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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 48h 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.

Key words: *Helicobacter suis* –antimicrobial susceptibility –zoonosis – gastric disease

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.

MATERIALS AND METHODS

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) (Additional 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% N_2, 10% CO_2, 5% O_2) 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 amphotericin 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).

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.

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 (CLSI, 2016b). Agar plates and broth were prepared to contain serial 2-fold dilutions of the antimicrobial agents. Then, 150 µl broth containing 5 x 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 48h 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 ΔC_t was at least 1 C_t higher than ΔcC_t (ΔC_t = C_t after incubation - C_t before incubation of the antimicrobial exposed *H. suis* isolate; ΔcC_t = C_t after incubation - C_t before incubation of the positive controls; C_t = 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).

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) (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 16-20h 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.

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) (Additional file 2). Three additional porcine *H. suis* isolates (P13/32, P13/35, and P13/36) and 7 non-human primate *H. suis* isolates (HSMf 331, HSMf 503b, HSMf 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).

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 (*pbp*) 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 nucleotide polymorphisms (SNPs) associated with amino acid substitutions were visualized. The online PredictSNP software was used to predict the effect of amino acid substitutions on protein activity. The available prediction tools were run with default parameters (i.e. PredictSNP, MAPP, PhD-SNP, PolyPhen-1 and -2, SIFT, SNAP, nsSNPAnalyzer, and PANTHER). The impact of SNPs on protein stability was also investigated using the online I-Mutant Suite 3.0 software, with temperature 37°C and pH 7. Only when the predicted free energy change (DDG) value was higher than 0.50 or lower than -0.50, an impact on protein stability was suspected. Finally, the ConSurf Server was consulted and BLAST using different databases and default parameters was run to investigate if the SNPs were located in conserved regions.

RESULTS

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 (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.

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 fluoroquinolones, 1 porcine isolate (HS4) for tetracycline and 1 primate isolate (HSMm R07055b) for spectinomycin. For lincomycin, 1 porcine isolate (HS5) and 2 primate isolates (HSMm R04052c and HSMm R07055b) did not belong to the wild type population (Additional file **3**). Compared to primate *H. suis* isolates, porcine isolates showed higher ampicillin, tetracycline, and doxycycline MIC 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).

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 (GGA (glycine) -> GAA (glutamate)) for HS10 (Additional 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) (Additional 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 (Additional file 1). For example, porcine isolates showed presence of a SNP at codon 622 of the *pbp2* gene (CCC (proline) -> CGC (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 (Additional 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) (Additional 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) (Additional 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 (Additional 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 (*RplW*), L29 (*RpmC*), and L32 (*RpmF*); and 30S ribosomal protein S3 (*RpsC*) genes indicated

that these amino acid substitutions potentially affect protein activity, although this was only observed for 1 to 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 (RplS), and L22 (RplV); or 30S Ribosomal protein S3 (RpsC), S8 (RspH), S11 (RpsK), and S20 (RpsT) (Additional 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 (*RpsS*) 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) (Additional 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 Additional 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 to 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) (Additional file 1).

For isolates HSMm R07055b, HSMm R04052c, and HS5, 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.

DISCUSSION

The agar dilution method recommended for *H. pylori* by the 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 ceftiofur was increased in an acidic environment, as described by 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) (Additional 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 8µg/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 EUCAST (2019). However, according to 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 (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* (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*, *RpsB*, and *RpsS*). 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; Spížek and Řezanka, 2004). The mechanism involved in decreased susceptibility of *H. suis* isolates HS5, HSMm R07055b, and HSMm R04052c to lincomycin, therefore, remains unclear.

CONCLUSION

In conclusion, this study shows 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.

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DECLARATION OF INTEREST

Declarations of interest: none.

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Antimicrobial agent	Strains with a MIC (µg/ml) of													
	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Ampicillin			HSMf 503b	P13/35 HSMf 331 R02019a R02019b R08041b	HS8 P13/28 R07055a	HS1 P13/04 P13/26 P14/09 HSMf 504/1 HSMf 504/2 HSMf 505/1 HSMf 505/2 R04052a R04052c R08041a	HS3 HS4 HS7 HS9 P13/24 P13/36 P14/06 R07055b R07102c	HS5 HS6 HS10 P14/10	P13/32 HS2					
Ceftiofur						HSMf 503b R08041b R02019a	HS2 HS4 P13/36 P14/06 P14/09 R07055a	P13/04 P13/24 P13/35 HSMf 505/2 R02019b	HS5 HS6 HS7 HS8 P13/26 P13/28 HSMf 331 HSMf 504/2 R07102c R08041a	HS1 P13/32 P14/10 HSMf 505/1 R04052a R04052c	HS3 HS9 HS10 HSMf 504/1 R07055b			
Clarithromycin	HSMf 503b	R02019b	HS5 HS6 HS8 P13/26 P13/28 P14/10 HSMf 504/2 HSMf 505/1 HSMf 505/2 R02019a R04052a R04052c R07102c R08041a R07055a R07055b	HS1 HS2 HS3 HS7 P13/04 P13/24 P13/32 P14/06 P14/09 HSMf 331 HSMf 504/1 R08041b	HS4 HS9 HS10 P13/35 P13/36									

	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Tylosin					P13/32 R02019a R02019b R07102c	P14/10 R07055a R08041b	HS3 HS7 HS9 P13/26 HSMf 331 HSMf 505/1	HS1 HS5 HS6 HS8 HS10 P13/04 P13/24 P13/28 P14/09 HSMf 503b HSMf 504/1 HSMf 504/2 R04052a R07055b R08041a	P13/35 P13/36 P14/06 R04052c	HS2 HS4 HSMf 505/2				
Azithromycin											HS8 R04052a R07102c	HS6 HS7 HS9 HSMf 503b HSMf 504/1 R08041a R08041b	HS1 HS4 HS5 HS10 P13/04 P13/26 HSMf 505/1 R02019b	HS2 HS3 P13/24 P13/28 P13/32 P13/35 P13/36 P14/06 P14/09 P14/10 HSMf 331 HSMf 504/2 HSMf 505/2 R04052c R02019a R07055a R07055b

	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Enrofloxacin	HS1 HS2 P13/04 P13/24 P13/26 P13/28 P13/32 P13/35 P13/36 P14/06 P14/09 P14/10 HSMf 503b HSMf 504/2 HSMf 505/1 HSMf 505/2 R04052a R04052c R07055a R07102c R08041b	HS3 HS4 HS5 HS7 HS8 HS9 HSMf 331 HSMf 504/1 R02019a R02019b R07055b R08041a						HS6	HS10					
Levofloxacin	HS8 HS9 HSMf 331 HSMf 503b R02019a R02019b R07055a R07102c R08041b	HS1 HS3 HS7 P13/04 P13/26 P13/28 P14/09 P14/10 HSMf 504/1 HSMf 504/2 HSMf 505/1 R04052a R04052c R07055b	HS2 P13/35 P13/36 P14/06 HSMf 505/2 R08041a	P13/24	HS4 HS5	P13/32			HS6		HS10			

	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Moxifloxacin	HS1 HS2 HS3 HS7 HS8 HS9 P13/04 P13/24 P13/26 P13/28 P14/06 P14/09 HSMf 331 HSMf 503b HSMf 504/1 HSMf 504/2 HSMf 505/1 HSMf 505/2 R02019a R02019b R04052a R04052c R07055a R07055b R07102c R08041a R08041b	P14/10	HS5 P13/35	HS4 P13/32 P13/36			HS6 HS10							
Gentamicin						HS3 P13/24 P13/26 P13/28 P13/36 P14/06 P14/09 HSMf 503b	HS1 P13/32 R02019a R04052a R07102c R08041a R08041b	HS2 HS4 HS5 HS6 HS7 HS8 HS9 HS10 P13/04 P13/35 HSMf 504/2 HSMf 505/1 R04052c R07055a R07055b	P14/10 HSMf 504/1 HSMf 505/2 R02019b	HSMf 331			

	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Spectinomycin				P13/26 P14/09	HS8 HSMf 504/1 R08041b	P13/32 P14/06 P14/10 R08041a	HS4 HS7 HS10	HS5 HS9 P13/28 HSMf 504/2 HSMf 505/1 HSMf 505/2 R07055a R07102c	HS1 HS6 P13/04 P13/24 P13/35 R02019a R02019b R04052a R04052c	P13/36 HS2 HS3 HSMf 503b	HSMf 331			R07055b
Neomycin							P14/09 P14/10	HS5 R02019a	HS3 HS8 P14/06	HS1 HS7 R07055b R08041b	HS6 HS9 P13/04 P13/24 P13/28 P13/35 P13/36 HSMf 505/2	HS10 P13/26 HSMf 504/1 R04052a R04052c R07055a R07102c R08041a	HS4 P13/32 HSMf 331 HSMf 504/2 HSMf 505/1 R02019b	HS2 HSMf 503b
Lincomycin							P13/24 P13/26 P13/28 P13/36 P14/06 P14/09 R07055a	HS1 HS3 P13/32 P14/10 HSMf 503b R02019b R08041b	HS4 HS6 HS7 HS8 P13/04 P13/35 HSMf 331 R02019a R07102c R08041a	HS2 HS9 HS10 HSMf 504/1 HSMf 504/2 HSMf 505/1 HSMf 505/2 R04052a			HS5 R04052c R07055b	
Tetracycline	R02019a R02019b R07055a R08041b HSMf 331	R04052a HSMf 505/2	HSMf 504/1 HSMf 504/2 R07055 b	HS3 HS7 P13/04 P13/32 HSMf 503b HSMf 505/1 R04052c R07102c R08041a	HS5 P13/35 P14/10	HS1 HS2 HS6 HS8 P13/26	HS9 P13/24 P13/28 P14/09	HS10 P13/36 P14/06			HS4			

	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128
Doxycycline		HSMf 503b	R02019a R02019b R07055a R07055b R08041b	HSMf 331 HSMf 504/1 R07102c	HS7 HSMf 504/2 HSMf 505/1 R04052a R08041a	HS6 HS8 P13/26 P13/28 P14/09 HSMf 505/2 R04052c	HS5 HS9 P13/04 P13/24 P13/35 P13/36 P14/06 P14/10	HS4 P13/32	HS2	HS3 HS10	HS1			
Metronidazole					P13/26	P13/28 P13/36 P14/06 P14/09	HS8 R02019a R07055a	P13/04 P13/24 P13/35	P13/32 HS7 HS10 HSMf 503b R02019b R07055b	HS6 HS2 R08041b	HS1 HS3 HS4 HS9 P14/10 HSMf 331 HSMf 505/1 R04052a R07102c R08041a	HS5 HSMf 504/1 HSMf 504/2 HSMf 505/2 R04052c		
Rifampicine	R02019a	R07055a R08041b HSMf 503b	HSMf 504/1 R02019b R07055b R07102c R08041a	HS1 HS8 P13/04 P13/28 P14/09 HSMf 504/2 HSMf 505/1 R04052a	HS2 HS6 HS7 HS10 P13/26 P13/32 P14/06 P14/10	HS9 P13/35 HSMf 331 HSMf 505/2 R04052c	HS5 P13/24 P13/36	HS3 HS4						

HS1-10: pig strains of *Helicobacter (H.) suis*
P13/04, P13/24, P13/26, P13/28, P13/32, P13/35, P13/36, P14/06, P14/09, P14/10: pig strains of *H. suis*
HSMf 331, HSMf 503b,HSMf 504/1, HSMf 504/2, HSMf 505/1, HSMf 505/2: Crab Eating Macaque strains of *H. suis*
HSMm R02019a, HSMm R02019b, HSMm R04052a, HSMm R04052c, HSMm R07055a, HSMm R07055b, HSMm R07102c, HSMm R08041a, HSMm R08041b: Rhesus Macaque strains of *H. suis*