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Use of a proposed antimicrobial susceptibility testing method for *Haemophilus parasuis*

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Abstract

The aim of this study was to examine the antimicrobial susceptibility of 97 *Haemophilus parasuis* cultured from Australian pigs. As there is no existing standard antimicrobial susceptibility technique available for *H. parasuis*, methods utilising the supplemented media, BA/SN for disc diffusion and test medium broth (TMB) for a microdilution technique, were initially evaluated with the reference strains recommended by the Clinical and Laboratory Standards Institute. The results of the media evaluation suggested that BA/SN and TMB can be used as suitable media for susceptibility testing of *H. parasuis*. The proposed microdilution technique was then used with 97 *H. parasuis* isolates and nine antimicrobial agents. The study found that Australian isolates showed elevated minimum inhibitory concentrations (MICs) for ampicillin (1%), penicillin (2%), erythromycin (7%), tulathromycin (9%), tilmicosin (22%), tetracycline (31%) and trimethoprim-sulfamethoxazole (40%). This study has described potential antimicrobial susceptibility methods for *H. parasuis* and has detected a low percentage of Australian *H. parasuis* isolates with elevated antimicrobial MICs.

Keywords: *Haemophilus parasuis;* Glässer's disease; Antimicrobial susceptibility testing; Test medium broth (TMB); BA/SN

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1. Introduction

Haemophilus parasuis is a commensal of the upper respiratory tract of pigs but is also the causative agent of Glässer's disease, which is characterised by polyserositis, polyarthritis and meningitis (Aragon et al., 2012; Jackson and Cockcroft, 2007). Glässer's disease is a significant challenge to the pig industry as it can cause economic losses in pig production (Aragon et al., 2012).

Antimicrobial agents such as beta-lactams, phenicols, macrolides, potentiated sulphonamides and tetracyclines, are recommended to control and treat *H. parasuis* infections (Karriker et al., 2013). Similar to other bacterial pathogens of pigs, the antimicrobial susceptibility of *H. parasuis* has evolved, thus, regular testing of susceptibility patterns is essential to ensure antimicrobials remain effective (Aarestrup et al., 2008). Antimicrobial resistance has been detected in *H. parasuis* isolated from China (Zhou et al., 2010), the Czech Republic (Nedbalcová and Kucerova et al., 2013), Denmark (Aarestrup et al., 2004), the United Kingdom and Spain (de la Fuente et al., 2007). While resistance has already been identified in other countries, current knowledge on the antimicrobial resistance of *H. parasuis* is unavailable in Australia. Thus, this study intended to determine the antimicrobial susceptibility of *H. parasuis* cultured from Australian pigs.

Additionally, this study was designed to perform preliminary evaluation of potential methods, using supplemented media, for the antimicrobial susceptibility testing of *H. parasuis* as no agreed standard method exists for this species. *Haemophilus parasuis* is a fastidious organism which does not grow in standard media recommended for other bacterial species as it requires V-factor (nicotinamide adenine

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dinucleotide or NAD) for growth *in vitro* (Rapp-Gabrielson et al., 2006). The veterinary fastidious medium (VFM) and chocolate Mueller-Hinton agar recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013) for other fastidious bacteria have not proven suitable for use with Australian isolates of *H. parasuis*.

2. Materials and Methods

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923 and ATCC 29213) and are the formal quality control strains when using the recommended media (Mueller-Hinton agar and broth) as defined by the CLSI (2013). These strains were used in the disc diffusion and microdilution techniques to test the capacity of BA/SN and TMB to function as media in antimicrobial susceptibility testing. The agar (BA/SN) was tested for disc diffusion, while the broth (TMB) was tested in the microdilution technique. Both BA/SN and TMB were used as they have a known capacity to support the growth of Australian *H. parasuis*. These media have been routinely used in the isolation and/or growth of haemophilic bacteria in the Microbiology Research Group, EcoSciences Precinct, Department of Agriculture, Fisheries and Forestry (DAFF), Queensland, Australia, for many years.

BA/SN was made of blood agar based medium, BBL™ Blood Agar Base (BD), supplemented with 0.0025% of NADH, 0.0005% of thiamine HCl, 1% of heat inactivated horse serum and 5% of oleic acid bovine albumin complex which consists of 4.75% bovine serum albumin in normal saline (with the normal saline containing 0.06% oleic acid and 5% 0.05N NaOH) (Sigma-Aldrich). TMB was prepared with 1% Biosate Peptone (BD), 1% sodium chloride (Merck Millipore, Australia), 0.1% starch (Merck Millipore), 0.1% glucose (Univar, Ajax Finechem), 0.05%

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yeast extract (Sigma-Aldrich) and was supplemented as for BA/SN except that 1% heat inactivated chicken serum was used in place of the horse serum.

The antimicrobial susceptibility testing using the proposed media (BA/SN and TMB) and standard media was done by conducting disc diffusion and broth microdilution techniques on the reference strains, *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923 and ATCC 29213), in accordance with the CLSI standard (CLSI, 2013). The reference strains were subjected to a panel of antimicrobials (five for *E. coli* and nine for *S. aureus*), using the test media (BA/SN and TMB) and standard media (Mueller Hinton agar and broth) in six independent repeats to determine repeatability. The antimicrobials used for disc diffusion were: ampicillin (10 µg), ceftiofur (30 µg), erythromycin (15 µg), florfenicol (30 µg), penicillin (10 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole combination (SXT) (30 µg), tilmicosin (15 µg) (all from Oxoid) and tulathromycin (30 µg) (Pfizer, Animal Health, USA). The same antimicrobials were used for microdilution, all sourced from Sigma Aldrich except for ceftiofur and tulathromycin (Pfizer). For microdilution, stock solutions were prepared according to the CLSI standard (CLSI, 2013) from pure powder form, except for erythromycin, which was a liquid standard solution obtained from the manufacturer (45703 from Sigma). Tulathromycin was prepared according to the manufacturer's recommendations (Pfizer).

A total of 97 *H. parasuis* isolates collected between the years 2002 and 2013 were selected from the culture collection of the Microbiology Research Group. All isolates were diagnostic submissions from Australian pig herds. The isolates, which represent 28% of the total available culture collection of Australian field isolates, came from New South Wales (21 isolates), Queensland (36 isolates), South Australia (15 isolates),

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Tasmania (1 isolate), Victoria (21 isolates) and Western Australia (3 isolates). The selection was made based on geographical and serovar diversity. All the isolates had been previously identified by species-specific polymerase chain reaction (Oliveira et al., 2001) and serotyped by either gel diffusion or indirect haemagglutination methods (Turni and Blackall, 2005).

The antimicrobial susceptibility of the 97 *H. parasuis* isolates was detected by determination of the MIC in duplicate using TMB. The antimicrobials used were ampicillin, ceftiofur, florfenicol, erythromycin, penicillin, SXT, tetracycline, tilmicosin and tulathromycin. The MIC was defined as the lowest antimicrobial concentration that inhibited bacterial growth.

3. Results and Discussion

A standard antimicrobial susceptibility technique for *H. parasuis* does not currently exist. Other studies have used veterinary fastidious media (VFM) or *Haemophilus* test medium (Aarestrup et al., 2004; de la Fuente et al., 2007; Zhou et al., 2010) and involved incubation under 5% CO_2. In this study, we used two NAD enriched media under aerobic conditions - BA/SN and TMB (agar and broth) - for disc diffusion and MIC, respectively. Both media have been previously described and used in antimicrobial susceptibility testing of *Avibacterium paragallinarum* (Blackall, 1988) and *H. parasuis* (Lancashire, et al., 2005). In this study, all tested antimicrobials (ampicillin, ceftiofur, florfenicol, SXT and tetracycline for *E. coli* and ampicillin, ceftiofur, erythromycin, florfenicol, penicillin, SXT, tetracycline, tilmicosin and tulathromycin for *S. aureus*) showed results within the acceptable ranges across the antimicrobials for disc diffusion and broth microdilution (MIC) techniques in six independent repeats using the test media (BA/SN and TMB, respectively) (Table 1 and 2). The only exceptions were the disc diffusion test for

ceftiofur for *E. coli*, which showed smaller zones of inhibition on the third repeat for both media (BA/SN and Mueller Hinton agar), and the disc diffusion test for SXT for *S. aureus* (on BA/SN only) where zones of inhibition one to two millimetres smaller than the acceptable range (24-32 mm) were recorded on five of the six repeats. The antimicrobial activity of trimethoprim and sulphonamide is influenced by the presence of antagonists (thymidine, thymine and folate) in the medium (CLSI, 2013). Some bacteria such as *E. coli* and *S. aureus* can utilise various amounts of exogenous thymidine and the effect of trimethoprim is correlated with the concentration of thymidine in the medium (Barry et al., 1984; Hamilton-Miller, 1988). In the current study, smaller zones of inhibition were observed around the SXT discs for *S. aureus* only (5/6 repeats) on the test medium. This agrees with the findings of Barry et al. (1984) who reported that *S. aureus* showed more sensitivity to trace amounts of antagonists than *E. coli* when testing for sulphonamide resistance. The trimethoprim and sulphonamide inhibitors have not been quantified in BA/SN, while Mueller-Hinton agar contains none of these inhibitors (CLSI, 2013).

As noted above, in one repeat (1/6), an error in the disc diffusion of *E. coli* against ceftiofur on both test and standard media was detected. This error could be due to experimental factors which can be considered insignificant due to the single occurrence of the error (King and Brown, 2001).

Overall, this initial evaluation of methods using the proposed media suggests that BA/SN and TMB have the potential to be suitable media for the performance of antimicrobial susceptibility techniques of *H. parasuis* for disc diffusion and MIC, respectively. Further, more extensive evaluations are required on both media.

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The MIC distribution of 97 *H. parasuis* isolates, the percentage of isolates with elevated antimicrobial MICs as well as the MIC_{50} and MIC_{90} are shown in Table 3. The MICs of the reference strains in each test run were within the CLSI acceptable quality control ranges. In the absence of agreed interpretation criteria, the MIC results were reviewed and a point that identified an elevated MIC was defined by the distribution of the MIC data – with those cut-off points being shown in Table 3.

A percentage of isolates with elevated ampicillin (1%), penicillin (2%), erythromycin (7%), tulathromycin (9%), tilmicosin (22%), tetracycline (31%) and SXT (40%) MICs were detected (Table 3). Although, the prevalence of elevated MICs to antimicrobials found in the *H. parasuis* isolates included in this study is low, some isolates showed high MICs to tilmicosin (21/97) and tulathromycin (9/97), the newer macrolides. Additionally, five isolates showed high MICs to all macrolides used in this study, including tulathromycin. Tulathromycin resistance has recently been identified in *H. parasuis* isolates from the Czech Republic (Nedbalcová and Kucerova 2013).

In summary, this study has identified potential antimicrobial susceptibility techniques for *H. parasuis* that might form the basis of a routine diagnostic technique to monitor antimicrobial susceptibility in this bacterial species following a full evaluation. Collaborative work on a full validation of these methods is now underway. This study also presented data on the occurrence of isolates of *H. parasuis* that show elevated MICs for key antimicrobial agents. National monitoring programs on antimicrobial susceptibility of important veterinary pathogens, using fully validated methodologies and agreed interpretation criteria, are necessary to allow veterinarians to prescribe the most rational treatment for bacterial infections.

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Table 1. Antimicrobial disk susceptibility test zone diameters (mm) for reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 using the standard media and the proposed media BA/SN in six independent repetitions.

	QC	Repeat 1		Repeat 2		Repeat 3		Repeat 4		Repeat 5		Repeat 6	
	ranges	MH	BA/SN	MH	BA/SN	MH	BA/SN	MH	BA/SN	MH	BA/SN	MH	BA/SN
E. coli ATCC 25922													
Ampicillin	16- 22	19	17	17	17	18	16	19	17	18	17	19	18
Ceftiofur	26- 31	27	26	27	27	23	24	27	26	26	26	28	28
Florfenicol	22- 28	23	22	23	22	22	22	24	26	22	22	26	26
SXT	23- 29	27	23	26	23	26	24	27	29	27	23	28	29
Tetracycline	18- 25	25	24	25	24	24	23	23	22	24	24	25	24
S. aureus ATCC 25923													
Ampicillin	27- 35	35	33	30	33	29	32	30	32	33	30	30	34
Ceftiofur	27- 31	30	30	27	29	28	30	27	29	27	30	28	27
Erythromycin	22- 30	24	25	23	23	23	23	24	24	23	25	26	26

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Florfenicol	22- 29	22	29	22	25	23	24	22	24	24	23	24	26
Penicillin	26- 37	31	36	37	35	34	34	30	34	33	36	33	37
SXT	24- 32	30	24	28	22	26	22	27	22	29	22	30	23
Tetracycline	24- 30	29	29	28	27	26	26	27	27	28	27	30	30
Tilmicosin	17- 21	17	19	17	19	17	18	17	18	18	18	18	19
Tulathromycin	18- 24	18	18	18	18	18	18	18	18	18	18	19	19

Acceptable ranges are provided by Clinical and Laboratory Standards Institute (CLSI, 2013).
MH- the standard media Mueller-Hinton agar.
SXT- Trimethoprim and sulfamethoxazole combination

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Table 2. Minimum inhibitory concentrations of antimicrobials (µg/ml) to reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 using the standard media and the proposed media BA/SN in six independent repetitions.

	QC	Repeat 1		Repeat 2		Repeat 3		Repeat 4		Repeat 5		Repeat 6	
	ranges	MHB	TMB	MHB	TMB	MHB	TMB	MHB	TMB	MHB	TMB	MHB	TMB
E. coli ATCC 25922													
Ampicillin	2- 8	4	4	2	2	4	4	4	4	2	2	2	2
Ceftiofur	0.25- 1	0.25	0.5	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
Florfenicol	2- 8	4	4	4	4	8	8	8	8	4	4	4	4
SXT	≤10	2.5	5	2.5	2.5	1.25	5	2.5	5	2.5	5	2.5	5
Tetracycline	0.5- 2	1	1	1	1	0.5	0.5	0.5	0.5	2	1	2	2
S. aureus ATCC 29213													
Ampicillin	0.5- 2	2	2	0.5	0.5	1	1	1	1	0.5	0.5	0.5	0.5
Ceftiofur	0.25- 1	0.5	0.25	0.5	0.5	0.5	0.5	0.25	0.25	0.25	0.25	0.25	0.25
Erythromycin	0.25- 1	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Florfenicol	2- 8	4	4	4	4	4	4	4	4	4	4	4	4

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Penicillin	0.25- 1	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
SXT	≤10	2.5	5	2.5	5	1.25	5	2.5	5	2.5	5	2.5	5
Tetracycline	0.12- 1	0.25	0.25	0.25	0.25	0.125	0.125	0.25	0.25	0.5	0.25	0.25	0.25
Tilmicosin	1.0- 4	1	1	1	1	1	1	1	1	1	1	1	1
Tulathromycin	2.0- 8	2	4	2	2	4	4	4	4	4	4	4	4

Acceptable ranges are provided by Clinical and Laboratory Standards Institute (CLSI, 2013).
MHB- Mueller-Hinton broth
SXT- Trimethoprim and sulfamethoxazole combination in a ratio of 1:19, the MIC of ≤10 is equivalent to ≤0.5/9.5 μg/ml.

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Table 3. Minimum inhibitory concentration distribution of 97 Australian *H. parasuis*.

Antimicrobial agents	Number of isolates with MIC (µg/ml) of								
	≤0.12	0.25	0.5	1	2	4	8	16	32
Ampicillin	69	11	9	6	1	1			
Ceftiofur	96	1							
Erythromycin		1	7	23	20	20	17	2	4
Florfenicol	10	54	27	6					
Penicillin	56	22	10	6	1	2			
SXT	37	12	9	18	15	6			
Tetracycline	3	21	33	10	13	5	3	4	5
Tilmicosin			11	29	19	10	7	9	5
Tulathromycin	3	12	26	23	16	6	2	8	

MIC_{50}, MIC_{90} - the lowest concentration of antimicrobial agent capable of inhibiting the growth of 50% and
SXT- trimethoprim and sulfamethoxazole in the ratio 1:19; test ranges are expressed as the trimethoprim cor
The isolates after the vertical lines were considered to have elevated antimicrobial MICs.

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3 Highlights

4 The use of BA/SN for disk diffusion testing of *Haemophilus parasuis* was evaluated.

5 Test medium broth (TMB) was evaluated for MIC tesing of *H. parasuis*.

6 TMB was used in the antimicrobial susceptibility testing of *H. parasuis*.

7 Low percentage of *H. parasuis* isolates with elevated antimicrobial MICs.

8

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