated at 55°C for 30 min. After all the cells had been lysed, 180 µl of AL Buffer was added and the sample incubated at 70°C for 10 min. Two hundred µl of absolute ethanol was immediately added to the sample and this was transferred to a DNeasy column. Column wash buffers AW1 and AW2 were added sequentially to the columns and centrifuged at 6,000 x g. The flow-through was discarded, and the DNA was eluted with elution buffer and stored at -20°C.

Multilocus sequence typing (MLST)

MLST was conducted as previously described (La *et al*, 2009). PCR assays were performed in 50 µl reactions consisting of 1x PCR buffer, 1.5 mM $MgSO_4$, 0.5 U HotStar HiFidelity DNA polymerase (Qiagen), 0.2 mM of each dNTP and 0.5 µM of forward and reverse primers. Cycling conditions involved an initial enzyme activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 15 s and primer extension at 72°C for 1 min. The products were separated by gel electrophoresis and visualised after staining with ethidium bromide. PCR products used for sequencing were purified with the UltraClean™ PCR Clean-up Kit (Mo Bio Laboratories) according to the manufacturer's instructions. The purified PCR products were used for sequencing. Sequencing reactions were performed in 10 µl volumes using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer's instructions. An annealing temperature of 50°C was used. The sequencing products were analysed using the Applied Biosystems 3730XL DNA analyser.

Data analysis

The raw sequences were edited and analysed using Geneious version 7.1.7 (Biomatters Ltd). For each locus the consensus sequences were aligned with the original *B. hyodysenteriae* strain WA1 sequence downloaded from the PubMLST database (www.pubmlst.org) and the aligned loci sequences were trimmed for subsequent MLST analysis as previously reported (La *et al*, 2009). Allele designations for each locus were obtained by a query search from the PubMLST website. New sequence type (ST) designations were assigned by Dr Tom La, the PubMLST curator for the *Brachyspira* MLST schemes.

Dendograms and minimum spanning trees (MST) were constructed from the data matrix of allelic mismatches using the UPGMA (unweighted-pair group method with allelic arithmetic means) method with 1000 bootstrap replicates using the PHYLOViZ software (Francisco *et al*, 2012). Colour coding of the MSTs was added to highlight the region where the isolates originated and source/supplier of the pigs.

2.4 Virulence Gene Testing of B. hyodysenteriae isolates

DNA preparation

High molecular weight DNA was extracted from 27 *B. hyodysenteriae* field isolates and four historical isolates as described above (section 2.3). The field isolates were select based on clinical information provided by the consulting veterinarians.

PCR for virulence genes

PCR tests targeting six plasmid-encoded genes recently described as possible virulence genes in *B. hyodysenteriae* (La *et al*, 2014) were used for the prediction of virulence in *B. hyodysenteriae* isolates. For each plasmid-encoded gene, three primer pairs were designed for PCR amplification. PCR assays were performed in 25 µl reactions, as described previously (section 2.1) with the exception that the annealing temperature used for each primer was set at 5 °C less than the optimal annealing temperature to allow for a moderate stringency reaction. Amplification products were electrophoresed through an agarose gel, stained with ethidium bromide and viewed over ultraviolet light. Isolates were predicted to have reduced virulence potential when two or more of the six virulence-associated genes gave a negative result.

3. Outcomes

3.1 Detection of Brachyspira species in Australian herds

A total of 611 samples, comprising 372 faecal samples and 239 colon samples were forwarded to Murdoch University for analysis during the period June 2014 to June 2015. These samples originated from 89 farms across five Australian States

(Victoria, New South Wales, South Australia, Queensland and Western Australia). "*B. hampsonii*" and "*B. suanatina*" were not found in any of the samples tested.

Brachyspira hyodysenteriae was detected in 83 (13.6 %) of the 611 samples originating from 27 of the 89 herds examined (30.3%). Of the 27 herds, six (22.2%) were reported as not showing signs of disease and five (18.5%) showed some mild signs of uncertain significance and aetiology. The other 16 herds were suspected of having SD based on clinical signs or past history. The herds without disease were sampled because they either were recorded as being "false positives" or epidemiologically linked to such herds in a previous study using a prototype SD serological ELISA test kit. They were considered false positive in the ELISA because no clinical disease was recorded. One of the herds without clinical signs but that was colonised was a breeding herd for a major Australian pig supplier.

A total of 56 *B. hyodysenteriae* isolates were successfully recovered in pure culture, of which a subset were further analysed (see below).

In addition, the potential pathogen *Brachyspira pilosicoli* was identified in 64 (10.5 %) of the 611 samples, *B. intermedia* (of uncertain clinical significance) in 56 (9.2 %) and non-pathogenic *B. innocens*/*B. murdochii* in eight (1.3 %) of the samples.

3.2 Antimicrobial Susceptibility Testing of B. hyodysenteriae isolates

The MIC values for 29 *B. hyodysenteriae* field isolates to tiamulin, tylosin, lincomycin and olaquindox are shown in Tables 2 to 5 respectively. A summary of resistance status of these isolates for the four antimicrobials is given in Table 6.

Tiamulin susceptibility was reasonably high (around 72% of isolates), however there appeared to be an increasing trend towards resistance (Table 2). A majority of the isolates (93%) were resistant to tylosin (Table 3). Few isolates (17.2%) were fully susceptible to lincomycin (Table 4), with around one quarter showing intermediate susceptibility and over half being fully resistant. Although no clear guidelines about how best to interpret the olaquindox MIC data are available for *Brachyspira* spp., the results indicate a similar pattern with around 62% resistant and 28% susceptible (Table 5). Three genetically distinct isolates were resistant to all four antimicrobials (isolates 9, 29 and 35) and one (isolate 37) was resistant to lincomycin, tylosin and olaquindox, and had intermediate resistance to tiamulin.

Table 2. MICs for tiamulin for the 29 *B. hyodysenteriae* isolate tested*

MIC (µg/ml) of tiamulin	State NSW	QLD	SA	VIC	WA	Total
≤ 0.25	9		3	6	4	22
> 0.25 ≤ 0.5						
> 0.5 ≤ 1		1		2	1	4
> 1 ≤ 4						
> 4 ≤ 8	1			2		3
> 8						
Total	10	1	3	10	5	**29**

*Grey shading indicates resistance

Table 3. MICs for tylosin for the 29 *B. hyodysenteriae* isolate tested*

MIC (µg/ml) of tylosin	State NSW	QLD	SA	VIC	WA	Total
≤ 1						
> 1 ≤ 5						
> 5 ≤ 25				2		2
> 25 ≤ 50	1				1	2
> 50 ≤ 75						
> 75 ≤ 100	1					1
> 100	8	1	3	8	4	24
Total	10	1	3	10	5	**29**

*Grey shading indicates resistance

Table 4. MICs for lincomycin for the 29 *B. hyodysenteriae* isolate tested*

MIC (µg/ml) of lincomycin	State NSW	QLD	SA	VIC	WA	Total
≤ 2	2				3	5
> 2 ≤ 4						
> 4 ≤ 16	2			3	1	6
> 16 ≤ 36	1			1		2
> 36 ≤ 72	1			2	1	4
> 72	4	1	3	4		12
Total	10	1	3	10	5	**29**

*Grey shading indicates resistance

Table 5. MICs for olaqunidox for the 29 *B. hyodysenteriae* isolate tested*

MIC (µg/ml) of olaquindox	State NSW	QLD	SA	VIC	WA	Total
≤ 0.1	1	1	1	3	2	8
> 0.1 ≤ 0.5	9		2	7		18
> 0.5 ≤ 2						
> 4						
Total	10	1	3	10	2**	**26**

*Grey shading indicates resistance
** 3 isolates not tested

Table 6. Classification of the 29 *B. hyodysenteriae* isolates collected as being susceptible, intermediate or resistant to the four antimicrobials

Antimicrobial	Susceptible (number of isolates)	Intermediate (number of isolates)	Resistant (number of isolates)
Tiamulin	21 (72.4%)	5 (17.2%)	3 (10.3%)
Tylosin	2 (6.9%)	-	27 (93.1%)
Lincomycin	5 (17.2%)	8 (27.6%)	16 (55.2%)
Olaquindox*	8 (27.6%)	na	18 (62.1%)

* breakpoints only categorise susceptible and resistant
na; not available

3.3 Multi-locus Sequence Typing of B. hyodysenteriae

The sequence types (ST) for 35 of the 56 *B. hyodysenteriae* isolates that were recovered are listed in Table 7. A total of 22 STs were identified, of which eight STs included multiple isolates (2-4 isolates), all of which originated from different farms. Multiple isolates were received from six farms (B, RL, Y, VD, L and BF), and in all seven cases these multiple isolates belonged to different STs. This was somewhat unanticipated, and is problematic on these farms as it could complicate control measures should the isolates have different antimicrobial susceptibility patterns and/or difference virulence potential.

All the STs identified in the study were unique and different from those previously identified for Australian and international isolates.

Table 7. MLST profiles for the 35 *B. hyodysenteriae* isolates tested from 26 farms. These included 31 field isolates recovered during this project (2014/2015) and four historical Australian isolates*

Isolate	Farm	State	MLST Profile					
			ST	*est*	*gdh*	*glp*K	*pgm*	*thi*
Isolate 1	Farm D	WA	ST150	3	4	13	9	3
Isolate 2	Farm RL	NSW	ST150	3	4	13	9	3
Isolate 3	Farm Y	WA	ST150	3	4	13	9	3
Isolate 4	Farm BC	QLD	ST151	3	4	17	9	3
Isolate 5	Farm B	NSW	ST152	3	4	33	3	3
Isolate 6	Farm SPC	Vic	ST152	3	4	33	9	3
Isolate 8	Farm RN	Vic	ST153	3	7	6	2	3
Isolate 9	Farm SM	Vic	ST154	3	7	17	9	3
Isolate 10**	Farm MT	Vic	ST154	3	7	17	9	3
Isolate 12	Farm WD	NSW	ST154	3	7	17	9	3
Isolate 13	Farm Y	WA	ST154	3	7	17	9	3
Isolate 14	Farm N	NSW	ST155	3	27	17	9	3
Isolate 15	Farm RL	NSW	ST156	10	4	13	9	3
Isolate 20	Farm W	NSW	ST156	10	4	13	9	3
Isolate 21**	Farm VD	Vic	ST157	10	10	8	2	12
Isolate 22**	Farm MD	Vic	ST158	10	14	6	2	3
Isolate 23**	Farm VD	Vic	ST158	10	14	6	2	3
Isolate 25	Farm GP	Vic	ST160	11	21	3	3	34
Isolate 26	Farm L	Vic	ST160	11	21	3	9	34
Isolate 27	Farm L	Vic	ST161	11	21	3	20	34
Isolate 28	Farm BK	Vic	ST162	11	21	32	3	1
Isolate 29	Farm G	NSW	ST162	11	21	32	3	1
Isolate 31	Farm WL	SA	ST162	11	21	32	3	1
Isolate 32	Farm M	Vic	ST162	11	21	32	9	1
Isolate 33	Farm BF	WA	ST163	14	1	13	2	2
Isolate 34	Farm BR	Vic	ST164	14	1	13	2	31
Isolate 35	Farm BO	Vic	ST165	14	1	13	3	2
Isolate 36	Farm WR	NSW	ST166	14	1	13	3	4
Isolate 37	Farm BP	Vic	ST167	14	1	13	9	2
Isolate 38	Farm B	NSW	ST167	3	4	33	3	3
Isolate 40	Farm KR	SA	ST169	14	17	16	2	6
Isolate 44	Farm KN	SA	ST170	14	17	16	3	6
Isolate 45	Farm B	NSW	ST171	22	27	3	3	1
Isolate 46	Farm B	NSW	ST172	23	28	34	2	32
Isolate 47	Farm BF	WA	ST173	24	29	35	19	33

*multiple isolates sharing an ST are outlined in grey

**historical isolate

A dendrogram demonstrating the relationships of the various STs to each other is shown as Figure 1. Minimum spanning trees (MSTs) showing the relationship of the *B. hyodysenteriae* isolates and the State from which they originated (Fig. 2) and the source of the pigs (Fig. 3) also are shown.

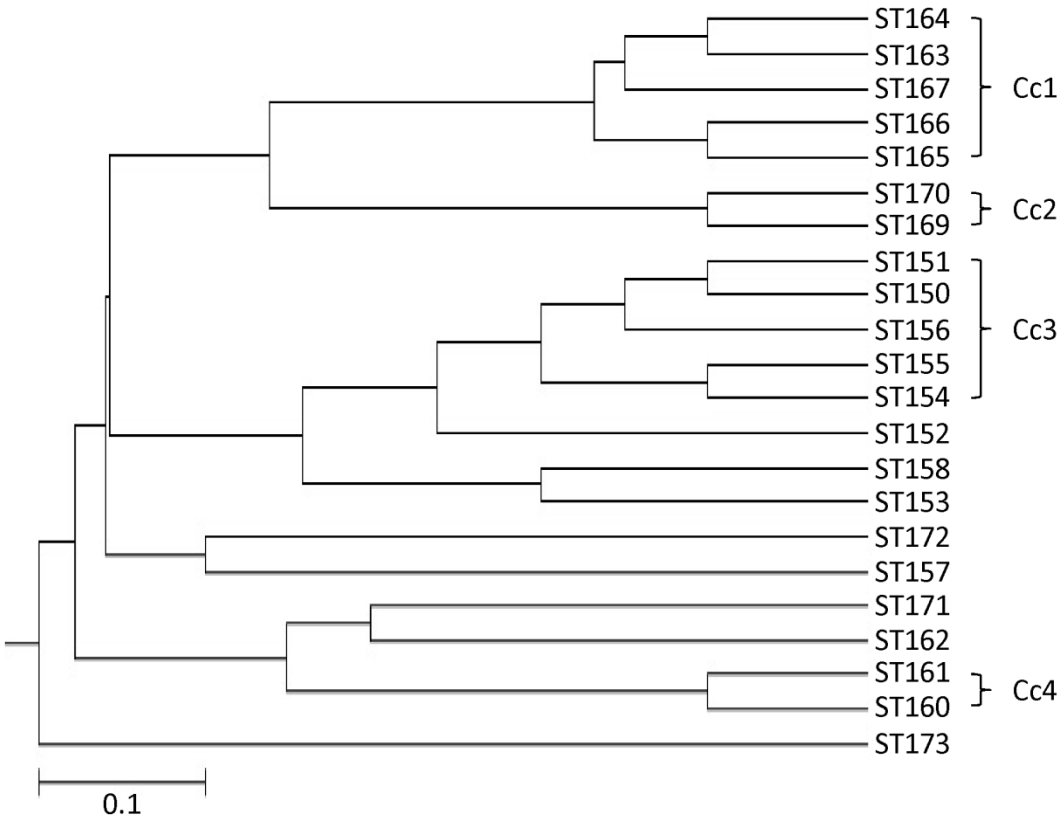

Figure 1. Dendrogram constructed from combined individual distance matrices of sequences of five genes *est*, *gdh*, *glp*K, *pgm* and *thi* from 35 *B. hyodysenteriae* isolates (31 field isolates and 4 historical isolates). Clusters of related isolates and individual unlinked isolates within the population are indicated as clonal complexes (Cc). The length of the scale bar represents 1 nucleotide substitution in 100 base pairs of the sequenced gene fragment.

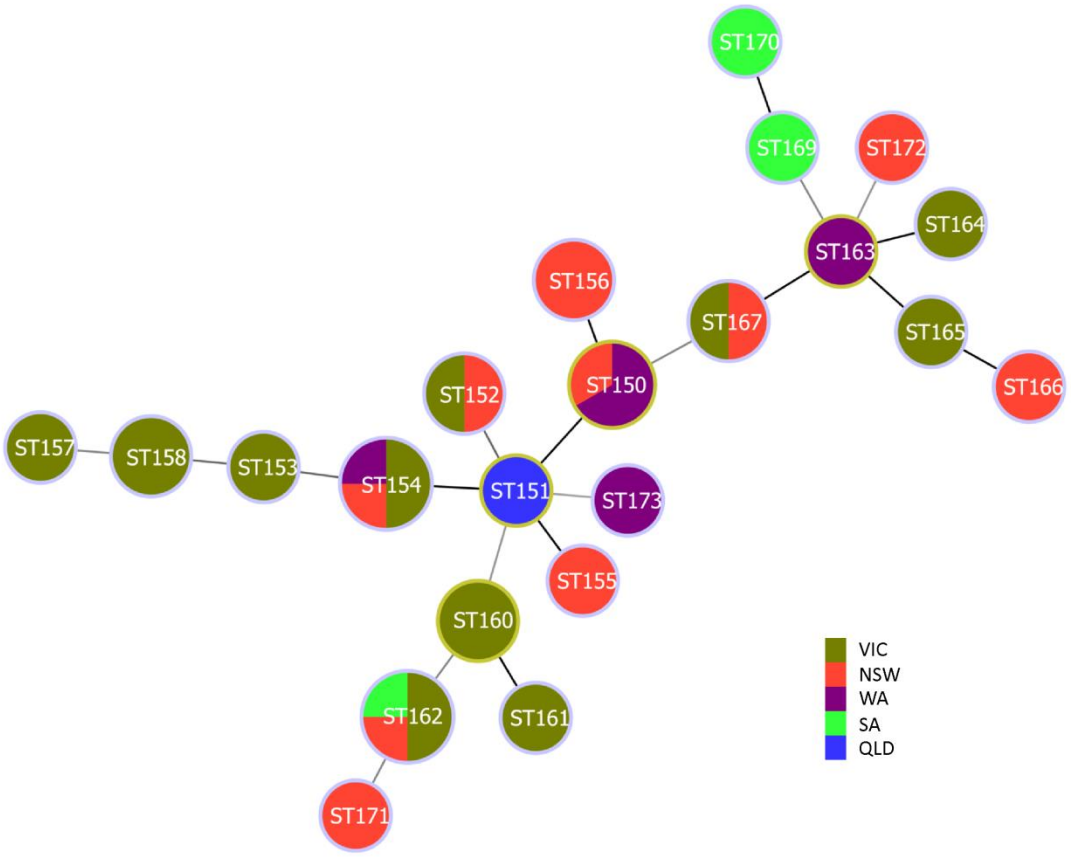

Figure 2. Minimum Spanning Tree (MST) analysis of 35 *B. hyodysenteriae* isolates contributing to 22 sequence types (ST) showing geographical distribution within Australia. In the MST, each node represents a different ST (labelled) and the colour represents the region of origin. Eight STs contained multiple isolates from different farms (larger circles).

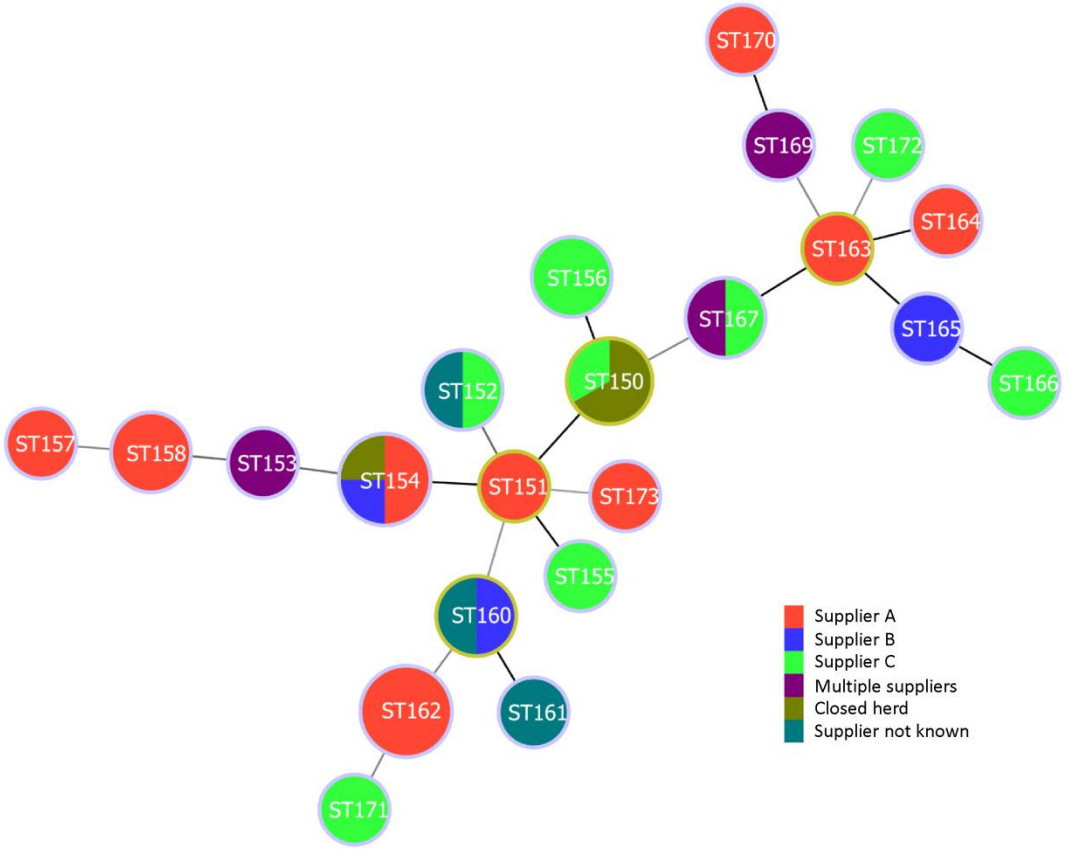

Figure 3. Minimum Spanning Tree (MST) analysis comparing the sequence types (ST) of the 35 *B. hyodysenteriae* isolates with the supplier of the pigs for the farm from which they were isolated. Each node represents a different ST (labelled) and the colour represents the pig supplier.

The majority of the STs that were shared by multiple isolates originated from different States and were isolated from farms with different pig suppliers. An exception was ST162 that was shared by four isolates from three States. These isolates also shared the same pig supplier (Supplier A), and one isolate originated from the breeding herd of Supplier A. The identification of the latter isolate initiated the analysis of four historical *B. hyodysenteriae* isolates that were linked to Supplier A in the early 1990s. No direct relationship was revealed from the MLST analysis of the historical isolates and current isolates recovered from farms populated by Supplier A. In addition, no correlation could be found linking the isolates from different farms to the pig supplier for those farms, where known. These findings suggest that *B. hyodysenteriae* isolates present in Australian herds are largely diverse and are unlikely to have originated from a single source of pigs. The diversity of the isolates could be attributed to ongoing genetic changes at the farm level (micro and macro-evolution), together with transmission of strains through indirect routes such as by trucks, personnel or through movement of potential reservoir species such as feral pigs, rodents, dogs and aquatic birds.

3.4 Virulence Gene Testing of B. hyodysenteriae Isolates

The results of the virulence PCR test applied to 31 *B. hyodysenteriae* isolates are shown in Table 8. The test predicts the virulence potential of *B. hyodysenteriae* on the basis of the absence of a block of six genes located on the plasmid of *B. hyodysenteriae*. These genes are believed to be associated with its ability to colonise the pig gut. For 22 isolates (71 %), the results of the virulence PCR test were concordant with the reported health status of the herds. Importantly, isolates from all five of the herds with mild disease and seven of the nine isolates from herds with no disease lacked the virulence genes. Historical isolate 23 had the virulence genes but came from a farm without clinical disease, but when it was subsequently used to experimentally infect pigs it induced severe colitis in four out of four challenged animals (Hampson *et al*, 1992). This might be explained by the use of antimicrobials or certain diets on the farm of origin masking the appearance of SD. Seven of the eight other isolates with discordant results were recovered from farms with disease but which lacked the putative virulence genes. In the case of farm Y (with disease) one isolate had the virulence genes and one did not. In farm B (with disease) three distinct isolates all lacked the virulence genes whilst another isolate had the genes. In both farms it is likely that the isolates with the virulence genes would have been the ones that induced the disease. In other discordant cases it is possible that other virulent isolates were present but were not isolated and tested.

Table 8. Results of the virulence PCR for 31 *B. hyodysenteriae* isolates including 27 collected as part of the current study and 4 historical isolates collected earlier

Isolate	Farm	Clinical SD[a]	Virulence PCR result[b]						Predicted virulence
			ORF 11	ORF 12	ORF 13	ORF 14	ORF 15	ORF 16	
Isolate 1	Farm D	Yes	N	N	P	P	P	P	Yes
Isolate 2	Farm RL	Yes	N	N	P	P	P	P	Yes
Isolate 3	Farm Y	Yes	N	N	P	P	P	P	Yes
Isolate 4	Farm BC	Yes	N	N	N	N	N	N	No
Isolate 5	Farm B	Yes	N	N	N	N	N	N	No
Isolate 6	Farm SPC	Mild	N	N	N	N	N	N	No
Isolate 8	Farm RN	Yes	N	N	P	P	P	P	Yes
Isolate 10*	Farm MT	No	N	N	N	N	N	N	No
Isolate 12	Farm WD	Yes	N	N	N	N	N	N	No
Isolate 13	Farm Y	Yes	N	N	N	N	N	N	No
Isolate 14	Farm N	Yes	N	N	N	N	N	N	No
Isolate 15	Farm RL	Yes	N	N	P	P	P	P	Yes
Isolate 20	Farm W	Yes	N	N	P	P	P	P	Yes
Isolate 21*	Farm VD	No	N	N	N	N	N	N	No
Isolate 22*	Farm MD	Yes	P	P	P	P	P	P	Yes
Isolate 23*	Farm VD	No	P	P	P	P	P	P	Yes
Isolate 25	Farm GP	Yes	P	P	P	P	P	P	Yes
Isolate 28	Farm BK	Yes	P	P	P	P	P	P	Yes
Isolate 29	Farm G	No	P	P	P	P	P	P	Yes

Isolate 31	Farm WL	No	N	N	N	N	N	N	No
Isolate 32	Farm M	No	N	N	N	N	N	N	No
Isolate 33	Farm BF	Mild	N	N	N	N	N	N	No
Isolate 34	Farm BR	No	N	N	N	N	N	N	No
Isolate 35	Farm BO	Mild	N	N	N	N	N	P	No
Isolate 37	Farm BP	No	N	N	N	N	N	N	No
Isolate 38	Farm B	Yes	N	N	N	N	N	N	No
Isolate 40	Farm KR	No	N	N	N	N	N	N	No
Isolate 44	Farm KN	Mild	N	N	N	N	N	N	No
Isolate 45	Farm B	Yes	P	P	P	P	P	P	Yes
Isolate 46	Farm B	Yes	N	N	N	N	N	N	No
Isolate 47	Farm BF	Mild	N	N	N	N	N	N	No

* historical isolate

[a] Yes; clinical SD observed, Mild; diarrhoea or mild lesions observed, No; no clinical SD observed

[b] N; negative, P; positive

Isolates marked in grey have disparate results for clinical signs and virulence prediction

4. Application of Research

4.1 Detection of novel pathogenic Brachyspira species in Australian herds

Identifying the presence of "*B. hampsonii*" or "*B. suanatina*" in Australia was the prime focus of the project. As no isolates of these species were recovered from the samples from 69 farms it can be concluded that if these species are present in Australia then they are at a very low prevalence. Overall these species are not likely to be a major issue for the Australian Pig Industry at this time and no further action is required beyond routine surveillance.

4.2 Presence of B. hyodysenteriae in Australian Herds

Prevalence

The study demonstrated that *B. hyodysenteriae* is widely distributed amongst Australian pig herds, and occurs in herds that do not have clinical signs, and in herds with mild disease of previously unknown aetiology. It is important that all herds routinely use specific laboratory testing to confirm their health status, and particularly so if they supply pigs to other herds.

Antimicrobial susceptibility

A variable and moderate level of resistance was found amongst the isolates tested. When compared to antimicrobial resistance patterns obtained for a Pork CRC funded study during 2006-2007, there has been an increase in resistance to tiamulin, lincomycin and olaquindox. Tylosin resistance remains almost universal. Three isolates were found to be multi-drug resistant and isolates of this sort represent a significant threat to the Australian pig industry.

Given the increasing trend in resistance to these drugs, it is important to obtain detailed information about the susceptibilities of isolates present on individual piggeries before control measures are implemented. Equally where susceptible isolates are present consideration should be made to eradicate the infection using the drugs that are still effective.

Multi-locus sequence typing (MLST) analysis

The study showed that there is considerable diversity amongst the *B. hyodysenteriae* isolates currently circulating in Australia. The MLST method was shown to be useful for studying this diversity and for tracking strains between farms that may be epidemiologically linked. Evidence for such transfer of strains between herds was obtained.

4.3 Virulence testing

This study supports the usefulness of the virulence prediction test that was developed at Murdoch University. It demonstrates that colonisation with predicted "avirulent" *B. hyodysenteriae* isolates occurs in Australia and can go undetected if the sole means of diagnosis is the appearance of clinical signs. This is of special concern for breeder herds as symptoms potentially may occur after animals are transferred to other farms where environmental conditions are different.

5. Conclusion

This study has demonstrated that "*B. hampsonii*" and "*B. suanatina*", the newly described agents of swine dysentery in North America and Europe, are unlikely to be present in Australia. On the other hand the classical agent *B. hyodysenteriae* is relatively common and widespread. The strains that are currently circulating generally are different from those found in the past, and are different from strains from other countries. Evidence was found for the likely transmission of strains between piggeries that are epidemiologically linked. More strains showed antimicrobial resistance than in the past, and of concern was the identification of three different multi-drug resistant strains. A major finding was identification of *B. hyodysenteriae* in farms that had no disease, or only mild disease of previously unknown aetiology. Strains from these farms were shown to lack plasmid-associated virulence genes, which potentially may reduce their ability to colonize. These results support the usefulness of this test, but also emphasize the need for routine testing of herds.

6. Limitations/Risks

In this report it has not been possible to provide details of individual herds because this data is confidential to the owners/managers of the herds and their veterinarians. The authors of the report are able to discuss individual cases only

when contacted by these individuals. This constraint does limit development of a national approach to controlling SD in Australia.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

1. A voluntary testing protocol endorsed by the Australian Pig Veterinarians should be developed that would identify sources of uninfected pigs and provide additional confidence of the health status of herds that supply pigs in Australia. This protocol could include a mixture of serological ELISA testing and culture and PCR of an agreed number of colonic samples as part of routine slaughter checks undertaken at least every six months.

2. To help control the spread of SD in Australia, ongoing surveillance for *B. hyodysenteriae* infections should be supported by the Industry, including confirmation of strain identity through MLST, determination of antimicrobial resistance and occurrence of strains predicted to have reduced virulence.

3. To confirm the validity of the PCR-based testing for virulence potential, strains of *B. hyodysenteriae* that are predicted to have reduced virulence based on the tests should be evaluated further in pigs experimentally infected under standard conditions.

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