

## Resistance Mechanism Against Fluoroquinolones in *Mycoplasma hyopneumoniae* Field Isolates

J. VICCA,<sup>1,2</sup> D. MAES,<sup>1</sup> T. STAKENBORG,<sup>3</sup> P. BUTAYE,<sup>3</sup> F. MINION,<sup>4</sup> J. PEETERS,<sup>3</sup>  
A. DE KRUIF,<sup>1</sup> A. DECOSTERE,<sup>2</sup> and F. HAESEBROUCK<sup>2</sup>

### ABSTRACT

The quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* of ten *Mycoplasma hyopneumoniae* field isolates that were either sensitive (5) or resistant (5) to the fluoroquinolones flumequine and enrofloxacin were characterized. In all five resistant isolates, one point mutation (C → A) in *parC* was found, resulting in an amino acid change from serine to tyrosine at position 80 (*Escherichia coli* numbering). For four of these isolates, this was the only mutation found. These isolates had a minimum inhibitory concentration (MIC) of enrofloxacin of 0.5 µg/ml, whereas for sensitive isolates the MIC of enrofloxacin was ≤0.06 µg/ml. One resistant isolate (Mh 20) had an extra mutation (C → T) in *gyrA* resulting in an amino acid change from alanine to valine at position 83 (*E. coli* numbering), leading to a further increase in the MIC of enrofloxacin (>1 µg/ml). No mutations resulting in an amino acid change were detected in the QRDR of the *gyrB* and *parE* genes of the selected isolates. This is the first description of the mechanism of stepwise resistance against fluoroquinolones in *M. hyopneumoniae*.

### INTRODUCTION

*MYCOPLASMA HYOPNEUMONIAE* is a major swine pathogen causing enzootic pneumonia, a chronic respiratory disease in pigs resulting in considerable economic losses. In a previous study,<sup>36</sup> conducted to determine the *in vitro* susceptibility of *M. hyopneumoniae* field isolates to frequently used antimicrobials in swine, 5 out of 21 isolates were found to be less susceptible or to be resistant to flumequine and enrofloxacin. This rather high frequency was unexpected because fluoroquinolone resistance does not often occur in swine respiratory pathogens.<sup>14,23,37</sup>

Fluoroquinolones are broad-spectrum antibiotics. Their use depends on the country regulations; fluoroquinolones are not allowed for use in pigs in the United States but are allowed in the European Union.<sup>38</sup> In Belgian pig herds, fluoroquinolones are frequently used as a prophylactic antibiotic during the suckling period,<sup>35</sup> mainly to prevent neonatal diarrhea. In older pigs, these antimicrobials are mainly used to treat individual animals with diarrhea, arthritis, meningitis, or respiratory symptoms.

The most frequently used fluoroquinolones in large animal veterinary medicine are flumequine and enrofloxacin.

Fluoroquinolones are known to have two enzyme targets in the bacterial cell belonging to the topoisomerases type 2, namely DNA gyrase and topoisomerase IV. The first enzyme catalyzes adenosine triphosphate (ATP)-dependent negative supercoiling of DNA; the latter enzyme is essential for chromosome segregation.<sup>12,18</sup> DNA gyrase is a tetramer composed of two GyrA and two GyrB subunits. Topoisomerase IV is similarly structured and is composed of two ParC and two ParE subunits. ParC is homologous to GyrA and ParE is homologous to GyrB. The primary target for fluoroquinolones in Gram-negative bacteria is the DNA gyrase, whereas in Gram-positive bacteria, including mycoplasmas, it seems to be topoisomerase IV.<sup>2,4,7,9,12,13,18,21</sup> However, some exceptions to this rule were found in *Streptococcus pneumoniae* and *Mycoplasma hominis* isolates for newer fluoroquinolones, such as sparfloxacin and gatifloxacin.<sup>3,11</sup> For *Mycoplasma gallisepticum*, the preferential target of enrofloxacin is DNA gyrase.<sup>33</sup> In several bacteria, mutations responsible for an increase in minimum inhibitory

<sup>1</sup>Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium.

<sup>2</sup>Department of Bacteriology, Pathology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium.

<sup>3</sup>CODA-CERVA, Veterinary and Agrochemical Research Centre, 1180 Brussels, Belgium.

<sup>4</sup>Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Ames, Iowa.

concentration (MIC)-value were found in the subunits of these target genes.<sup>4,6,38,40,41</sup>

In the present study, the mechanism responsible for fluoroquinolone resistance in *M. hyopneumoniae* field isolates<sup>37</sup> was determined. Therefore, the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* were sequenced.

## MATERIALS AND METHODS

### *M. hyopneumoniae* isolates

The 10 *M. hyopneumoniae* field isolates selected for this study were obtained between 2000 and 2002 from slaughter pigs from 10 different Belgian farrow-to-finish pig herds and were previously used for MIC determination.<sup>36</sup> Isolate selection for this study was based on the MIC value: five isolates with the highest and five isolates with the lowest MIC values for flumequine and enrofloxacin were retained. The MIC values of flumequine and enrofloxacin for four isolates (Mh 4, 8, 14, and 17) were >16 µg/ml and 0.5 µg/ml, respectively. For isolate Mh 20, the MIC of flumequine was >16 µg/ml and that of enrofloxacin >1 µg/ml. The other five isolates (Mh 7, 10, 11, 15, and 19) were susceptible to flumequine (MIC ≤2 µg/ml) and enrofloxacin (MIC ≤0.06 µg/ml).<sup>37</sup>

### DNA extraction and PCR amplification

The *M. hyopneumoniae* isolates were grown in nonselective Friis medium and subsequently centrifuged at 5,000 × g for 10 min. DNA was extracted using the DNeasy Tissue kit (Qiagen, Westburg, Leusden, The Netherlands) according to the manufacturers' instructions. Without further purification, an aliquot of the supernatant containing DNA was used as a template for PCR amplification.

To sequence parts of the DNA gyrase subunits, *gyrA* and *gyrB*, and the topoisomerase subunits, *parC* and *parE*, containing the QRDR, primers were designed based on the *M. hyopneumoniae* genome sequence of reference strain 232.<sup>25</sup> Oligonucleotides MhgyrAfor (5'-CTKCCRGATGTCCGWGATGG-3') and MhgyrArev (5'-GTSGGAAARTCYGGCYCCGG-3') were used to amplify a 557-bp *gyrA* fragment between positions 487 and 1,043 (*Escherichia coli* coordinates). A 937-bp *gyrB* fragment between positions 1,994 and 3,437 (*E. coli* coordinates) was amplified with primers MhgyrBfor (5'-ACATTCATAACCCTGAAGGC-3') and MhgyrBrev (5'-GTCTCTCAAAGTTGTTCCGG-3'). To amplify the QRDR of *parC*, primers MhparCfor (5'-ATTCAGTAATTAATTC-3') and MhparCrev (5'-TCTTCAAGGTAAATTTGCTG-3') were selected to amplify a 1,309-bp fragment between positions 19 and 1,313 (*E. coli* coordinates) and a 735-bp *parE* fragment between positions 1,046 and 1,765 (*E. coli* coordinates) was amplified using the primers MhparEfor (5'-ATTCTTGAATTTGTTGGGC-3') and MhparErev (5'-CCCAAGTCCTTATAGCGC-3'). DNA amplification was performed with a DNA thermal cycler (model 9600 GeneAmp PCR system, Perkin-Elmer, Zaventem, Belgium). Each 50-µl PCR mixture contained 25 µl of Mastermix (Invitrogen, Belgium), 2 µM for both primers, and a 2.5-µl DNA sample. Water was added to a total volume of 50 µl. For all amplification reactions, the same PCR running conditions were used, con-

sisting of an initial cycle of 5 min denaturation at 94°C, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of elongation at 72°C. After amplification, 5 µl of amplicon was mixed with 3 µl of sample buffer (50% glycerol, 1 mM Cresol Red). This mixture was electrophoresed in a 1.5% agarose gel for 75 min at 175 V in 0.5× TBE (0.45 M Tris-HCl, 0.45 M boric acid, 0.01 M EDTA).

### Sequencing

After purification of the PCR product with the Qiaquick PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands), both strands of the PCR product were sequenced using the Big Dye Terminator v3-1 cycle sequencing kit (Applied Biosystems, Lennik, Belgium) on a ABI Prism™ 310 Genetic Analyser. The electropherograms were exported and converted to the sequence analysis software, Kodon® (Applied Maths, Sint-Martens-Latem, Belgium). The nucleic acid sequences of the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* of the susceptible *M. hyopneumoniae* isolates were compared with those of the isolates with a MIC of >16 µg/ml and ≥0.5 µg/ml for flumequine and enrofloxacin, respectively. The deduced amino acid sequences of susceptible isolate Mh 7 and resistant isolate Mh 20 were compared with the sequences of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *E. coli*, and, to date, fully sequenced human and veterinary *Mycoplasma* species. The percentage of identity between the susceptible Mh 7 isolate, the other organisms and the GenBank accession numbers are listed in Table 1.

## RESULTS

### PCR amplification and sequences of PCR products

Each of the selected forward and reverse primer pairs amplified one PCR product. An acquired C264A transition (*E. coli* numbering) was found in the *parC* gene of all five isolates with MIC values >16 µg/ml and ≥0.5 µg/ml for flumequine and enrofloxacin, respectively. This corresponds to an amino acid change from serine to tyrosine at position 80 (*E. coli* numbering). An additional transition was found in isolate Mh 20. The MIC of enrofloxacin for this isolate was >1 µg/ml, whereas it was 0.5 µg/ml for the other resistant isolates. This additional transition, C635T, was found in *gyrA*, resulting in an amino acid change from alanine to valine at position 83 (*E. coli* numbering) (Table 2). In the same isolate, another substitution, T630A, was found in *gyrA*. However, this substitution did not result in an amino acid change. Other silent substitutions in the QRDR of *gyrA* were found in isolate Mh 7 (G651A) and in isolates Mh 15, 19, and 20 (G759A). In the QRDR of *gyrB*, silent substitutions were found in isolates Mh 4, 8, 11, 14, 17, and 20 (T2529A) and isolates Mh 10 and 19 (G2577A). No silent substitutions were found in the QRDR of *parC*. In the QRDR of *parE*, two silent substitutions were found: C315T in isolates Mh 7 and 15, and G345A in isolates Mh 7, 11, and 15. The identity for the QRDR of the four fluoroquinolone target genes at DNA level was very high for all *M. hyopneumoniae* isolates; 96.30%, 98.80%, 98.78%, and 97.53% for the QRDR of *gyrA*, *gyrB*, *parC*, and *parE*, respectively.

TABLE 1. PERCENTAGE OF IDENTITY AND GENBANK ACCESSION NUMBERS OF THE QUINOLONE RESISTANCE-DETERMINING REGION OF *gyrA*, *gyrB*, *parC*, AND *parE* AMINO ACID SEQUENCES OF TO DATE FULLY SEQUENCED *MYCOPLASMA* SPECIES, *S. AUREUS*, *S. PNEUMONIAE*, AND *E. COLI*

	Percent identify with <i>M. hyopneumoniae</i> (Mh 7, susceptible isolate)				GenBank accession number			
	<i>GyrA</i>	<i>GyrB</i>	<i>ParC</i>	<i>ParE</i>	<i>GyrA</i>	<i>GyrB</i>	<i>ParC</i>	<i>ParE</i>
<i>M. hominis</i>	83.95	69.87	82.92	68.67	U59880	X77529	AF036961	AF036961
<i>M. genitalium</i>	65.43	59.04	68.29	61.44	U09251	U09251	U25549	U25549
<i>M. gallisepticum</i>	66.67	71.08	68.29	72.29	U18306	U18306	AF372652	AF372652
<i>M. mobile</i>	82.72	74.70	80.49	69.88	NC 006908	NC 006908	NC 006908	NC 06908
<i>M. mycoides</i> subsp. <i>mycoides</i> SC	79.01	66.26	75.61	67.47	NC 005364	NC 005364	NC 005364	NC 005364
<i>M. penetrans</i>	72.84	68.67	71.95	66.26	AP004170	AP004170	AP004172	AP004172
<i>M. pneumoniae</i>	65.43	60.24	67.07	55.42	X53555	X53555	AE000004	AE000004
<i>M. pulmonis</i>	82.72	74.70	79.27	71.08	AL445563	AL445565	NC 002771	NC 002771
<i>S. aureus</i>	79.01	62.65	69.51	60.24	D10489	D10489	L25288	L25288
<i>S. pneumoniae</i>	74.07	6506	65.85	60.24	AF065152	X83917	AF065151	AF065153
<i>E. coli</i>	75.31	55.42	58.54	52.01	X06373	X04341	M58408	M58409

## DISCUSSION

Resistance in *M. hyopneumoniae* field isolates was first described in a previous study where 5 of 21 isolates were found to be resistant to flumequine and less susceptible or resistant to enrofloxacin.<sup>36</sup> This prevalence of fluoroquinolone resistance is higher than the resistance rate found in other bacterial swine pathogens like *Streptococcus suis*,<sup>1,23</sup> *Arcanobacterium pyogenes*,<sup>42</sup> *Pasteurella multocida*, and *Mannheimia haemolytica*.<sup>37</sup> Several mechanisms for fluoroquinolone resistance have been described in different bacterial species. These include alterations in the two drug target enzymes, namely DNA gyrase and topoisomerase IV, changes in drug permeation through modifications in the outer membrane proteins, induction of active efflux systems, modifications in the peptidoglycan layer or the outer membrane proteins,<sup>19,28,29</sup> and plasmid-correlated

quinolone resistance.<sup>15,24</sup> Although the existence of energy-dependent efflux systems has recently been described for *M. hominis*,<sup>31</sup> acquired resistance in *Mycoplasma* species is usually due to alterations in the target enzymes. In the present study, the QRDRs of the four target genes *gyrA*, *gyrB*, *parC*, and *parE* were sequenced in fluoroquinolone-susceptible and -resistant *M. hyopneumoniae* isolates. The amino acid change at position 80 (*E. coli* numbering) in *parC*, observed in all five resistant isolates, is the most common mutation related to fluoroquinolone resistance in Gram-positive bacteria,<sup>10,20,27,39</sup> including *M. hominis*.<sup>5</sup> For four of the *M. hyopneumoniae* isolates, this was the only mutation found and it resulted in at least an eight-fold increase in the MIC of flumequine and enrofloxacin. Such isolates are considered to be resistant to flumequine (MIC >16 µg/ml),<sup>14</sup> whereas they are still considered to be susceptible to enrofloxacin (MIC = 0.5 µg/ml).<sup>26</sup>

TABLE 2. MIC VALUES FOR FLUMEQUINE AND ENROFLOXACIN AND THE AMINO ACID MUTATIONS IN *gyrA* AND *parC* OF THE FLUOROQUINOLONE-RESISTANT FIELD ISOLATES OF *M. HYOPNEUMONIAE*

<i>Mh</i> isolate	MIC (µg/ml)		Amino acid change (codon)	
	Flumequine	Enrofloxacin	<i>gyrA</i> 83 <sup>a</sup>	<i>parC</i> 80
Mh 7	2	0.06	—	—
Mh 10	2	0.06	—	—
Mh 11	1	0.03	—	—
Mh 15	2	0.06	—	—
Mh 19	2	0.06	—	—
Mh 4	>16	0.5	—	S(TCT) → Y(TAT)
Mh 8	>16	0.5	—	S(TCT) → Y(TAT)
Mh 14	>16	0.5	—	S(TCT) → Y(TAT)
Mh 17	>16	0.5	—	S(TCT) → Y(TAT)
Mh 20	>16	>1	A(GCT) → V(GTT)	S(TCT) → Y(TAT)

<sup>a</sup>Amino acid position according to *E. coli* numbering.

In a recent study, *M. hyopneumoniae* clones with an eight-fold increase in MIC values of enrofloxacin were reisolated from pigs that had been treated with marbofloxacin after experimental infection with *M. hyopneumoniae*.<sup>22</sup> In these clones a point mutation in *parC* was detected, resulting in amino acid changes at positions 80, 84, or 116. Our study demonstrates that mutations in *parC*, resulting in amino acid changes, also occur under field conditions, warranting further monitoring of fluoroquinolone resistance in porcine *Mycoplasma* species. *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis*, two other pathogenic mycoplasmas in swine, also appeared to exhibit a high resistance rate against flumequine (100% and 85% resistance, respectively), although they were found to be fully susceptible to enrofloxacin.<sup>14</sup>

The occurrence of low-level resistance against fluoroquinolones after a single mutation in *parC* has been described earlier for *Enterococcus faecalis*, *S. aureus*, and *S. pneumoniae*, whereas high-level resistant isolates had mutations in both *parC* and *gyrA*.<sup>8,20,30,34</sup> In *M. bovirhinis*, however, a single mutation in *parC* (position 80) resulted in different MIC profiles, including low- and high-level resistant isolates.<sup>16</sup> The authors suggested that the differences in MIC might have been caused by the level of expression of the quinolone efflux transporter.

One isolate, Mh 20, had an extra mutation (C → T) in *gyrA* at position 635, resulting in an amino acid change from alanine to valine at position 83 (*E. coli* numbering), another hot spot for fluoroquinolone resistance.<sup>17</sup> This was associated with at least a four-fold increase in MIC of enrofloxacin (MIC >1 µg/ml) compared to isolates with only a mutation in *parC* and demonstrates stepwise resistance development against fluoroquinolones in *M. hyopneumoniae* for the first time.

As in fluoroquinolone-resistant *M. hominis*, *Ureaplasma urealyticum*, and *Acholeplasma laidlawii* isolates, no mutations were found in the QRDR of *gyrB* in *M. hyopneumoniae*. Such mutations have been described in *in vitro*-selected resistant *M. gallisepticum* isolates.<sup>32</sup> Also, no mutations resulting in amino acid changes were found in the QRDR of *parE* of the *M. hyopneumoniae* isolates. In clinical isolates of *M. hominis*, however, a mutation resulting in an amino acid substitution in *parE* was previously observed.<sup>6</sup> The absence of amino acid changes in *GyrB* and *ParE* of fluoroquinolone-resistant *M. hyopneumoniae* isolates is in agreement with other studies reporting that amino acid changes in *GyrB* or *ParE* occur less frequently than in *GyrA* and *ParC*.<sup>17</sup>

In conclusion, topoisomerase IV of *M. hyopneumoniae* seems to be the primary target for fluoroquinolones (flumequine and enrofloxacin), with position 80 in *parC* as the hot spot. A single mutation in *parC* is sufficient to reach resistance to flumequine, whereas a second mutation in the secondary target, DNA gyrase (*gyrA*), is necessary to make *M. hyopneumoniae* resistant to enrofloxacin.

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Address reprint requests to:

Dr. Jo Vicca  
KaHo St. Lieven Polytechnics  
Hospitalstraat 23  
9100 St. Niklaas  
Belgium

E-mail: Jo.Vicca@kahosl.be

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