Antimicrobial susceptibility pattern of *Helicobacter suis* isolates from pigs and macaques

H. Berlamonta,b,s, A. Smetb,1, S. De Bruykerea, F. Boyenæ, R. Ducatellea, F. Haesebroucka,*,2, C. De Wittea,*,2

a Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
b Laboratory of Experimental Medicine and Pediatrics, Faculty of Medicine and Health Sciences, Antwerp University, Antwerp, Belgium

**ARTICLE INFO**

**Keywords:**
Helicobacter suis  
Antimicrobial susceptibility  
Zoonosis  
Gastric disease

**ABSTRACT**

*Helicobacter suis* is a fastidious, Gram negative bacterium that colonizes the stomach of pigs and non-human primates. It has also been associated with gastric disease in humans. A combined agar and broth dilution method was used to analyze the activity of 15 antimicrobial agents against 20 and 15 *H. suis* isolates obtained from pigs and macaques, respectively. After 48 h microaerobic incubation, minimal inhibitory concentrations (MICs) were determined by software-assisted calculation of bacterial growth as determined by quantitative real-time PCR.

A monomodal distribution of MICs was seen for β-lactam antibiotics, macrolides, gentamicin, neomycin, doxycycline, metronidazole, and rifampicin. Presence of a bimodal distribution of MICs indicated that 2 porcine isolates did not belong to the wild type population (WTP) for fluoroquinolones. This was also the case for 1 porcine isolate for tetracycline, 1 porcine and 2 primate isolates for lincomycin, and 1 primate isolate for spectinomycin. Single nucleotide polymorphisms (SNPs) were present in the *gyrA* gene of the isolates not belonging to the WTP for fluoroquinolones and in ribosomal protein encoding genes of the isolates not belonging to the WTP for tetracycline and spectinomycin. MICs of ampicillin, tetracycline and doxycycline were higher for porcine *H. suis* isolates compared to primate isolates and in these porcine isolates SNPs were detected in genes encoding penicillin binding and ribosomal proteins.

This study indicates that acquired resistance occasionally occurs in *H. suis* isolates and that zoonotically important porcine isolates may be intrinsically less susceptible to β-lactam antibiotics and tetracyclines than primate isolates.

1. Introduction

*Helicobacter suis* naturally colonizes the stomach of pigs worldwide, causing gastritis and a decreased daily weight gain (Haesebrouck et al., 2009). Recent findings also indicate that *H. suis* is involved in the development of porcine gastric ulceration through its effects on gastric acid secretion and on the gastric microbial composition (De Witte et al., 2018a, 2019). *H. suis* also is the most prevalent non-*H. pylori* Helicobacter species (NHPH) in human patients and infection has been associated with gastritis, peptic ulceration, and low grade mucosa-associated lymphoid tissue lymphoma (Haesebrouck et al., 2009). Transmission from pigs to humans may occur through direct or indirect contact with *H. suis*-infected pigs or through consumption of raw or undercooked pork or contaminated water (De Cooman et al., 2013).

Most likely, non-human primates constitute the original hosts of *H. suis* (Flahou et al., 2018). A possible host jump from macaques to pigs happened between 100,000 and 15,000 years ago, after which domestication may have had a significant impact on the spread of *H. suis* in the pig population. In rhesus (*Macaca mulatta*) and cynomolgus (crab-eating/Java macaque; *Macaca fascicularis*) macaques, *H. suis* infection generally remains asymptomatic, although mild gastritis can be present (Flahou et al., 2018). So far, the prevalence of *H. suis* in wild macaque populations has not been assessed, but in captive animals it has been described to be rather high (Martin et al., 2013).

Since gastric Helicobacter spp. infections in human patients are associated with severe pathologies (Haesebrouck et al., 2009), an
appropriate treatment should be selected. However, not much is known on the antimicrobial susceptibility pattern of gastric NHPH, including *H. suis*. Antimicrobial treatment of gastric NHPH infections in humans is based on clinical experience and mostly, treatment schemes applied for eradicating *H. pylori* are used (Kaklikkaya et al., 2002). These usually consist of a combination of a proton pump inhibitor with 2 or 3 antibiotics selected from clarithromycin, amoxicillin, metronidazole, tetracycline, and/or levofloxacin (Chey et al., 2017).

For *H. suis*, standard antimicrobial susceptibility assays are unsuitable for minimal inhibitory concentration (MIC) determinations since *H. suis* only grows in an enriched biphasic medium with an acidic pH (Baele et al., 2008). Therefore, Vermoote et al. (2011) developed a combined agar and broth dilution method to analyze the antimicrobial susceptibility pattern of porcine *H. suis* isolates. In that study, acquired resistance to enrofloxacin was demonstrated in one out of 9 *H. suis* isolates investigated. The aim of the present study was to determine the intrinsic susceptibility of *H. suis* and the presence and mechanisms of acquired resistance in a larger collection of isolates obtained from both pigs and non-human primates. This may eventually help to improve the management of *H. suis* infections.

2. Materials and methods

2.1. *H. suis* isolates

Thirty-five *H. suis* isolates were included in this study, of which 20 were isolated from the gastric mucosa of slaughter pigs from different herds (i.e. 6–8 months old pigs and adult sows) and 15 from the gastric mucosa of 6 cynomolgus monkeys (*Macaca fascicularis*) and 9 rhesus monkeys (*Macaca mulatta*) (Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands) (Supplementary file 2). The porcine isolates HS1-9 were the same as used in the study of Vermoote et al. (2011). All isolates were obtained according to the method described by Baele et al. (2008) and were shown to be genetically different by multilocus sequence typing (Liang et al., 2013). The bacteria were grown under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) at 37 °C on biphasic Brucella culture plates (Becton-Dickinson, Erembodegem, Belgium) supplemented with 20% inactivated fetal calf serum (Hyclone, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 5 mg/l amoxicillin B (Sigma-Aldrich, Saint Louis, Missouri, USA), *Campylobacter* selective supplement (Skirrow, Oxoid, Basingstoke, United Kingdom; containing 10 mg/l vancomycin, 5 mg/l trimethoprim lactate, and 2500 U/l Polymyxin B), and Vitox supplement (Oxoid). Brucella broth (Oxoid) was added on top of the agar to obtain biphasic culture conditions. The pH of both agar and broth was adjusted to 5 by adding HCl to a final concentration of 0.05%. Isolates were passaged at least twice to ensure reliable growth. After incubation, the bacteria were harvested and the number of viable (motile) bacteria/ml was microscopically determined using an improved Neubauer counting chamber (Sigma-Aldrich).

2.2. Reference strains

*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213, grown overnight on Columbia agar (Oxoid) supplemented with 5% defibrinated sheep blood (E&O laboratories, Bonnybridge, Scotland), were included as reference strains.

2.3. Antimicrobial susceptibility testing of *H. suis*

Testing for antimicrobial susceptibility was performed as described by Vermoote et al. (2011). In brief, the susceptibility to β-lactam antibiotics (i.e. ampicillin and ceftiofur), macrolides (i.e. clarithromycin and tylosin), fluoroquinolones (i.e. enrofloxacin, levofloxacin, and moxifloxacin), aminoglycosides (i.e. gentamicin and neomycin), aminocyclitols (i.e. spectinomycin), lincosamides (i.e. lincomycin), tetracyclines (i.e. doxycycline and tetracycline), nitroimidazole antibiotics (i.e. metronidazole), and rifamycins (i.e. rifampicin) was determined using a combined agar and broth dilution method in 24-well plates (Greiner Bio-On, Frickenhausen, Germany). The composition and pH (5) of the agar and broth were the same as described above, except that no amphotericin B and *Campylobacter* selective supplement were added. All antimicrobials were supplied by Sigma-Aldrich as standard powders with known potencies, with the exception of ceftiofur, which was purchased from Pfizer (Puurs, Belgium). The antimicrobial compounds were dissolved and diluted according to the standards of the Clinical and Laboratory Standards Institute (Clinical Laboratory Standards Institute (CLSI), 2016b). Agar plates and broth were prepared to contain serial 2-fold dilutions of the antimicrobial agents. Then, 150 μl broth containing 5 × 10⁻⁷ viable bacteria/ml of each *H. suis* isolate was added. Each well contained 250 μl broth and 400 μl agar, with final concentrations of the antimicrobial compounds ranging from 0.003 to 128 μg/ml. Wells containing agar and broth free of the tested antimicrobials, but with *H. suis* bacteria, were included as positive controls. In addition, wells without antibiotics and bacteria were used to verify sterility of used media (negative control). All samples were tested in duplicate and incubated during 48 h under microaerobic conditions at 37 °C.

After incubation, 100 μl of the broth containing *H. suis* was taken and bacterial DNA was isolated using PrepMan ultra sample preparation reagent (Applied Biosystems, CA, USA), according to the manufacturer’s guidelines. *H. suis* was quantified using a species-specific, quantitative real-time PCR (RT-PCR) based on the ureA gene (De Witte et al., 2018b). The MIC was determined as the lowest concentration of an antimicrobial agent for which ΔCt was at least 1 Ct higher than ΔCt₀ (ΔCt = Ct after incubation - Ct before incubation of the antimicrobial exposed *H. suis* isolate; ΔCt₀ = Ct after incubation - Ct before incubation of the positive controls; Ct = threshold cycle value). This is the lowest concentration of the antimicrobial agent with at least 50% less bacterial growth compared to controls without antimicrobials (De Witte et al., 2018b).

2.4. Antimicrobial susceptibility testing of reference strains

Suspensions with a density of 0.5 McFarland standard were prepared from overnight-grown reference strains *E. coli* and *S. aureus*. Two different MIC assays were performed for the reference strains in order to determine the impact of culture and pH conditions: (i) an assay in the biphasic and microaerobic conditions at pH 5, similar as described for *H. suis* testing (combined agar and broth dilution method) and (ii) the broth microdilution method according to the CLSI standards (i.e. unsupplemented Mueller-Hinton broth, pH 7) (Clinical Laboratory Standards Institute (CLSI), 2016a). Wells containing agar and broth free of the tested antimicrobials, but with the reference bacteria, were included as positive controls. Wells containing agar and broth free of the tested antimicrobials and bacteria were included as controls for contamination. After 16–20 h incubation under aerobic conditions at 37 °C, plates were examined visually for the presence of bacterial growth, indicated by presence of turbidity. The MIC was determined as the lowest concentration of an antimicrobial agent for which turbidity was absent. All samples were tested in duplicate.

2.5. Whole genome sequencing

Genome sequences of 10 porcine *H. suis* isolates (HS1-HS10) were already available from the ftp NCBI database (Smet et al., 2018) (Supplementary file 2). Three additional porcine *H. suis* isolates (P13/32, P13/35, and P13/36) and 7 non-human primate *H. suis* isolates (HSFM 331, HSFM 503b, HSFM 505/2, HSMm R02019b, HSMm R04052c, HSMm R07055b, and HSMm R08041b) were selected based on their divergent MIC values of certain antimicrobial agents compared to the other isolates and whole genome sequencing was performed as
previously described (De Witte et al., 2017). Gene finding and automatic annotation were performed using the Rapid Annotation Subsystems Technology (RAST) server (Smet et al., 2018).

2.6. Antimicrobial resistance mechanisms

The ABRicate software tool was used to screen H. suis genomes for the presence of acquired antimicrobial resistance genes. In addition, online databases containing a large set of acquired antimicrobial resistance genes (i.e. Resfinder, ARG-ANNOT, CARD, NCBI Bacterial Antimicrobial Resistance Reference Gene Database, EcOH, PlasmidFinder, and VFDB) were consulted. H. suis genomes were blasted against these antimicrobial gene resistance databases using default parameters. Data on antimicrobial resistance mechanisms of H. pylori reported in the literature was also collected, such as presence of chromosomal point mutations. Homology search for presence of similar point mutations in H. suis was performed using BLAST with default parameters.

Screening was performed in all the isolates belonging or not to the wild type population (Schwarz et al., 2010). Point mutations in the 16S rRNA, 23S rRNA, 30S and 50S ribosomal protein genes were checked for their association with tetracycline, spectinomycin, and lincomycin resistance. For fluoroquinolone resistance, point mutations in the gyrA and gyrB genes were explored and for β-lactam antibiotic resistance, point mutations in penicillin binding protein encoding (pnp) genes were investigated. Jalview 2.10.5 was used for visualization and further analysis of the different gene sequence alignments. First, cDNA and protein translation was performed, after which the presence of single point mutations in penicillin binding proteinencoding (pbp) genes was determined. When using the combined agar and broth dilution method at pH 5 (Clinical Laboratory Standards Institute (CLSI), 2016b). The MIC endpoints of doxycycline, tetracycline, metronidazole, and rifampicin fell within acceptable quality ranges when using the combined agar and broth dilution method at pH 5.

When tested in accordance with CLSI standards, the MIC endpoints of all antimicrobial agents fell within acceptable quality control ranges for both reference strains.

3. Results

3.1. Activity of antibiotics at different pH

When using the combined agar and broth dilution method at pH 5 on the E. coli and S. aureus reference strains, the MICs of clarithromycin, lincomycin, and spectinomycin were 2 times 2-fold dilutions, those of enrofloxacin and tylosin 3 times 2-fold dilutions, those of gentamicin, levofloxacin, and neomycin 4 times 2-fold dilutions, and those of moxifloxacin 5 times 2-fold dilutions higher than the highest value of the acceptable quality control range of the CLSI standards. MICs of ceftiofur were one 2-fold dilution and those of ampicillin 2 times 2-fold dilutions lower than the lowest value of the acceptable quality control ranges of the CLSI standards when using the combined agar and broth dilution method at pH 5.

3.2. MIC distribution

All growth control (positive control) plates showed sufficient growth and all sterility control (negative control) plates did not show any microbial growth.

The MICs for all H. suis isolates are summarized in Table 1. MIC values obtained for HS1-9 were identical as reported by Vermoote et al. (2011) or only differed by one 2-fold dilution.

A monomodal distribution of MICs was seen for β-lactam antibiotics (i.e. ampicillin and ceftiofur), macrolides (i.e. clarithromycin and tylosin), gentamicin, neomycin, doxycycline, metronidazole, and rifampicin. A bimodal distribution was observed for the fluoroquinolones (i.e. enrofloxacin, levofloxacin, and moxifloxacin), spectinomycin, lincomycin, and tetracycline, indicating that the porcine and primate H. suis isolates that fell in the higher MIC-ranges did not belong to the wild type population for these antimicrobial agents. More specifically, 2 porcine isolates (HS6 and HS10) did not belong to the wild type population for the fluoroquinolones, 1 porcine isolate (HS4) for tetracycline and 1 primate isolate (HSMm R07055b) for spectinomycin. For lincomycin, 1 porcine isolate (HSS) and 2 primate isolates (HSMm R04052c and HSMm R07055b) did not belong to the wild type population (Supplementary file 3). Compared to primate H. suis isolates, porcine isolates showed higher ampicillin, tetracycline, and doxycycline MIC

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Number of H. suis isolates with a MIC (μg/ml)</th>
<th>0.03</th>
<th>0.06</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>&gt;128</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftiofur</td>
<td></td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td></td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tylosin</td>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td></td>
<td>21</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td></td>
<td>9</td>
<td>12</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td>27</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>8</td>
<td>7</td>
<td>15</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neomycin</td>
<td></td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectinomycin</td>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lincomycin</td>
<td></td>
<td>12</td>
<td>3</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H. suis isolates showing acquired antimicrobial resistance are indicated in bold and red. See Supplementary file 3 for more detailed information.
values. More specifically, for the porcine isolates, the MICs of ampicillin varied between 0.25–8 µg/ml (median: 2 µg/ml), those of tetracycline between 0.25–32 µg/ml (median: 1 µg/ml), and those of doxycycline between 0.5–32 µg/ml (median: 2 µg/ml). For the primate isolates, the MICs of ampicillin varied between 0.125–2 µg/ml (median: 1 µg/ml), those of tetracycline between 0.03–0.250 µg/ml (median: 0.125 µg/ml), and those of doxycycline between 0.06–1 µg/ml (median: 0.25 µg/ml).

3.3. Antimicrobial resistance mechanisms

According to the different antimicrobial gene resistance databases, none of the H. suis isolates showed presence of known acquired antimicrobial resistance genes. Point mutations in the 16S rRNA and 23S rRNA genes associated with resistance in H. pylori or other pathogens against tetracycline and macrolides, respectively, were not detected in H. suis isolates not belonging to the wild type population for these antimicrobials.

Multidrug efflux pump subunit AcrB as well as multidrug export protein EmrA, MdtC and MepA encoding genes were found in all H. suis isolates.

When searching for presence of known point mutations associated with fluoroquinolone resistance in H. pylori and Enterobacteriaceae, SNPs were detected in the gyrA gene of porcine H. suis isolates HS6 and HS10 not belonging to the wild type population for fluoroquinolones. These SNPs were present at codon 78 (AGT (serine) - > AGG (arginine)) for HS6 and HS10 and also at codon 669 (GG (glutamyl) - > GA (glutamate)) for HS10 (Supplementary file 1). Using BLAST, serine was mainly present at codon 78 in other bacterial species, while various amino acids were found at codon 669. This was confirmed by the ConSurf Sever, giving a conservation score of 5 and 1 for codon 78 and 669, respectively. All PredictSNP tools, with the exception of PolyPhen-1, showed that the amino acid substitution at codon 78 may affect the activity of gyrA, with an average accuracy of 69%. Conversely, only the PhD-SNP tool indicated that the amino acid substitution at codon 669 may affect the activity of gyrA, with an accuracy of 68%, while the other tools indicated a neutral effect with an average accuracy of 75%.

Using I-Mutant 3.0, the amino acid substitution at codon 669 was predicted to decrease protein stability (DDG: -0.62, reliability index (RI): 3), while the amino acid substitution at codon 78 did not affect protein stability (DDG: 0.07, RI: 4) (Supplementary file 1).

Several SNPs were found in the pbp1A and pbp2 encoding genes of all porcine H. suis isolates showing higher MICs of ampicillin, but not in non-human primate isolates showing lower MICs for this antimicrobial (Supplementary file 1). For example, porcine isolates showed presence of a SNP at codon 622 of the pbp2 gene (GCC (proline) - > GCG (arginine)). Using BLAST, proline was present at this position in other bacterial species and the ConSurf Server gave a conservation score of 6. Two PredictSNP tools, PolyPhen-1 and -2, indicated that this amino acid substitution may affect the activity of pbp2, with an average accuracy of 57%. Using the I-Mutant 3.0 tool, this amino acid substitution was predicted to decrease pbp2 protein stability (DDG: -0.48, RI: 1). Analysis of the other SNPs present in pbp1A and pbp2 genes indicated that these amino acid substitutions most likely did not affect protein activity or stability (Supplementary file 1).

In addition, unique SNPs were also detected in the c-terminal region of the pbp1A gene at codon 318 (GCA (alanine) - > ACA (threonine)) and at codon 452 (AAC (asparagine) - > AAA (lysine)) of porcine H. suis isolates P13/32 and HS2 showing the highest MICs for ampicillin (i.e. 8 µg/ml) (Supplementary file 1). Using BLAST, various amino acids were present at codon 318 and 452 in other bacterial species and the ConSurf Server gave conservation scores below 5. Only the PolyPhen-2 tool indicated that the amino acid substitution at codon 318 may affect the activity of pbp1A, with an accuracy of 47%, while the other tools indicated a neutral effect with an average accuracy of 69%. None of the PredictSNP tools showed that the amino acid substitution at codon 452 may affect pbp1A activity. Using the I-Mutant 3.0 tool, the amino acid substitution at codon 318 was predicted to decrease pbp1A protein stability (DDG: -0.63, RI: 5), while this was not observed for the substitution at codon 452 (DDG: -0.46, RI:6) (Supplementary file 1).

Several SNPs were found in 30S and 50S ribosomal protein genes of all porcine H. suis isolates showing higher MICs of ampicillin, tetracycline and doxycycline, but not in primate isolates showing lower MICs for these antimicrobials (Supplementary file 1). For example, porcine isolates showed presence of a SNP at codon 159 of the 30S ribosomal protein S1 gene (RpsA) (TTT (phenylalanine) - > TGT (cysteine)). Using BLAST, various amino acids were present at this position in other bacterial species, but never cysteine. All PredictSNP tools showed that this amino acid substitution may affect the activity of RpsA, with an average accuracy of 61%. In addition, a SNP was also present at codon 102 of the 30S ribosomal protein S2 (RpsB) (TTT (phenylalanine) - > CTT (lysine)). Using BLAST and the ConSurf Server, only a limited number of amino acids were present at this position in other bacterial species, including lysine. Five PredictSNP tools showed that this amino acid substitution may affect the activity of RpsB, with an average accuracy of 58%. Using I-Mutant 3.0 tool, this amino acid substitution was predicted to decrease RpsB protein stability (DDG: -1.28, RI: 2). Analysis of SNPs present in 50S ribosomal protein L3 (RplC), L5 (RplE), L6 (RplF), L23 (RpmC), and L32 (RpmT); and 30S ribosomal protein S3 (RpsC) genes indicated that these amino acid substitutions potentially affect protein activity, although this was only observed for 1–4 PredictSNP tools, while the other tools indicated a neutral effect. None of the PredictSNP tools indicated an impact of the presence of SNPs on the activity of 50S ribosomal protein L1 (RplA), L4 (RplD), L9 (RplI), L13 (RplM), L15 (RplO), L16 (RplP), L19 (RpsL), and L22 (RplV); or 30S ribosomal protein S3 (RpsC), S8 (RspH), S11 (RpsK), and S20 (RpsT) (Supplementary file 1).

Unique for the porcine H. suis isolate HS4, not belonging to the wild type population for tetracycline, was the presence of serine at codon 8 of the 30S ribosomal protein S19 (Rps9) gene, while glycine was present in all other H. suis isolates. Using BLAST, glycine was mainly present at this position in other bacterial species and the ConSurf Server gave a conservation score of 7. With an average accuracy of 72%, all predictSNP tools showed that this amino acid substitution may affect the activity of RpsS. According to the I-Mutant 3.0 tool, this substitution also decreased protein stability (DDG: -1.15, RI: 7). Furthermore, valine was present at codon 189 of the 50S ribosomal protein L1 (RplA) gene and at codon 240 of the 30S ribosomal protein S2 (RpsB) gene of HS4, whereas methionine was present at these positions for all other H. suis isolates. BLAST analysis showed presence of various amino acids at these positions in the RplA and RpsB genes of other bacterial species and the ConSurf Server gave conservation scores below 5. One PredictSNP tool, MAPP, showed with 41% accuracy that the amino acid substitution at codon 189 affects the activity of RplA, while the other prediction tools showed a neutral effect with an average accuracy of 75%. None of the PredictSNP tools indicated an impact on the activity of RplB. The I-Mutant 3.0 tool showed that the substitution at codon 189 affects RplA protein stability (DDG: -1.10, RI: 8), similarly to the substitution at codon 240 of RplB (DDG: -0.65, RI: 4) (Supplementary file 1).

SNPs unique for the non-human primate H. suis isolate HSMm R07055b, not belonging to the wild type population for spectinomycin, were also found and are shown in Supplementary file 1. Analysis of SNPs present in 50S ribosomal protein L10 (RplJ), L12 (RplL) and L25 (RplY); and in 30S ribosomal protein S1 (RpsA), S6 (RpsF), and S15 (RpsO) genes indicated that the amino acid substitutions potentially affect protein activity, although this prediction was only observed for 1–4 PredictSNP tools, while the other tools indicated a neutral effect. Using BLAST, various amino acids were present at these positions in other bacterial species and the ConSurf Server gave conservation scores below 5, with the exception of RplL, where a conservation score of 7 was obtained at codon 100. According to the I-Mutant 3.0 tool, the substitution at codon 100 decreased RplL protein stability (DDG: -0.49, RI: 7) (Supplementary file 1).

For isolates HSMm R07055b, HSMm R04052c, and HSS, not
belonging to the wild type population for lincomycin, no SNPs were detected which were unique for these isolates and could be related to lincomycin resistance.

4. Discussion

The agar dilution method recommended for H. pylori by the Clinical Laboratory Standards Institute (CLSI) (2016a) cannot be used for antimicrobial susceptibility testing of H. suis, as this bacterium only grows in a biphasic medium with an acidic pH. Furthermore, visual inspection of presence of turbidity is not reliable to determine H. suis growth. Here, we confirm the feasibility of a combined agar and broth dilution method followed by qPCR to determine the antimicrobial susceptibility of H. suis (Vermoote et al., 2011). Nevertheless, as cultivation of H. suis is labor-intensive and time-consuming and since isolation of H. suis is often not successful, the clinical application of this technique remains questionable.

The use of the combined agar and broth dilution method may have influenced the results, as medium composition and pH variations have a clear impact on in vitro antimicrobial activity (Butaye et al., 2000). In comparison with the CLSI standards, MIC endpoints for the reference strains E. coli and S. aureus were clearly higher for lincomycin, macrolides, fluoroquinolones, and aminoglycosides when using the H. suis susceptibility assay conditions at pH 5. The presence of dextrose, casein, and/or other components in Brucella broth in combination with the acidic pH may have contributed to the decreased activity of these antimicrobials. Conversely, the antimicrobial activity of ampicillin and cefotiofur was increased in an acidic environment, as described by Clinical Laboratory Standards Institute (CLSI) (2016b). Interpretation of MIC determinations should be done with caution and the MIC values of several antimicrobial agents obtained here may underestimate or overestimate (for beta-lactam antibiotics) their in vivo activity. Also, the gastric environment is far more complex than the in vitro environment, further complicating the activity prediction of these antimicrobials in the stomach.

Although the activity of ampicillin was increased in the low-pH medium, the obtained MICs for H. suis were higher than those described for H. pylori, H. bizzozeronii, H. felis, and H. salomonis (Hachem et al., 1996; Van den Bulck et al., 2005) (Supplementary file 4). Furthermore, MICs of ampicillin were also higher for porcine than for primate isolates. For 2 porcine isolates MICs were even as high as 8 μg/ml. These findings may indicate an intrinsic, decreased susceptibility of porcine H. suis isolates for aminobenzyl penicillins, which are often used for treatment of human patients infected with gastric Helicobacter spp. For metronidazole, 24 H. suis isolates showed MICs > 8 μg/ml, which is the clinical breakpoint for H. pylori according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2019). However, according to Clinical Laboratory Standards Institute (CLSI) (2016a), in vitro determination of such breakpoints is not reliable to predict metronidazole therapy failure in vivo. In general, the therapeutic significance of our findings remains to be determined.

In the present study, mainly the epidemiological criterion for interpretation of MICs was used to determine which isolates did not belong to the wild type population (Schwarz et al., 2010). The epidemiological criterion defines microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to the antimicrobial agent in question, regardless the clinical context (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2019). Monomodal MIC distributions indicated that all H. suis isolates fell within the wild type range, whereas bimodal MIC distributions indicated that H. suis isolates that fell in the higher MIC-ranges did not belong to the wild type population. Although this criterion does not necessarily predict the outcome of treatment of an infection with non-wild type isolates and even though in vitro activity may differ from in vivo activity, it may indicate that certain isolates acquired mechanisms rendering them less susceptible than the normal bacterial population to the antimicrobial agent tested. Porcine isolates not belonging to the wild type population were detected for fluoroquinolones (2 isolates), tetracycline (1 isolate), and lincomycin (1 isolate). One and 2 primate isolates did not belong to the wild type population for spectinomycin and lincomycin, respectively. This indicates that acquired resistance and/or decreased susceptibility occasionally occur in H. suis isolates. As pigs or porcine products may constitute sources of infection for humans, this should be kept in mind when dealing with a human patient infected with H. suis. Indeed, for all these isolates, MICs were clearly higher than for the wild type population. Furthermore, for tetracycline (32 μg/ml), which activity was not affected by the low-pH medium used here, MICs clearly exceeded the CLSI approved clinical breakpoint (≥ 2 μg/ml) for H. pylori (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2019).

Unlike E. coli, Salmonella enterica and Campylobacter spp., no antimicrobial gene resistance database is available for Helicobacter spp., making it difficult to determine antimicrobial resistance mechanisms. As such, we investigated the presence of SNPs in H. suis isolates showing higher MICs than the other isolates. To predict the impact of SNPs on protein activity and stability, several tools (Jalview 2.10.5, PredictSNP, I-Mutant 3.0, and ConSurf) were used. Nevertheless, further investigation is necessary, as these results do not necessarily imply a causal relationship between the presence of SNPs and antimicrobial resistance.

Presence of SNPs in the pbp1A and pbp2 encoding genes may have contributed to the reduced susceptibility of porcine H. suis isolates for ampicillin. Amino acid substitutions in pbp1 have already been described to induce amoxicillin resistance in H. pylori, by affecting the binding site of amoxicillin (Qureshi et al., 2011).

Here, we demonstrated that porcine H. suis isolates showed higher MICs for tetracycline and doxycycline compared to primate isolates, indicating a decreased susceptibility for tetracycline antimicrobials. SNPs unique for porcine H. suis isolates were found in several ribosomal protein encoding genes and might be associated with this decreased susceptibility, as indicated by the software tools. Acquired resistance in H. pylori to tetracycline has been associated with point mutations in the 16S rRNA gene (Smith et al., 2014). Here, SNPs unique for isolate HS4, not belonging to the wild type population for tetracycline, were identified in 3 ribosomal protein genes (i.e. RpLA, RpPB, and RpPS). Furthermore, software tools demonstrated that the amino acid substitutions affect protein activity and stability, especially for RpsS. This may indicate that these point mutations are involved in decreased susceptibility of this isolate for tetracycline.

Vermoote et al. (2011) showed acquired resistance of a porcine H. suis isolate HS6 to enrofloxacin, which was linked to the presence of a point mutation at position 78 in the QRDR region of gyrA. Here, we showed acquired resistance of HS6 to a larger set of fluoroquinolones, which was also observed for porcine isolate HS10. An identical point mutation was found in the gyrA gene of both isolates and PredictSNP tools predicted an impact of this SNP on gyrA activity. Similarly, point mutations in the QRDR region of gyrA have been associated with fluoroquinolone resistance in H. pylori strains (Smith et al., 2014). In addition, a point mutation leading to glycine to glutamic acid substitution was found at position 669 in the gyrA gene of HS10 showing higher MICs for enrofloxacin and levofloxacin compared to HS4 (i.e. 8 vs 4 μg/ml, 32 vs 8 μg/ml, resp.). Such mutation may have further decreased the susceptibility of HS10 to fluoroquinolones, as glycine and glutamic acid possess a different charge which may subsequently affect the activity of the DNA gyrase enzyme. Indeed, the I-Mutant 3.0 tool predicted a decreased gyrA stability due to this amino acid substitution.

No mutations in the 23S rRNA gene, as described in lincomycin resistant H. pylori strains, were found in the H. suis isolates HS5, HSMm R07055b, and HSMm R04052c not belonging to the wild type population for this antibiotic (Wang and Taylor, 1998). Furthermore, no SNPs unique for these isolates were detected in the different 50S and 30S ribosomal protein genes (Wang and Taylor, 1998; Spižek and
The mechanism involved in decreased susceptibility of *H. suis* isolates HSS, HSMm R07055b, and HSMm R04052c to lincomycin, therefore, remains unclear.

5. Conclusion

In conclusion, this study shows that acquired resistance occasionally occurs in *H. suis* isolates and that zootonically important porcine isolates may be intrinsically less susceptible to β-lactam antibiotics and tetracyclines than primate isolates.

Declaration of Competing Interest

None.

Acknowledgement

This research was supported by the Research Fund of Ghent University, Belgium (BOF GOA 01G01014).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108459.

References


Haesebrouck, F., Pasmans, F., Flahou, B., Chiers, K., Baele, M., Meysn, T., Decostere, A., Ducatelle, R., 2009. Gastric helicobacters in domestic animals and nonhuman pri-


Schwarz, S., Silley, P., Simjee, S., Woodford, N., van Dijikkeren, E., Johnson, A.P., Gastra, W., 2010. Assessing the antimicrobial susceptibility of bacteria obtained from ani-


Smith, S.M., Morain, C.O., Mccarnara, D., Smith, S.M., Morain, C.O., Mccarnara, D., 2014. Antimicrobial susceptibility testing for *Helicobacter pylori* in times of increasing an-
tibiotic resistance. World J. Gastroenterol. 20, 9912–9921.


