DETECTION AND STRAIN TYPING OF *BRACHYSPIRA HYODYSENTERIAE* TO SUPPORT SWINE DYSENTERY ERADICATION AND CONTROL

Report prepared for the
Co-operative Research Centre for an Internationally Competitive Pork Industry

Professor David Hampson

School of Veterinary and Biomedical Sciences
Murdoch University
South Street, Murdoch
Western Australia 6150

July 2008

Established and supported under the Australian Government's Cooperative

Executive Summary

This project ran from April 2006 until June 2008. It aimed to help reduce the cost of pig production by enhancing control of swine dysentery in key Australian herds. Control of this important disease would improve herd feed conversion, reduce mortalities, and improve pig welfare. To achieve this aim, the objectives were to:

1. receive diagnostic samples from herds belonging to the Industry Partners, and from other Australian herds with swine dysentery (SD)
2. subject the samples to culture and polymerase chain reaction (PCR) testing to identify *Brachyspira hyodysenteriae*, the aetiological agent of SD
3. test Australian *B. hyodysenteriae* isolates for their susceptibility to a range of antimicrobial agents
4. develop a new strain typing system for *B. hyodysenteriae* using multilocus sequence typing (MLST)
5. To look for potential new reservoirs of *B. hyodysenteriae* that could threaten herd biosecurity

During the study a total of 842 diagnostic samples were received and were subjected to selective anaerobic culture and to polymerase chain reaction (PCR) amplification for *B. hyodysenteriae.* A total of 115 (13.7%) of the samples were PCR positive for *B. hyodysenteriae*, and 47 isolates of *B. hyodysenteriae* were recovered in pure culture. The PCR positive samples came from 16 of the 22 sources submitting samples.

The susceptibility of Australian *B. hyodysenteriae* isolates to antimicrobials was assessed in agar dilution tests. The 47 isolates from 2006-2007 and 13 other isolates from this period were tested against dilutions of eight antimicrobial agents, and the minimum inhibitory concentration (MIC) for each antimicrobial was calculated. Another 87 isolates that had been collected between 2002 and 2006 also were tested with four of these antimicrobials. Resistance to tylosin was almost universal amongst the isolates, while all isolates were susceptible to dimetronidazole. Unfortunately the latter drug has recently been withdrawn for use for SD control in Australia. Resistance to lincomycin or tiamulin was observed in a number of isolates. These are both important drugs for SD control, and it is significant that resistance to tiamulin has emerged since the last Australian survey was conducted in 2001. Most of the resistant isolates were recovered from Queensland, although one isolate from Victoria was resistant to both of these drugs, as well as to other antimicrobials. Most of the isolates appeared to be either susceptible or "moderately susceptible" to monensin and olaquindox, but interpretation of the data was difficult. Most isolates were susceptible to tetracycline and ampicillin, although these are not routinely used for SD control. Overall there should be concern about the increasing resistance to lincomycin and tiamulin, although it was reassuring to find that monesin still appears to be useful for inhibiting *B. hyodysenteriae* growth. Attempts should be made to register this drug for more routine use in SD control in Australia.

A new multilocus sequence typing (MLST) method was developed for differentiating between *B. hyodysenteriae* isolates, based on examining allelic (sequence) differences at eight conserved loci on the *B. hyodysenteriae* genome. The method was established using 50 well-characterized reference stains, and was shown to be more discriminatory than previous methods available to type these *B. hyodysenteriae* strains. The method also has the advantage that once the allelic data is entered into an accessible database other investigators will be able to compare their isolates with those in the database, and they will be able to add new data. This will be an important resource that can be used to help trace the routes of transmission of individual strains between piggeries, and across Australia. Data from the 60 isolates collected during 2006-2007 is still being assembled, and will be added to the database to determine the relationship of these strains to the reference strains used in the current analysis. Most of the latter strains were of Australian origin and were collected in the 1980s and 1990s, so this analysis will be particularly interesting to determine to what extent Australian *B. hyodysenteriae* strains have changed in the last 20 years.

In the final part of the project faecal samples were collected from 222 feral pigs that had been trapped in three regions in the southeast of Western Australia. *B. hyodysenteriae* was detected by PCR in 18 (8.1%) of the pigs, from all three areas, and an isolate was obtained from a subset of 61 samples that were cultured. Feral pigs are known to travel large distances and to enter piggeries, and this is the first time that they have been shown to carry *B. hyodysenteriae*. This new knowledge will be important when reviewing and upgrading the biosecurity of Australian piggeries.

Contents

Background

Swine dysentery (SD) is a severe mucohaemorrhagic colitis of pigs resulting from infection of the caecum and colon with the anaerobic intestinal spirochaetal bacterium *Brachyspira hyodysenteriae* (Hampson et al., 2006). SD is widespread and problematic in many swine-rearing countries. It remains a common problem in Australia, including in the large QAF and APF groups, and has long been considered to be one of the most economically important infectious diseases in the Australian Pig Industry. SD can severely depress feed conversion efficiency in the grower/finisher phases, its control requires considerable antimicrobial use, and the presence of the disease impinges on animal welfare.

In recent years the control of SD in many countries, including Australia, has been compromised by the emergence of strains of *B. hyodysenteriae* with reduced susceptibilities to a range of potential therapeutic antimicrobials (Karlsson et al., 2002). Moreover, the presence of resistant strains and the need to more effectively control the disease has led to an increased interest in improving understanding of the molecular epidemiology of *B. hyodysenteriae* infections.

Although Australian pig veterinarians are well aware of the importance of SD, attempts to control or eradicate the disease have not always been successful. There has been a lack of technical support available to accurately diagnose the disease, to determine whether there are single or multiple strains present in a given herd or group of herds, and to identify the antimicrobial susceptibilities of the organisms using appropriate testing methods. The inability to answer questions relating to potential reservoirs of infection in the environment, and how re-infection can occur following eradication attempts also has been frustrating. Much of the difficulty lies in the nature of the spirochaete – as it is a fastidious anaerobe with specialised growth requirements, and because of the specific nature of the methods needed to characterise and type these bacteria. These techniques are not routinely available in veterinary diagnostic laboratories in Australia.

Strategy

This project involved a partnership between staff at Murdoch University, QAF meats and the APF group. Laboratory facilities and staff at Murdoch University were made available for detecting, isolating, identifying, strain typing and determining antimicrobial sensitivities of *B. hyodysenteriae* isolates. The initial focus of the work was on sites from the two main industry partners, where is SD was known to be present. Subsequently samples from other piggeries were examined and tested. In addition to identifying and testing antimicrobial sensitivities, a more rapid and accurate strain typing method for *B. hyodysenteriae* called multilocus sequence typing (MLST) was developed. This new approach was possible as a consequence of the Murdoch laboratory recently determining the complete genomic sequence of *B. hyodysenteriae.* The development of new, easy and fast methods for strain typing *B. hyodysenteriae* should greatly assist in future control programs for swine dysentery.

Finally, an opportunity became available to investigate faecal samples from feral pigs, and this was extended into a larger investigation once it was realised that some of the feral pigs were carrying *B. hyodysenteriae.*

Aim and objectives

The aim of this project was to help reduce the cost of pig production by enhancing control of swine dysentery (SD) in key Australian herds. Control of this important disease would improve herd feed conversion, reduce mortalities, and increase pig welfare.

In order to achieve this aim the objectives of the work were to:

1. receive diagnostic samples from herds belonging to the Industry Partners, and from other Australian herds with SD
2. subject the samples to appropriate specialised culture and polymerase chain reaction (PCR) testing to identify *B. hyodysenteriae*
3. test Australian *B. hyodysenteriae* isolates for their susceptibility to a range of antimicrobial agent
4. develop a new strain typing system for *B. hyodysenteriae* using multilocus sequence typing (MLST)
5. look for potential new reservoirs of *B. hyodysenteriae*

Testing diagnostic samples

Methods

Spirochaete isolation and identification

During the project a total of 842 faecal samples were received for diagnostic testing. These included 489 samples collected from herds in the APF and QAF groups, and 353 from pigs from another 20 herds in different States of Australia.

The samples were plated onto selective Trypticase Soy Agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) plates containing 5% (vol/vol) defibrinated ovine blood, 400 µg of spectinomycin per ml, and 25 µg each of colistin and vancomycin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) per ml (Jenkinson and Wingar, 1981). The plates were incubated for 5 to 7 days at 37°C in an anaerobic environment of 94% H_2 and 6% CO_2 generated with anaerobic Gaspak plus sachets (BBL). The plates were examined for the presence of a low, flat, spreading growth and associated haemolysis. Surface growth was picked off, resuspending in phosphate-buffered saline, and examined under a phase-contrast microscope. Spirochaetes were confirmed as *B. hyodysenteriae* on the basis of their strong beta haemolysis, positive indole reactivity and amplification of surface growth or isolated spirochaetes in a species-specific PCR reaction amplifying a portion of the spirochaete's NADH oxidase gene (La et al., 2003). The spirochaetes then were propagated at 37°C in Kunkle's pre-reduced anaerobic broth containing 2 % (vol/vol) foetal bovine serum and a 1 % (vol/vol) ethanolic cholesterol solution (Kunkle et al., 1986). Aliquots containing active spirochaetal cells in mid-log phase were then checked for purity, removed, transferred into individual sterile storage tubes and stored frozen at -80°C for later use.

Results

A total of 115 of the 842 samples (13.7%) were positive in the PCR reaction for *B. hyodysenteriae*. These came from 16 of the 22 sources submitting samples. A total of 47 *B. hyodysenteriae* isolates were successfully obtained in pure culture.

Discussion

Overall, fewer samples were obtained from the Industry Partners than was originally planned. This is because the majority of samples were of normal appearance and were negative for *B. hyodysenteriae*, in turn due to the Industry Partners having implemented new programs of medication to control the disease before the project started. As a result, additional samples from pigs with clinical signs were obtained from other piggeries around Australia - in order to obtain sufficient isolates to make the rest of the study more useful.

Overall 13.7% of samples were PCR positive, and *B. hyodysenteriae* was isolated from 40% of these positive samples. Isolation of *B. hyodysenteriae* is a slow and difficult process, and even when spirochaetes can be seen on

the plate they can become lost or overgrown on subsequent subculturing. Nevertheless, sufficient isolates were obtained to allow them to be used in other parts of the overall study.

Antimicrobial susceptibility testing

Introduction

The purpose of this part of the study was to examine the antimicrobial susceptibilities of the *B. hyodysenteriae* isolates obtained in the earlier work. Besides the four most commonly used antimicrobials worldwide (lincomycin, tiamulin, dimetronidazole and tylosin), four other antimicrobials (monensin, olaquindox, teracycline and ampicillin) were tested at the request of individual Australian veterinarians who attended a meeting on swine dysentery organised by the Pork CRC in May 2007. For comparison with the isolates collected in 2006-2007, a further set of Australian isolates recovered between 2002 and 2006 was also tested against the four "core" antimicrobials.

Methods

Isolates

A set of 60 Australian isolates of *B. hyodysenteriae* collected as part of the current study in 2006-2007, including the 47 recovered from the diagnostic samples described above, eight from Queensland (provided by the Toowoomba Veterinary Laboratory) and five from Victoria/New South Wales (provided by the Bendigo Veterinary Laboratory), were tested for their susceptibility to eight antimicrobials (shown in Table 1). An additional 89 Australian isolates that had been recovered or received by our laboratory in the period 2002-2006 also were tested for their susceptibilities to four antimicrobials.

Growth of spirochaetes

The spirochaetes to be tested were removed from -80°C storage. For preparation of the inoculum, isolates were plated onto TSA with 5% defibrinated ovine blood and incubated in an atmosphere of 94% H_2, 6% CO_2 at 37°C for 5 days. The cells were gently resuspended in 1 ml of sterile phosphate buffered saline and then counted using a haemocytometer and phase-contrast microscope.

Susceptibility testing

The names and sources of the antimicrobials used in the susceptibility testing are shown in Table 1. Stock solutions were made by mixing the resuspended antimicrobial powders with sterile diluent.

Table 1: Antimicrobials used in susceptibility testing

Antimicrobial	Manufacturer
Lincomycin*	Sigma, St. Louis, MO
Tylosin*	Sigma, St. Louis, MO
Dimetridazole*	Sigma, St. Louis, MO

Antimicrobial	Manufacturer
Ampicillin	Sigma, St. Louis, MO
Tiamulin*	Riedal- de Haen, Germany
Monensin	Riedal- de Haen, Germany
Olaquindox	Riedal- de Haen, Germany
Tetracycline	Progen, Qld, Australia

* The four "core" antimicrobials used throughout the study

The strains were tested in duplicate in agar dilution assays against the antimicrobials. Antimicrobial susceptibility plates were made using Trypticase Soy Agar (TSA), supplemented with 5% defibrinated ovine blood. For each antimicrobial tested the desired concentration was obtained by adding the appropriate amount of each stock solution to the agar immediately before pouring the plates (Table 2). The antimicrobial sensitivity plates and control plates containing TSA with 5% ovine blood but no antimicrobials were dried for 15 min at 37°C before inoculation with the measured amount of test organisms. The inoculum was allowed to dry before incubating in an atmosphere of 94% H_2 and 6% CO_2 at 37°C for three days.

Table 2: Antimicrobial concentrations used in the agar dilution antimicrobial sensitivity test

Antimicrobial	Concentrations (µg/ml)
Lincomycin	1, 4, 20, 36, 80, 150
Tiamulin	0.5, 1, 2, 4, 6, 10
Tylosin	2, 25, 50, 100, 200, 250
Dimetridazole	0.1, 0.5, 1, 4, 10, 16
Monensin	0.1, 0.5, 1, 4, 10, 16
Olaquindox	0.1, 0.5, 2, 4, 10, 16
Tetracycline	2, 4, 6, 8, 12, 16
Ampicillin	6, 8, 12, 16, 24, 32

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 B78^T was included in each batch of tests. Growth of the strains on the control and sensitivity plates was checked visually after five days incubation. Zones of haemolysis were present around growth on the control plates, 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. The minimum inhibitory concentration (MIC) values were recorded as being in the range between the highest sensitive concentration and the lowest resistant concentration. The MIC ranges of the antimicrobials at which 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}, respectively) also were calculated. MIC breakpoints used to assist interpretation of the results are shown in Table 3. For tiamulin, lincomycin, tylosin and metronidazole, values identified for *B. hyodysenteriae* by Rønne and Szancer (1990) were applied, whilst for tetracycline and ampicillin MIC breakpoints for Gram negative enteric veterinary pathogens were used (National Committee for Clinical Laboratory Standards, 1999). Values for monensin and olaquindox were derived from the publications of Molnar (1996) and Uezato et al (2004).

Table 3: MIC breakpoints (mg/l) for in-vitro antimicrobial susceptibility tests as used to assist in interpretation in the study

Antimicrobial	Sensitive	Intermediate	Resistant	Publication
Lincomycin	≤4	>4 ≤36	>36	Ronne & Szancer, 1990
Tiamulin	≤1	>1 ≤4	>4	Ronne & Szancer, 1990
Tylosin	≤1	>1 ≤4	>4	Ronne & Szancer, 1990
Dimetridazole	≤4	>4 ≤16	>16	Ronne & Szancer, 1990
Monensin	0.4	3.12	12.5	Molnar, 1996
Olaquindox	<0.1	-	-	Uezato *et al*, 2004
Tetracycline	≤4	8	≥16	NCCLS Guidelines, 1997
Ampicillin	≤8	16	>32	NCCLS Guidelines, 1997

Results

Summaries of the MIC values for the 60 isolates collected in 2006-2007 for each of the eight antimicrobials tested are shown in Tables 4-12 respectively, and a summary of the MIC50 and MIC90 results for all the antimicrobials are shown in Table 13. Summaries of the MIC values for the other 89 isolates from the 2002-2006 period for the four antimicrobials are shown in Tables 14-17, respectively. A summary of the antimicrobial resistance status of the 89 isolates is given in Table 18, and the MIC_{50} and MIC_{90} results are presented in Table 19. A summary of the data for all 149 isolates for the 2002-2007 period to the four "core" antimicrobials is shown in Table 20.

Table 4: Summary of the MICs for lincomycin for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Lincomycin	WA	NSW	SA	Vic	Qld	
<1					1	1
>1 <4	13				5	18
>4 <20	5	2	1	1		9
>20 <36	16	1	1	2	2	22
>36 <80	8			1		9
>150			1			1
Total	42	3	3	4	8	**60**

WA, Western Australia; NSW, New South Wales; SA, South Australia; Vic, Victoria; Qld, Queensland.

Table 5: Summary of the MICs for tiamulin for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Tiamulin	WA	NSW	SA	Vic	Qld	
<0.5	41	3	2	3	8	57
>0.5 <1	1					1
>1 <2			1			1
>10				1		1
Total	42	3	3	4	8	**60**

Table 6: Summary of the MICs for tylosin for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Tylosin	WA	NSW	SA	Vic	Qld	
>250	42	3	3	4	8	60
Total	42	3	3	4	8	**60**

Table 7: Summary of the MICs for dimetridazole for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Dimetridazole	WA	NSW	SA	Vic	Qld	
<0.1					5	5
>0.1 <0.5	21	1	1		2	25
>0.5 <1	8	2	2	4	1	17
>1 <4	13					13
Total	42	3	3	4	8	**60**

Table 8: Summary of the MICs for monensin for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Monensin	WA	NSW	SA	Vic	Qld	
<0.1	1					1
>0.1 <0.5	3				1	4
>0.5 <1	21	2	2	4	7	36
>1 <4	17	1	1			19
Total	42	3	3	4	8	**60**

Table 9: Summary of the MICs for olaquindox for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Olaquindox	WA	NSW	SA	Vic	Qld	
<0.1	4	1		1	7	13
>0.1 <0.5	37	2	3	2	1	45
>0.5 <2	1					1
>16				1		1
Total	42	3	3	4	8	**60**

Table 10: Summary of the MICs for tetracycline for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Tetracycline	WA	NSW	SA	Vic	Qld	
<2	40	1			5	46
>4 <6	2	1	1	4	3	11
>6 <8		1	1			2
>16			1			1
Total	42	3	3	4	8	**60**

Table 11: Summary of the MICs for ampicillin for the 60 isolates collected in 2006-2007

MIC (µg/ml)	State					Total
Ampicillin	WA	NSW	SA	Vic	Qld	
<6	39	1	2		4	46
>12 <16	1	1		1	4	7
>16 <24	2	1		1		4
>24 <32				1		1
>32			1	1		2
Total	42	3	3	4	8	**60**

Table 12: Classification of the 60 isolates collected in 2006-2007 as being susceptible, intermediate or resistant to the eight antimicrobials

Antimicrobial	Susceptible No. (%) of *B. hyodysenteriae* isolates	Intermediate No. (%) of *B. hyodysenteriae* isolates	Resistant No. (%) of *B. hyodysenteriae* isolates
Lincomycin	19 (31.6%)	31 (51.6%)	10 (16.6%)
Tiamulin	58 (96.6%)	1 (1.6%)	1 (1.6%)
Tylosin	-	-	60 (100.0%)
Dimetridazole	60 (100.0%)	-	-
Monensin	5 (8.3%)	55 (91.6%)	-
Olaquindox	13 (21.6%)	46 (76.6%)	1 (1.6%)
Tetracycline	46 (76.6%)	13 (21.6%)	1 (1.6%)
Ampicillin	46 (76.6%)	12 (20.0%)	2 (3.3%)

Table 13: MIC_{50} and MIC_{90} data for the 60 isolates collected in 2006-2007

Antimicrobial	MIC_{50}	MIC_{90}
Dimetridazole	>0.1 <0.5	>1 <4
Lincomycin	>4 <20	>36 <80
Tiamulin	<0.5	<0.5
Tylosin	>250	>250
Olaquindox	>0.1 <0.5	>0.1 <0.5
Monensin	>0.5 <1	>1 <4
Tetracycline	<2	>4 <6
Ampicillin	<6	>16 <24

Table 14: Summary of the MICs for lincomycin for the 89 *B. hyodysenteriae* isolates from the period 2002 – 2006.

MIC (ug/ml)	State				Total
Lincomycin	WA	Qld	Vic	SA	
<1	22	0	0	0	22
> 1 < 4	0	4	0	0	4
> 4 < 20	10	22	0	1	33
> 20 < 36	19	4	1	0	24
> 36 < 80	3	2	0	0	5
> 80 < 150	1	0	0	0	1
Total	55	32	1	1	**89**

Table 15: Summary of the MICs for tiamulin for the 89 *B. hyodysenteriae* isolates from the period 2002 – 2006.

MIC (ug/ml)	State				Total
Tiamulin	WA	Qld	Vic	SA	
< 0.5	5	11	0	0	16
> 0.5 < 1	11	9	1	0	21
> 2 < 4	38	2	0	1	41
> 6 < 10	1	7	0	0	8
> 10	0	3	0	0	3
Total	55	32	1	1	**89**

Table 16: Summary of the MICs for tylosin for the 89 *B. hyodysenteriae* isolates from the period 2002 – 2006.

MIC (ug/ml)	State				Total
Tylosin	WA	Qld	Vic	SA	
< 2	2	0	0	0	2
> 2 < 25	28	0	0	1	29
> 50 < 100	21	8	1	0	30
> 100 < 200	4	1	0	0	5
> 200	0	9	0	0	9
NT	0	14	0	0	14
Total	55	32	1	1	**89**

NT, not tested

Table 17: Summary of the MICs for dimetridazole for the 89 *B. hyodysenteriae* isolates from the period 2002 – 2006.

MIC (ug/ml)	State				Total
Dimetridazole	WA	Qld	Vic	SA	
< 0.1	3	17	0	0	20
> 0.1 < 0.5	16	2	0	1	19
> 1 < 4	36	8	0	0	44
NT	0	5	1	0	6
Total	55	32	1	1	**89**

Table 18: Classification of the 89 *B. hyodysenteriae* isolates from the period 2002 – 2006 as being susceptible, intermediate or resistant to four antimicrobials

Antimicrobial	Susceptible	Intermediate	Resistant
Lincomycin	26 (29.2%)	57 (64%)	6 (6.7%)
Tiamulin	37 (41.6%)	41 (46.1%)	11 (12.4%)
Tylosin*	-	2 (2.7%)	73 (97.3%)
Dimetridazole^{+}	83 (100%)	-	-

* 14 isolates not tested; $^{+}$ 6 isolates not tested

Table 19: MIC_{50} and MIC_{90} data for the 89 isolates collected in 2002-2006

Antimicrobial	MIC_{50}	MIC_{90}
Lincomycin	>4 <20	>20 <36
Tiamulin	>2 <4	<6 <10
Tylosin	>50 <100	>200
Dimetridazole	>1 <4	>1 <4

Table 20: Classification of all 149 Australian isolates of *B. hyodysenteriae* collected in the period 2002-2007 as being susceptible, intermediate or resistant to four antimicrobials

Antimicrobial	Susceptible	Intermediate	Resistant
Lincomycin	45 (32.9%)	88 (59.1%)	16 (10.7%)
Tiamulin	95 (63.8%)	42 (28.2%)	12 (8%)
Tylosin*	-	2 (1.5%)	133 (98.5%)
Dimetridazole^{+}	143 (100%)	-	-

* 14 isolates not tested; $^{+}$ 6 isolates not tested

Discussion

The antimicrobial resistance patterns observed were variable. When considering the four "core" antimicrobials, only around one third of isolates were fully susceptible to lincomycin. Around 50-60% of isolates showed intermediate susceptibility and 11-17% were fully resistant. There was a slight tendency for more of the isolates from the 2006-2007 period to be resistant than isolates from the 2002-2006 period, although interpretation is difficult because the isolates were drawn from different regions as well as different times. In particular, although isolates from WA predominated in both sets, 36% of the isolates tested from the period 2002-2006 originated from Queensland. An increasing trend for resistance to lincomycin however also has been recorded in Australian isolates collected before 2002 (Karlsson et al., 2002).

The presence of tiamulin resistant isolates in the post-2002 period was of concern, as none were found in a survey of pre-2002 isolates from Australia (Karlsson et al., 2002). Resistance of *B. hyodysenteriae* isolates to tiamulin is an increasing problem in a number of European countries, and severely reduces options for control of SD in these countries (Karlsson et al., 2003, 2004; Lobova et al., 2004). Interestingly, in this study only one resistant and one intermediate isolate were found in 2006-2007, compared to the substantial numbers found in 2002-2006. This is likely mainly to reflect the different origins of the isolates, rather than necessarily indicating that these resistant spirochaetes are no longer commonly circulating in Australian herds. Ten of the 11 resistant isolates from the 2002-2006 period originated from herds in Queensland, whilst the single resistant isolate from the 2006-2007 set came from Victoria. The latter isolate also was resistant to lincomycin, tylosin, olaquindox and ampicillin. It remained susceptible to monensin, and this drug is used to control SD in the herd of origin. If this strain also developed resistance to monensin it would be very difficult to control.

As is found worldwide, resistance of *B. hyodysenteriae* isolates to tylosin was almost universal. The point mutation that causes tylosin resistance at position 2058 in the 23S rRNA gene is also known to increase the MICs for the lincosamide antibiotics (Karlsson et al., 1999), and this also may help to explain the trend for the increased MICs to lincomycin that were found.

Dimetridazole, the fourth "core' antimicrobial, was active against all isolates, although some isolates were showing an elevation of the MIC. Unfortunately dimetridazole recently has been withdrawn for use in the treatment of SD in Australia. This will present an extra burden on Australian pig producers who have previously relied on this drug to help control SD.

When considering the other four antimicrobials that were only tested on the 89 isolates from 2006-200, all had low MICs for monensin and were likely still to be susceptible – although 92% had MICs that would be considered to be "intermediate". Interpretation is difficult as there are no good guidelines for interpreting results for this drug. The reported study by Molnar (1996) was limited, as testing was done by disc diffusion – and this method is know to be unreliable for testing anaerobic bacteria. Molnar also did not try to set any clear guidelines for interpreting results.

Olaquindox showed a similar profile to monensin, with most isolates having low MICs, although one isolate certainly was resistant. Unfortunately once again there are no clear guidelines about how best to interpret the MIC data. The current results however will provide a useful benchmark for monitoring the situation with this drug in Australia in coming years.

For both tetracycline and ampicillin there were only a small minority of resistant isolates, with most appearing to be susceptible. These drugs are not used routinely for the control of swine dysentery, and are probably best reserved for other respiratory or systemic infections for which they are still widely used. Their overuse would encourage selection for resistant strains of *B. hyodysenteriae.*

Overall this study has provided updated information about the susceptibility of some recent Australian isolates of *B. hyodysenteriae* to antimicrobial drugs. Nevertheless, it is important to obtain detailed information about the susceptibilities of strains present on a given piggery before control measures are implemented. In particular, the existence of strains that are susceptible to lincomycin and tiamulin should be a matter of concern for the Australian Pig Industry. Monitoring of susceptibility to monensin is also important, as this drug is being used off label to help control SD in some Australian herds. The data from this study has been supplied to the manufacturer of the monensin that is being used, with a view to these results being used to support registration of this drug for the treatment of SD in Australia.

Multilocus sequence typing (MLST) analysis

Introduction

The purpose of this component of the work was to develop a specific multilocus sequence typing (MLST) system for *B. hyodysenteriae*, based on modifications to the scheme previously described for the whole genus (Rasback et al., 2007). MLST is now regarded as being one of the best and most robust methods to study the molecular epidemiology of bacterial species (Urwin and Maiden, 2003). To evaluate the method with *B. hyodysenteriae*, it was applied to a collection of strains that had previously been analysed by multilocus enzyme electrophoresis (MLEE), and most of which also previously had been analysed by pulsed field gel electrophoresis (PFGE), DNA restriction endonuclease analysis (REA) and/or serotyping, and the results for the different typing systems were compared.

Methods

Brachyspira hyodysenteriae strains

A total of 50 strains of *B. hyodysenteriae* were obtained as frozen stock from the culture collection at the Reference Centre for Intestinal Spirochetes at Murdoch University. These were selected as representatives of a range of MLEE electrophoretic types previously established in our laboratory (Lee et al., 1993; Trott et al., 1997). The majority of these strains also had been analysed in our laboratory by PFGE (Atyeo et al., 1999), REA (Combs et al., 1992) and serotyping (Hampson et al., 1989; Combs et al., 1992; Hampson et al., 1997). These reference strains originated from different States of Australia (n=37), the USA (n=6), Canada (n=3) and the UK (n=3). The names of the strains, their origins and previous results of MLEE, PFGE, REA and serotyping are presented in Table 21.

Bacterial culture

Spirochaete strains were propagated at 37^{o}C in Kunkle's pre-reduced anaerobic broth containing 2 % (vol/vol) foetal bovine serum and a 1 % (vol/vol) ethanolic cholesterol solution (Kunkle et al., 1986). Cells were harvested from mid-log phase culture by centrifuging at 10,000 g, and counted in a hemocytometer chamber under a phase contrast microscope.

DNA extraction

The DNeasy kit (QIAGEN Pty Ltd, Doncaster, Australia) was used to extract chromosomal DNA, using the Gram-negative bacterial protocol. Ten ml of a 10^{8} cells/ml culture of *B. hyodysenteriae* was harvested by centrifugation at 5,000 x g. The cell pellet was resuspended in 180 µl of lysis buffer containing 20 µl of proteinase K (10 mg/ml) and incubation at 55^{o}C for 30 min. After all the cells had been lysed, 180 µl of AL Buffer was added and the sample incubated at 70^{o}C for 10 min. Two hundred µl of absolute ethanol was immediately added to the sample and this was transferred to DNeasy minicolumn. Ethanol (70%, vol/vol)-based buffers AW1 and AW2 were added sequentially to the columns and centrifuged at 6,000 x g. The

supernatants were discarded, and the DNA was resuspended in sterile water and stored at -20°C.

Multilocus sequence typing

Seven of the eight MLST loci previously described for use with the genus *Brachyspira* (Rasback et al., 2007) were selected for this study. These were the genes encoding alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and acetyl-CoA acetyltransferase (*thi*). As the DNA mismatch repair protein gene (*mutS*) was not discriminatory in the previous study it was replaced with the NADH oxidase gene (*nox*). The primers and PCR conditions used were as previously described, except that primer pair NOX-F259 (TGGTACATGGGCAGCAAAAAC) and NOX-R1249 (CAAATACGCATAGCGTTAG) were used for amplification of a 991 bp. segment of the *nox* gene, and only primer pairs ADH-F206 and ADH-R757 were used for *adh*, and ALP-F354 and ALP-R1262 for *alp*.

PCRs were performed in a 25µl reaction mixture with *Taq* DNA polymerase (Biotech International). Each PCR reaction set included a positive control represented by *B. hyodysenteriae* WA1 and a negative control consisting of double distilled water. The PCR program was 95°C for 3 min, followed by 33 cycles at 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, followed by an extension period of 7 min at 72°C before cooling to 14°C. The PCR products were purified with the UltraClean™ PCR Clean-up Kit according to the manufacturer's instructions (Mo Bio Laboratories, Inc., Carlsbad, CA). For weak bands, a QIAquick PCR Purification Kit (QIAGEN) was used to increase the amount of DNA in the eluate. For cycle sequencing, an annealing temperature of 43-45 °C was used with 1/8 of the amount of Big dye. Sequencing was performed with a 3730 DNA analyser (Applied Biosystems and Hitachi, Ltd, Foster City, CA).

Data analysis

The sequences were edited and analysed by using NTI Vector 9.0 (http://www.invitrogen.com). Each unique nucleotide sequence was assigned a unique allele number. The allelic profile for each isolate was determined and consisted of a line listing the allele number for each gene in turn. Isolates were assigned a sequence type (ST) according to their allelic profiles. Isolates were considered genetically identical and hence of the same ST if they were identical at all eight loci. An MLST dendrogram was constructed from the data matrix of allelic mismatches with START, applying the unweighted pair group method with averages. Isolates were grouped into clonal complexes (CC) by BURST implemented in the program START, if they differed at no more than two loci (Jolley et al., 2001). Simpson's index of diversity (D) was calculated for the results of MLST, MLEE, PFGE and REA results, as previously described (Hunter and Gaston, 1988).

Results

Sequence types and population structure analysis

The sequence types (ST) of all strains are listed in Table 21. In total, 36 STs were identified. The relationships of the various STs to each other are depicted in the dendrogram (Figure 1).

Diversity

A summary of the diversity obtained by the MLST analysis is contrasted with the diversity achieved using MLEE, PFGE, REA and serotyping in table 22. The MLST method was more discriminatory for strain typing than the other methods.

Table 21: *Brachyspira hyodysenteriae* strain names and origin, sequence type (ST) in MLST, and comparison with previous results of MLEE, PFGE, REA and serotyping.

				Previous typing results			
Strain	Origin	ST (nt)	ST (aa)	ET	PFGE	REA	Serogroup
B234	USA	25	24	1 (1)	NT	A	A
NSW3	NSW	8	26	4 (3)	A1	H2	B
WA1	WA	10	10	4 (3)	A1	H1	B
WA4	WA	9	7	4 (3)	A1	H1	B
WA8	WA	9	7	4 (3)	A1	H1	B
Vic35	VIC	4	4	4 (3)	A2	H1	B
WA2	WA	12	9	4 (3)	A2	H1	B
WA9	WA	9	7	4 (3)	A2	H1	B
Vic36	VIC	19	2	6 (4)	B1	H5	B
WA26	WA	6	11	6 (4)	B2	H5	B
Vic31	VIC	7	7	10 (7)	C1	H9	B
Q14	Q	7	7	10 (7)	C1	H9	G
Q17	Q	11	8	10 (7)	C2	H10	B
Q18	Q	29	22	10 (7)	C3	H10	G
Q22	Q	9	7	10 (7)	C3	H11	G

Strain	Origin	ST (nt)	ST (aa)	Previous typing results			
				ET	PFGE	REA	Serogroup
Vic30	VIC	18	1	11 (8)	D1	H8	B
Vic32	VIC	18	1	11 (8)	D1	H8	B
B78^{T}	USA	27	21	12 (9)	E1	A	A
FMV89.3323	Canada	33	17	12 (9)	NT	NT	K
Vic2	VIC	5	7	12 (9)	E2	H6	H
WA28	WA	3	11	16 (11)	F1	H4	A
Q10	Q	7	7	16 (11)	F1	H9	A
Q11	Q	7	7	16 (11)	F1	H9	B
B204	USA	26	23	20 (15)	NT	C	B
R301	USA	1	12	20 (15)	NT	NT	A
ACK300/8	USA	36	5	20 (15)	NT	NT	B
P18A	UK	32	25	21 (13)	NT	E2	D
KF9	UK	2	13	22 (12)	NT	G	E
VS1	UK	30	16	39	NT	NT	F
WA5	WA	9	7	25 (14)	G1	I1	E
WA6	WA	29	22	25 (14)	G1	I2	E
B169	Canada	35	11	27 (16)	NT	D	C
FM88.90	Canada	28	20	28	NT	NT	J
B6933	USA	34	14	35 (21)	NT	NT	A
Q1	Q	22	15	35 (21)	J1	L2	D
Q3	Q	22	15	35 (21)	J1	L2	D
Q8	Q	22	15	35 (21)	J2	L3	D
Q9	Q	22	15	35 (21)	J2	L3	D
SA1	SA	20	15	36 (22)	K1	L8	D

Strain	Origin	ST (nt)	ST (aa)	Previous typing results			
				ET	PFGE	REA	Serogroup
SA2	SA	20	15	36 (22)	K1	L8	D
SA2206	SA	16	11	50 (29)	NT	NT	A
NSW2	NSW	17	6	36 (22)	K2	L9	D
WA14	WA	24	5	38 (19)	I1	J	A
WA27	WA	23	19	38 (19)	I2	J	A
B8044	USA	21	18	42	NT	NT	B
Vic23	VIC	14	3	43 (18)	H1	L5	D
Vic24	VIC	15	3	43 (18)	H2	L5	D
Vic25	VIC	13	3	43 (18)	H2	L5	D
Vic33	VIC	13	3	43 (18)	H3	L5	NT
Vic38	VIC	31	27	47 (26)	L1	M	B

Nt. Nucleotide level; aa. Amino acid level

NT. Not tested

NSW, New South Wales; WA, Western Australia; Vic, Victoria; Q, Queensland; SA, South Australia

ET, electrophoretic type in multilocus enzyme electrophoresis (Trott et al., 1997), with results for Lee et al. (1993) in parenthesis. PFGE, pulse field gel electrophoresis type (Atyeo et al., 1999). REA, restriction endonuclease analysis type (Combs et al., 1992). Serogroup, as defined by Hampson et al. (1989; 1997)

Table 22: Comparison of the Simpson's Index of Diversity for MLST and other previously described methods of sub-species differentiation. The Simpson's Index of Diversity was calculated as described by Simpson (1949). The higher the index value the more discriminatory is the technique.

Method	Number of types	Frequency (%) of the largest type	Simpson's Index of Diversity
MLST	36	10%	0.959
PFGE	22	11%	0.944
REA	23	12%	0.938
MLEE (Trott et al., 1997)	19	14%	0.922
MLEE (Lee et al., 1993)	18	14%	0.920
Serotyping	29	36%	0.775

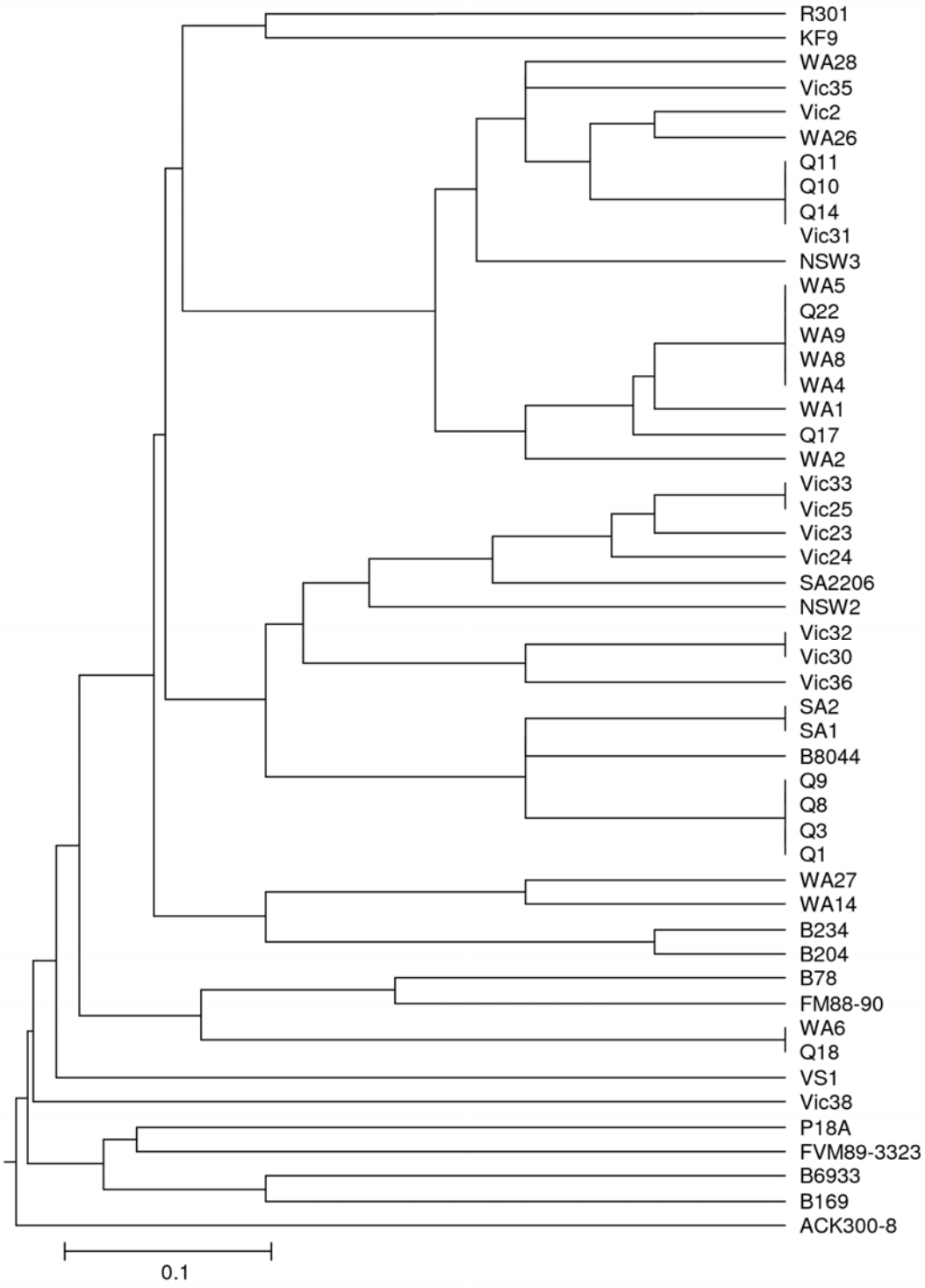

Figure 1: Dendrogram constructed from combined individual distance matrices of sequences from 50 *B. hyodysenteriae* isolates of with all eight genes *adh*, *alp*, *est*, *gdh*, *glpK*, *nox*, *pgm* and *thi* successfully sequenced. The length of the scale bar represents 1 nucleotide substitution in 100 base pairs of the sequenced gene fragment.

Discussion

The MLST scheme for *B. hyodysenteriae* that is described here is the first that has been developed for this spirochaete species. A scheme for the whole genus previously has been developed in our laboratory (Rasback et al., 2007), but its purpose was to differentiate species in the genus rather than as a strain typing method for the individual species in the genus. As used here it is clearly a highly discriminatory strain typing method, which is better for this purpose than any previously described method. Moreover, the MLST technique allows the construction of trees that can be used to look at relatedness between isolates, rather than just scoring them as being different.

The other major advantage of MLST is that as the data are sequence-based, the information is portable – that is, other investigators can use the technique on their isolates, and then directly compare their results to those obtained here. Later in 2008 the data that we have generated about the sequences of the eight loci will be deposited into a database that can be accessed externally. We have also almost completed the sequencing of the eight loci for the 60 Australian *B. hyodysenteriae* isolates that were recovered in 2006-2007. This information will be added to the database, and a new tree will be constructed to look at the relationship of these recent isolates to those of the older Australian isolates that were used in development of the typing scheme. These data will be published once the work is completed.

The data from the reference strains indicates the existence of considerable diversity amongst strains of the species, with a wide range of different genetic types of *B. hyodysenteriae* being present. There was a strong tendency for the Australian strains to be both diverse and genetically distinct from the non-Australian strains, and this suggests that Australian strains have evolved considerably and independently since they were originally introduced into the country. In future work it would be useful to try to establish whether the different genetic groups that were identified in Australia all have the same pathogenic potential. Differences in virulence potential between strains may help to explain why SD can be much more severe and difficult to control on some farms than on others.

Reservoirs of B. hyodysenteriae

Introduction

Feral pigs are widespread in many countries, and are known to cause a variety of economic and environmental problems. For example, in Australia they destroy crops and pastures, have a negative impact on native fauna and flora, and may eat young livestock (Choquenot et al., 1996). They also are known to act as reservoirs of zoonotic bacteria, and of other pathogens that potentially may be transmitted to pigs in commercial piggeries (Gresham et al., 2002; Baums et al., 2007; Jay et al., 2007). This is especially problematic as feral pigs often range over large distances (Hampton et al., 2004), and they may break through fences to enter piggeries.

Previously, by using selective anaerobic culture on 48 faecal samples from Swedish feral boar populations, no evidence for the presence of *Brachyspira* species was found (Jacobson et al., 2005), and it has been assumed that feral pigs do not carry *Brachyspira* species.

The aim of the current study was to determine whether in fact feral pigs in Australia may carry *B. hyodysenteriae,* and hence may represent a potential threat to the health status of commercial piggeries in Australia.

Methods

Source of samples

Faeces or colonic contents were collected from 222 feral pigs that were captured over an 18-month period as part of a control program run by the Western Australian Department of Environment and Conservation (DEC) and Water Corporation. These animals belonged to feral populations living in forest and scrubland in three locations to the east and southeast of Perth. A total of 32 samples (14.4%) were from weaner pigs (< 10 weeks old), 38 (17.1%) were from adult pigs (> 1 yr old) and the remaining 152 samples (68.5%) were from "juvenile" pigs between the other two age groups. The pigs were caught in traps and a licensed DEC or Water Corporation officer killed them by shooting. All samples were collected at post-mortem in the field, and were kept at 4^{o}C until processed in the laboratory within 1-2 days. The pigs that were sampled were reported to have no obvious evidence of diarrhoea.

Extraction of DNA and PCR

DNA was extracted from the samples with the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany), as previously described (La et al., 2003). The purified DNA was amplified by a hot-start PCR (M-PCR), as previously described (La et al., 2006). This amplified a a 354-bp portion of the *B. hyodysenteriae* NADH oxidase gene (*nox*). Briefly, amplification mixtures consisted of 1× PCR buffer (containing 1.5 mM $MgCl_2$), 1.25 U of HotStar*Taq* DNA polymerase (QIAGEN GmbH), 0.1 mM each deoxynucleoside triphosphate (Amersham Pharmacia Biotech AB, Uppsala, Sweden), 0.3 µM of the primer pairs and 2.5 µl of chromosomal template DNA. The cycling

conditions used involved an initial 15-min HotStar*Taq* DNA polymerase activation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 90 s, and primer extension at 68°C for 2 min, with a final 10 min at 68°C. The PCR products were subjected to electrophoresis in 1.5% (wt/vol) agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide, and viewed over UV light.

Brachyspira culture

A sub-set of 61 (27.5%) samples were plated onto selective Trypticase Soy Agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) plates containing 5% (vol/vol) defibrinated ovine blood, 400 µg of spectinomycin per ml, and 25 µg each of colistin and vancomycin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) per ml (Jenkinson and Wingar, 1981). The plates were incubated for 5 to 7 days at 37°C in an anaerobic environment of 94% H_2 and 6% CO_2 generated with anaerobic Gaspak plus sachets (BBL). The plates were examined for the presence of a low, flat, spreading growth and associated haemolysis. Surface growth was picked off, re-suspending in phosphate-buffered saline, and examined under a phase-contrast microscope. The surface growth on any plates suspected to have spirochaete growth were subjected to a PCR reaction for *B. hyodysenteriae*, as previously described (La et al., 2003).

Sequencing of 16S rDNA from B. hyodysenteriae

DNA extracts from five faecal samples that were PCR positive for *B. hyodysenteriae,* and the single *B. hyodysenteriae* isolate were amplified in a general *Brachyspira* species 16S rRNA gene PCR, as previously described (Phillips et al., 2005). The samples that were analysed were selected to represent two from each of the three study areas. Briefly, the primers Brachy-16S-F (5¤-TGAGTAACACGTAGGTAATC-3¤) and Brachy-16S-R (5¤-GCTAACGACTTCAGGTAAAAC-3¤) were used to amplify a 1309-bp portion of the gene from base position 118 to 1427. Sequencing of the PCR product was performed in duplicate using the same primers. Sequence results were edited, compiled and compared using Vector NTI version 7 (Invitrogen, Carlsbad, CA, USA).

The 16S rDNA sequences were compared, and a 1250 bp nucleotide sequence aligned with 16S rDNA sequences for *B. hyodysenteriae* and other *Brachyspira* species available in the GenBank sequence database using ClustalX (Thompson et al., 1997). A dendrogram was created with MEGA version 4, using the Maximum Composite Likelihood Method to calculate phylogenic distance values (Tamura et al., 2001).

Results

PCR results

A total of 18 (8.1%) samples were positive for *B. hyodysenteriae*. *B. hyodysenteriae* was found in pigs from all three regions. Eight of the 152 samples (5.3%) from juvenile pigs, one of the samples from weaner pigs (3.1%) and nine (23.7%) from adult pigs were positive for *B. hyodysenteriae*.

Selective culture

Brachyspira hyodysenteriae was isolated from one of the 61 cultured samples, from a juvenile pig. The isolate was strongly beta-haemolytic, and was designated "FP/6". DNA extracted from the isolate was amplified in the *nox* gene PCR for *B. hyodysenteriae.*

Sequencing of Brachyspira 16S rDNA

The 1309-bp 16S rDNA sequences from the *B. hyodysenteriae* isolate and from the five faecal samples that were amplified and sequenced were consistent with them being from *B. hyodysenteriae.* Their relationships to each other, to the type strain B78^T, reference strain B204, and to other *Brachyspira* species over 1250-bp are illustrated in Figure 2. The sequences from the feral pigs formed three groups, differing by one or two base pairs. The sequences of samples FP/3 and FP/4 were identical to each other and to that of type strain B78^T, whilst the sequences of FP/2 and the isolate FP/6 were the same as that the reference strain B204.

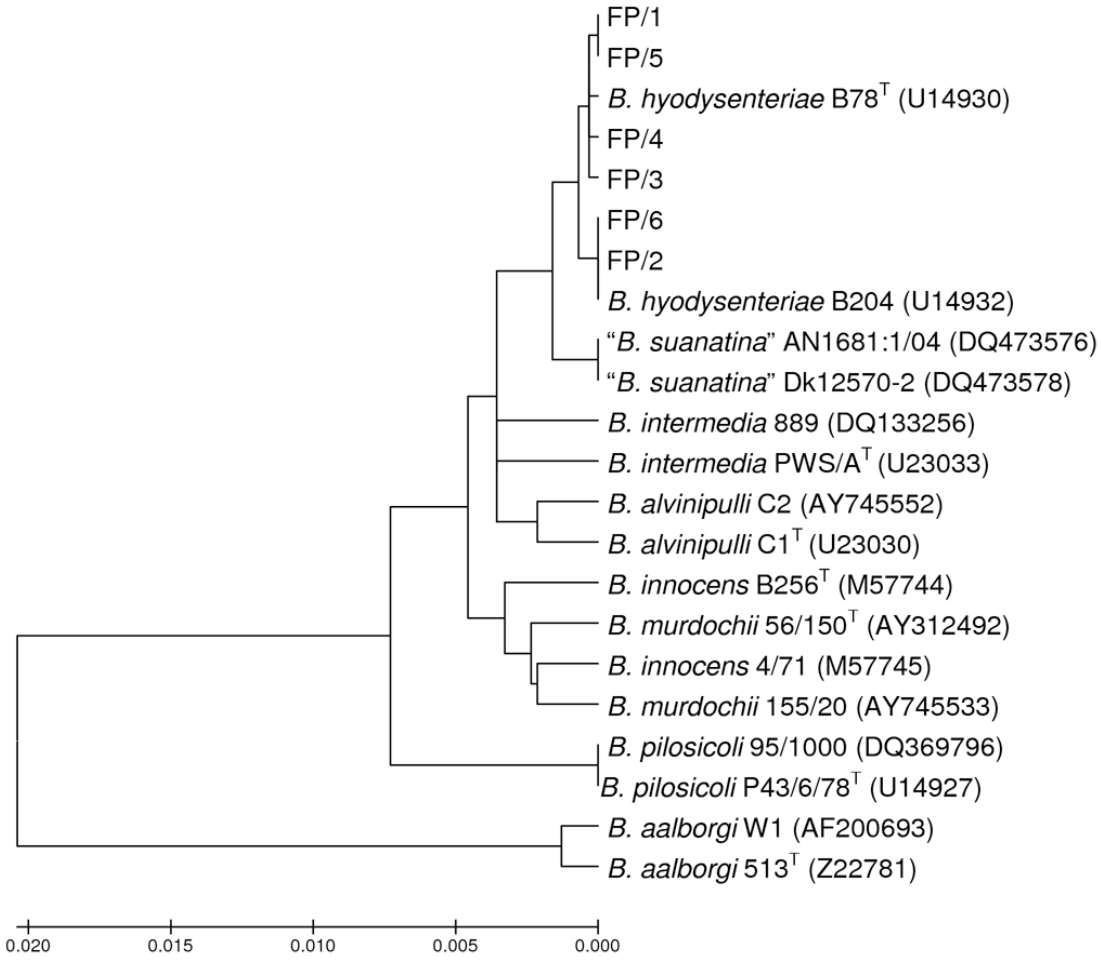

Figure 2: Dendrogram showing the relationship of the 1250-bp 16S rDNA sequences of the *B. hyodysenteriae* samples from feral pigs (FP/1 through

FP/6) with sequences from the type strain ($B78^T$) and a reference strain (B204) of *B. hyodysenteriae* and other *Brachyspira* species strains. The scale bar shows the number of base substitutions per site.

Discussion

In this study evidence was obtained that feral pigs in three locations in Western Australia were colonized with *B. hyodysenteriae.* None of the animals had obvious diarrhoea at the time of trapping, and it was not clear whether the pathogen was affecting the health of the feral pigs.

This is the first report of *B. hyodysenteriae* being found in feral pigs, and the rates of colonization were surprisingly high, particularly in the adult pigs (23.7%). The PCR results were confirmed by the results of sequence analysis of *Brachyspira* 16S rDNA that was amplified from a subset of samples. A single strongly haemolytic isolate was also isolated from one of a subset of the samples, further confirming the validity of the PCR results. The minor differences (1-2 bp) in the 16S rDNA sequence that were found between the six samples suggests the presence of at least three different strains, although analysis by other strain typing methods is required to investigate this diversity further. The single isolate is currently being analysed by the new MLST method.

Besides pigs, *B. hyodysenteriae* has been found in rats and mice living on infected piggeries (Joens and Kinyon, 1982; Hampson et al., 1991), and in captive rheas (Jensen et al., 1996) and commercial laying chickens (Feberwee et al., 2008). The only wildlife species so far that has been identified as being naturally colonized with *B. hyodysenteriae* are feral mallards, and to date this has only been demonstrated in Scandinavia (Jansson et al., 2004). It is possible that the feral pigs may have come into contact with water sources contaminated with faeces from feral mallards that were carrying the spirochaete, or there may be other animal or environmental reservoirs of this pathogen. Alternatively, and perhaps more likely, these feral pigs may have had contact with other pigs that were carrying the spirochaete. For example, populations of feral pig in these areas have been supplemented through illegal translocations or release of pigs by recreational hunters (Spencer and Hampton, 2005). These released animals are likely to have originated from commercial or domestic sources where they may have been exposed to various pathogens. In addition, the feral pigs could have had intermittent contact with pigs in commercial piggeries within their territories, particularly those in outdoor piggeries where access is easier. This possibility could be analysed further by comparing individual strains of *B. hyodysenteriae* from feral pigs with those of strains recovered from commercial pigs located within the geographic vicinity of the feral populations. The existence of transmission of the spirochaete between feral and commercial pigs would highlight this as an important threat to farm biosecurity. Based on our findings, it is likely that *B. hyodysenteriae* may be present in feral pigs in other locations, and further studies are required to determine the extent of this problem in other regions of Australia.

Impact on Industry

This study has confirmed that SD remains widespread in Australia, and that many different genetic types of the causative spirochaete are present. The availability of a new and robust typing method for the spirochaete means that veterinarians who support the Industry will be able to keep track of different strains of the spirochaete – particularly those that show resistance to the major antimicrobial drugs. This knowledge will help to control and reduce transmission of such strains within and between herds.

The demonstration that many Australian strains are increasingly resistant to antimicrobials should now encourage the industry to attempt to eradicate the disease from as many farms as is possible while there are still effective antimicrobials available. The industry should also consider investing in exploring other means of control, including development of vaccines and using the pigs' diet to modulate the disease. The demonstration that monensin is effective at inhibiting the growth of Australian isolates of *B. hyodysenteriae* also has already encouraged the manufacturer to consider seeking registration of this drug for SD control in Australia.

Finally, the demonstration that feral pigs can carry the spirochaete should further encourage Australian producers to improve biosecurity measures around their farms to help exclude feral pigs.

No specific opportunities for commercial development of the results of this study were identified. The benefits to the Industry will flow from the new knowledge that has been generated.

Conclusions and recommendations

Swine dysentery remains an important disease in Australia. This study has shown that Australian strains of the causative spirochaete are genetically diverse, and differ from many of those found overseas. Additional data from the recently collected isolates will be useful to get an impression of how Australian strains have evolved and changed in the last 20 years.

It is clear that antimicrobial resistance in *B. hyodysenteriae* is an important issue in Australia, with many strains being resistant to one or more of the major classes of drugs that are used to control SD. In particular, the emergence of tiamulin resistance in Australian strains in the last six years is a matter of concern. The laboratory data that were collected support the field observation that monensin is useful for the control of SD, with no frank resistance being detected. This will encourage the registration and use of this antimicrobial in Australia, although it will be important to keep monitoring the susceptibility of isolates in coming years.

In this project the first observation of carriage of *B. hyodysenteriae* by feral pigs was made. Carriage was common and widespread in the southeast of Western Australia, and further work is required to determine if a similar situation exists in other parts of Australia. This finding should encourage pig producers to increase biosecurity measures to exclude the possibility of feral pigs entering their properties.

The major recommendation from this work is that the industry maintains a specialised laboratory with the expertise to isolate, type and determine the antimicrobial sensitivity of Australian isolates of *B. hyodysenteriae*. Currently there is no continuity in this work, and such a laboratory would have an important role in monitoring the situation in Australia in relation to the emergence of new strains and changes in their antimicrobial sensitivities. Work supporting alternative methods to control SD (vaccine development, dietary investigations and alternative antimicrobials) is also required.

Bibliography

Atyeo, R.F., Oxberry, S.L., Hampson, D.J., 1999. Analysis of *Serpulina hyodysenteriae* strain variation and its molecular epidemiology using pulsed-field gel electrophoresis. Epidemiol. Infect. 123, 133-8.

Baums, C.G., Verkühlen, G.J., Rehm, T., Silva, L.M., Beyerbach, M., Pohlmeyer, K., Valentin-Weigand, P., 2007. Prevalence of *Streptococcus suis* genotypes in wild boars of Northwestern Germany. Appl. Environ. Microbiol. 73, 711-717.

Choquenot, D., McIlroy, J., Korn, T., 1996. Managing vertebrate pests: feral pigs. Bureau of Resource Sciences, Australian Government Publishing Services, Canberra.

Combs, B. G., Hampson, D.J., Harders, S.J., 1992. Typing of Australian isolates of *Treponema hyodysenteriae* by serology and by DNA restriction endonuclease analysis. Vet. Microbiol. 31, 273-85.

Dugourd, D., Jacques, M., Bigras-Poulin, M., Harel, J., 1996. Characterisation of *Serpulina hyodysenteriae* isolates of serogroups 8 and 9 by random amplification of polymorphic DNA analysis. Vet. Microbiol. 48, 305-14.

Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.M., Wellenberg, G.J., Dwars, R.M., Landman, W.J., 2008. Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. J. Clin. Microbiol. 46, 593-600.

Gresham, C.S., Gresham C.A., Duffy, M.J., Faulkner, C.T., Patton, S., 2002. Increased prevalence of *Brucella suis* and pseudorabies virus antibodies in adults of an isolated feral swine population in coastal South Carolina. J. Wildl. Dis. 38, 653-656.

Hampson, D.J., Duhamel, G.E., 2006. Porcine colonic spirochetosis/intestinal spirochetosis. Chapter 46. In: Straw BE, Zimmerman JJ, D'Allaire S & Taylor DJ (Eds), Diseases of Swine. Blackwell Publishing, Oxford, UK. pp. 755-768.

Hampson, D. J., J. R. L. Mhoma, B. G. Combs, and J. R. Buddle. 1989. Proposed revisions to the serological typing systems for *Treponema hyodysenteriae*. Epidemiol. Infect. 102, 75-84.

Hampson, D.J., Combs, B.G., Harders, S.J., Connaughton, I.D., Fahy, V.A., 1991. Isolation of *Treponema hyodysenteriae* from a wild rat living on a piggery. Aust. Vet. J. 68, 308.

Hampson, D. J., Atyeo, R.F., Combs, B.G., 1997. Swine dysentery. In: Hampson, D.J., Stanton, T.B. (Eds), Intestinal Spirochaetes in Domestic Animals and Humans. CAB International, Wallingford, UK. pp. 175-209.

Hampson, D.J., Fellström, C., Thomson. J,R., 2006. Swine dysentery. Chapter 48. In: Straw BE, Zimmerman JJ, D'Allaire S & Taylor DJ (Eds), Diseases of Swine. Blackwell Publishing, Oxford, UK. pp. 785-805.

Hampton, J., Pluske, J.R., Spencer, P.B.S., 2004. A preliminary genetic

study of social biology of feral pigs in south-western Australia and the implications for management. Wildlife Res. 31, 375-381.

Harel, J., M. Belanger, C. Forget, and M. Jacques. 1994. Characterisation of *Serpulina hyodysenteriae* isolates of serotype 8 and 9 from Quebec by restriction endonuclease fingerprinting and ribotyping. Can. J. Vet. Res. 58, 302-5.

Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465-2466

Jacobson, M., Gerth Löfstedt, M., Holmgren, N., Lundeheim, N., Fellström, C., 2005. The prevalences of *Brachyspira* spp. and *Lawsonia intracellularis* in Swedish piglet producing herds and wild boar population. J. Vet. Med. B. 52, 386-391.

Jansson, D.S., Johansson, K.E., Olofsson, T., Råsbäck, T., Vågsholm, I., Pettersson, B., Gunnarsson, A., Fellström, C., 2004. *Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). J. Med. Microbiol. 53, 293-300.

Jay, M.T., Cooley, M., Carychao, D., Wiscomb, G.W., Sweitzer, R.A., Crawford-Miksza, L, Farrar, J.A., Lau, D.K., O'Connell, J., Millington, A,. Asmundson, R.V., Atwill, E.R., Mandrell, R.E., 2007. *Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast. Emerg. Infect. Dis. 13, 1908-1911.

Jenkinson, S.R., Wingar, C.R., 1981. Selective medium for the isolation of *Treponema hyodysenteriae*. Vet. Rec. 109, 384-385.

Jensen, N.S., Stanton, T.B., Swayne, D.E., 1996. Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*). Vet. Microbiol. 52, 259-269.

Joens, L.A., Kinyon, J.M., 1982. Isolation of *Treponema hyodysenteriae* from wild rodents. J. Clin. Microbiol. 15, 994-997.

Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17, 1230-1231.

Karlsson, M., Fellström, A., Heldtander, M.U., Johansson, K.E. and Franklin, A., 1999. Genetic basis of macrolide and lincosamide resistance in *Brachyspira* (*Serpulina*) *hyodysenteriae.* FEMS Microbiol. Letts. 172, 255-260.

Karlsson, M., Oxberry, S.L., Hampson, D.J., 2002. Antimicrobial susceptibility testing of Australian isolates of *Brachyspira hyodysenteriae* using a new broth dilution method. Vet. Microbiol. 84, 123-33.

Karlsson, M., C. Fellström, A. Gunnarsson, A. Landén, and A. Franklin. 2003. Antimicrobial susceptibility testing of porcine *Brachyspira* (*Serpulina*) species isolates. J. Clin. Microbiol. 41, 2596-604.

Karlsson, M., A. Aspán, A. Landén, and A. Franklin. 2004. Further characterization of porcine *Brachyspira hyodysenteriae* isolates with decreased susceptibility to tiamulin. J. Med. Microbiol. 53, 281-5.

Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244-1245.

Kunkle, R. A., D. L. Harris, and J. M. Kinyon. 1986. Autoclaved liquid medium for propagation of *Treponema hyodysenteriae*. J. Clin. Microbiol. 24, 669-671.

La, T., Phillips, N.D., Hampson, D.J., 2003. Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. J. Clin. Microbiol. 41, 3372-3375.

La, T., Collins, A.M., Phillips, N.D., Oksa, A., Hampson, D.J., 2006. Development of a multiplex-PCR for rapid detection of the enteric pathogens *Lawsonia intracellularis, Brachyspira hyodysenteriae,* and *Brachyspira pilosicoli* in porcine faeces. Lett. Appl. Microbiol. 42, 284-288.

Lee, J. I., D. J. Hampson, B. G. Combs, and A. J. Lymbery. 1993. Genetic relationships between isolates of *Serpulina* (*Treponema*) *hyodysenteriae*, and comparison of methods for their subspecific differentiation. Vet. Microbiol. 34, 35-46.

Lobová, D., Smola, J., Cizek A., 2004. Decreased susceptibility to tiamulin and valnemulin among Czech isolates of *Brachyspira hyodysenteriae*. J Med Microbiol. 53, 287-91.

Maiden, M.C., J. A. Bygraves, E, Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA. 95, 3140-3145.

Matson, E. G., M. G. Thompson, S. B. Humphrey, R. L. Zuerner, and T. B. Stanton. 2005. Identification of genes of VSH-1, a prophage-like gene transfer agent of *Brachyspira hyodysenteriae*. J Bacteriol. 187, 5885-5892.

Molnar, L., 1996. Sensitivity of *Serpulina hyodysenteriae* isolated in Hungary to chemotherapeutic drugs. Vet. Rec. 138, 158-160.

Nathues, H., Oliveira, C.J., Wurm, M., Grosse Beilage, E., Givisiez, P.E., 2007. Simultaneous detection of *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Lawsonia intracellularis* in porcine faeces and tissue samples by multiplex-PCR. J. Vet. Med. A. 54, 532-8.

Phillips, N.D., La, T., Hampson, D.J., 2005. A cross-sectional study to investigate the occurrence and distribution of intestinal spirochaetes (*Brachyspira* spp.) in three flocks of laying hens. Vet. Microbiol. 105, 189-98.

Råsbäck, T., K-E. Johansson, D. S. Jansson, C. Fellström, Y. Alikhani, T. La, D. S. Dunn, and D. J. Hampson. 2007. Development of a multilocus sequence typing scheme for intestinal spirochaetes of the genus *Brachyspira.* Microbiology 153, 4074-4087.

Rønne, H., Szancer, J., 1990. *Proceedings of the International Pig Veterinary Society Congress*, Lausanne, Switzerland. p. 126.

Simpson, E.H. 1949, Measurement of Diversity, Nature 163, 688.

Spencer, P.B.S., Hampton, J.O., 2005. Illegal translocations and genetic structure of feral pigs in Western Australia, J. Wildlife Man. 69, 377-384.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 10,1596-1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Trott, D. J., Oxberry, S.L., Hampson, D.J., 1997. Evidence for *Serpulina hyodysenteriae* being recombinant, with an epidemic population structure. Microbiology 143, 3357-3365.

Uezato, K., Kinjo, E., and Adachi, Y., 2004. *In vitro* susceptibility of 31 antimicrobial agents to 37 isolates of *Brachyspira hyodysenteriae* isolated from pigs in Okinawa prefecture. J. Vet. Med. Sci. 66, 307-309.

Urwin, R., and M. C. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol. 11, 479-487.