Molecular characterization of fluoroquinolone resistance in *Haemophilus parasuis* isolated from pigs in South China

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Objectives: To perform molecular characterization of fluoroquinolone-resistant *Haemophilus parasuis* isolated from South China.

Methods: *H. parasuis* isolates were investigated for quinolone and fluoroquinolone susceptibility and screened for plasmid-mediated quinolone resistance (PMQR) determinants by PCR amplification and DNA sequence analysis. Additionally, quinolone resistance-determining region (QRDR) mutations of DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) were determined. The genetic relatedness among the strains was analysed by PFGE.

Results: These *H. parasuis* isolates showed higher MIC values of nalidixic acid, enrofloxacin, ciprofloxacin, levofloxacin, norfloxacin and lomefloxacin. Moreover, *qnrA1*, *qnrB6* and *aac(6′)-Ib-cr* were present in 2.61%, 0.87% and 2.61% of the 115 isolates, respectively. One strain possessed both *aac(6′)-Ib-cr* and *qnrA1*. Mutation analysis of QRDRs showed that the resistant strains carried at least one mutation in *gyrA* (at codon 83 or 87), but no mutation was detected in *gyrB*. PFGE analysis showed great genetic diversity among these resistant *H. parasuis* strains.

Conclusions: The data presented here highlight the presence of *qnr* and *aac(6′)-Ib-cr* genes in *H. parasuis* strains from South China. Moreover, the *gyrA* (at codon 83 or 87) mutation is linked to fluoroquinolone resistance in *H. parasuis*. Transferable PMQR determinants and multiple target gene mutations play important roles in the fluoroquinolone resistance of *H. parasuis*. These data provide important insights into the mechanism of fluoroquinolone resistance in *H. parasuis*, thereby highlighting the usefulness of fluoroquinolones for the treatment and control of this infection.

Keywords: *H. parasuis*, plasmid-mediated quinolone resistance, quinolone resistance-determining region, PFGE

Introduction

*Haemophilus parasuis* is the causative agent of Glässer's disease, characterized by fibrinous polyserositis, arthritis and meningitis. This disease has produced large losses in the pig industry worldwide. However, no universal vaccines are available to provide effective cross-protective immunity due to serovar diversity and a high number of non-typeable *H. parasuis* strains. Therefore, antimicrobial agents have become increasingly important to fight this pathogen. Unfortunately, the extended use of fluoroquinolones in veterinary medicine has encouraged an increase in resistance to fluoroquinolones in *H. parasuis* in many countries. Therefore, the objective of this work was to investigate the presence of transferable plasmid-mediated quinolone resistance (PMQR) genes, quinolone resistance-determining region (QRDR) mutations and the genetic relatedness of *H. parasuis* strains in South China to provide a scientific basis for further study of the fluoroquinolone resistance mechanism in *H. parasuis*.

Materials and methods

Bacterial isolates

All *H. parasuis* strains were isolated from diseased swine suffering polyserositis, pneumonia or meningitis in South China between September 2008 and February 2010. Identification of these 115 isolates was
carried out by biochemical tests (NAD dependency; absence of haemolysis; and urease, oxidase and catalase tests) and 16S diagnostic PCR.\textsuperscript{1,3}

**Antimicrobial susceptibility testing**

The MIC values of nalidixic acid, ciprofloxacin, levofloxacin, enrofloxacin, norfloxacin and lomefloxacin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) were determined in fastidious medium (tryptic soy broth (TSB) (OXOID, Inc., UK) + 5% bovine serum + 10 µg/mL NAD (Sigma, Inc., USA)) using custom-designed 96-well microtitre plates. All plates were inoculated following the guidelines of the CLSI (formerly the NCCLS).\textsuperscript{4} The microdilution plates were incubated in an atmosphere containing 5% CO\textsubscript{2} at 37°C for 24 h. The MIC value was defined as the lowest concentration exhibiting no visible growth. Ranges of susceptibility were recorded along with the MIC that inhibited 50% (MIC\textsubscript{50}) and 90% (MIC\textsubscript{90}) of the isolates. The reference strains *Actinobacillus pleuropneumoniae* ATCC 27090 and *Escherichia coli* ATCC 25922 served as quality controls for MIC determinations.

**Detection of PMQR genes**

PCR amplification of PMQR genes (*qnrA, qnrB, qnrS, qnrC, qnrD, aac(6\textsuperscript{-})-Ib-cr* and *qepA*) was performed with ExTaq DNA polymerase (TaKaRa, Inc., China) and previously described primers.\textsuperscript{5,6} Purified PCR products were directly sequenced or cloned into pGEM-T (TaKaRa, Inc., China) and then sequenced (model ABI3730; Applied Biosystems, Inc., USA).

**Detection of mutations in QRDRs of gyrA, gyrB, parC and parE**

To determine the QRDR mutations in *H. parasuis*, the 20 isolates that showed higher MICs of nalidixic acid, enrofloxacin, levofloxacin, ciprofloxacin, norfloxacin and lomefloxacin were analysed for *gyrA, gyrB, parC* and *parE* sequences using the primers listed in Table S1 (available as Supplementary data at JAC Online). In addition, these genes were also amplified in 15 reference strains that presented lower MICs of the six fluoroquinolones tested. The primers were designed based on the sequences of *H. parasuis gyrA, gyrB, parC* and *parE* genes (GenBank accession number NC-011852). The amplified products were then sequenced as described above.

**PFGE**

To gain insight into the genomic relatedness of *H. parasuis* strains, macro-restriction CpaI (TakaRa, Inc., China) analysis resolved by PFGE was applied to 20 resistant strains and 6 PMQR-positive strains. The PFGE typing was carried out using a Chef Mapper electrophoresis system (Bio-Rad, USA). The separation conditions were 2.16–63.8 s for 21 h. *Salmonella enterica* serovar Braenderup H9812 served as the size standard, which was digested with XbaI (TakaRa, Inc., China). Interpretation of the PFGE patterns was accomplished using BioNumerics 4.0 software (Applied Maths Inc., USA).

**Results and discussion**

**Quinolone and fluoroquinolone resistance in *H. parasuis***

A summary of the MIC values of nalidixic acid, enrofloxacin, ciprofloxacin, levofloxacin, norfloxacin and lomefloxacin found in this study is shown in Table S2 (available as Supplementary data at JAC Online). Compared with other studies, we found that different fluoroquinolone susceptibilities of *H. parasuis* have been observed in different countries over time.\textsuperscript{2,3} Furthermore, the MICs of nalidixic acid and lomefloxacin for *H. parasuis* were determined for the first time. The MIC\textsubscript{50} and MIC\textsubscript{90} of these antimicrobial agents showed 4- to 8-fold increases compared with previous studies.\textsuperscript{2,3} Our study demonstrating higher MICs for *H. parasuis* isolated during 2008–2010 in South China should better reflect the current status of quinolone and fluoroquinolone susceptibility. This phenomenon could be related to excessive use of quinolones and fluoroquinolones for the treatment or prevention of infectious diseases in pig husbandry in South China. Therefore, prudent measures for these agents’ usage and active surveillance should be established.

**Identification of PMQR genes**

In this study, *qnrA1, qnrB6* and *aac(6\textsuperscript{-})-Ib-cr*, detected in *H. parasuis* for the first time, were present in 3 (2.61%), 1 (0.87%) and 3 (2.61%) isolates, respectively. The *qnrA* and *qnrB* amplicon sequences showed 100% identity to the reported *qnrA1* and *qnrB6* from *Klebsiella pneumoniae* plasmid pGDKA1 (GenBank accession number EU772251) and *Shigella sonnei* strain 136 (GenBank accession number GQ914054), respectively. The sequences for all of the amplicons of the *aac(6\textsuperscript{-})-Ib-cr* fragment were indistinguishable and demonstrated 100% sequence identity to the reported *aac(6\textsuperscript{-})-Ib-cr* from *E. coli* plasmid pEC L8 (GenBank accession number GU371928). However, other PMQR genes (*qnrS, qnrC, qnrD* and *qepA*) were not found in these 115 isolates. Additionally, one strain was positive for both *aac(6\textsuperscript{-})-Ib-cr* and *qnrA1*, and the MIC of ciprofloxacin was 16 mg/L. This strain awaits further study. To date, PMQR genes (*qnrA, qnrB, qnrS, qnrD* and *qepA*) have been reported in veterinary clinical isolates in China.\textsuperscript{7,8} Additionally, most *qnr* genes are located on a Tn-like sequence or integron in a conjugative plasmid, which has facilitated the rapid spread and increase in fluoroquinolone resistance in bacteria.\textsuperscript{9} Thus, the dissemination of PMQR genes in the pig industry cannot be ignored and the mechanism of their spread in *H. parasuis* requires further study.

**Analysis of QRDR mutations**

In the present study, several QRDR mutations were found among resistant *H. parasuis* strains (Table 1). It is worth noting that at least one mutation in *gyrA* was present in 20 resistant strains and 90% of isolates had two mutations in *gyrA*, but no *gyrB* mutations were found in the 15 reference strains. This finding suggests that *gyrA* mutations (S83Y, S83F, D87Y, D87N and D87G) are closely correlated with fluoroquinolone resistance in *H. parasuis*. The fluoroquinolone resistance levels of the strains with double *gyrA* mutations were higher than those with a single *gyrA* mutation, indicating that the increasing MICs of fluoroquinolones for *H. parasuis* are also associated with stepwise accumulation of *gyrA* mutations.\textsuperscript{10} double *parC* mutations (Y577C, V648I, E678D, S669F, A464V and A466S) were found both in susceptible and resistant strains, indicating that these mutations are possibly not directly related to fluoroquinolone resistance of *H. parasuis*. In addition, *parE* mutations (S283G, A227T and G241S) were mainly found in *qnr*-positive strains in this study. Many previous studies have demonstrated that topoisomerase IV is the secondary target for fluoroquinolones, and a *parC* mutation related to fluoroquinolone resistance in other Gram-negative bacteria, such as *Salmonella* and *E. coli*, has been shown.\textsuperscript{5,6} However,
in A. pleuropneumoniae, parE mutation was possibly not involved in enrofloxacin resistance. Thus, further studies are needed to evaluate whether parC and parE mutations are involved in fluoroquinolone resistance in H. parasuis. In contrast, gyrB mutations were not detected in this study. Overall, the QRDR analysis suggested that DNA gyrase (gyrA) is a primary target of fluoroquinolone resistance in H. parasuis, as in other Gram-negative bacteria.

**PFGE**

In this study, PFGE revealed great genomic heterogeneity among the 20 resistant isolates and PMQR-positive isolates [Figure 1 and Figure S1 (available as Supplementary data at JAC Online)]. Of the 20 isolates, 3 isolates from different farms were clonally related (Figure S1). Interestingly, one of the three clonally related strains carried a qnrA gene, along with the genetic diversity between the six PMQR-positive strains, indicates that the PMQR determinants may be obtained by each strain through transferable elements (such as plasmids, transposons etc.). So the spread of mechanisms of fluoroquinolone resistance in H. parasuis needs to be further investigated.

In conclusion, here, for the first time, we describe H. parasuis containing qnrA, qnrB andaac(6′)-Ib-cr in South China. The data from this study suggest that fluoroquinolone resistance of H. parasuis is mainly due to the transferable PMQR determinants and multiple target gene mutations. These findings provide a

### Table 1. Mutations in the QRDRs of and the MICs for 15 reference and 20 H. parasuis isolates.

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>Mutations in QRDRs</th>
<th>MICs (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gyrA</td>
<td>parC</td>
</tr>
<tr>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10 (50%)</td>
<td>S83F, D87N</td>
<td>—</td>
</tr>
<tr>
<td>3 (15%)</td>
<td>S83Y, D87N</td>
<td>—</td>
</tr>
<tr>
<td>1&lt;sup&gt;c&lt;/sup&gt; (5%)</td>
<td>S83F</td>
<td>S283G, A227T, G241S</td>
</tr>
<tr>
<td>1&lt;sup&gt;d&lt;/sup&gt; (5%)</td>
<td>D87G</td>
<td>Y577C, S669F, A227T</td>
</tr>
<tr>
<td>1 (5%)</td>
<td>S83F, D87Y</td>
<td>E678D</td>
</tr>
<tr>
<td>1 (5%)</td>
<td>S83F, D87N</td>
<td>E678D</td>
</tr>
<tr>
<td>1 (5%)</td>
<td>S83F, D87N</td>
<td>A464V, A466S, Y577C</td>
</tr>
</tbody>
</table>
| NAL, nalidixic acid; LVX, levofloxacin; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin; LOM, lomefloxacin. 
<sup>ab</sup>Some of the H. parasuis reference strains also had mutations in parC. 
<sup>b</sup>Fifteen H. parasuis reference strains. 
<sup>c</sup>One strain carryingaac(6′)-Ib-cr. 
<sup>d</sup>One strain carrying qnrB6.

**Figure 1.** Dendrogram of patterns generated by PFGE of H. parasuis isolates containing PMQR determinants together with mutations in QRDRs and MIC values of ciprofloxacin (CIP).
scientific basis for further study of the mechanism of fluoroquinolone resistance in H. parasuis.

**GenBank accession numbers**
The nucleotide sequences of qnr, aac(6′)-Ib-cr and the variant gyrA genes reported here were deposited in the GenBank database with the accession numbers HQ117876–HQ117882 and HQ332468–HQ332483.

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**Transparency declarations**
None to declare.

**Supplementary data**
Table S1, Table S2 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**