Role of Mutations in DNA Gyrase and Topoisomerase IV in Fluoroquinolones-Resistance of *Mycoplasma gallisepticum* Obtained in vitro and in vivo

1Jian Li, 2Dian-Hong Lu, 1Zhi-Jie Liu, 1Xiao-Hua Zhang, 1Fei-Long Wei, 1Ya-Hong Liu and 1Hong-Xia Jiang

1College of Veterinary Medicine, Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, South China Agricultural University, 510642 Guangzhou, People’s Republic of China

2Laboratory of Clinical Microbiology, Guangdong Academy of Agriculture Sciences, Institute of Veterinary Medicine, 510640 Guangzhou, People’s Republic of China

**Abstract:** The role of mutations in genes for GyrA, GyrB, ParC and ParE in fluoroquinolone-resistance in *Mycoplasma gallisepticum* obtained in vitro and in vivo was studied. *M. gallisepticum* mutants were generated by stepwise selection in either 1/2×MIC or escalating concentration of enrofloxacin or ciprofloxacin. Seven fluoroquinolone-resistant clinical isolates of *M. gallisepticum* recovered from commercial poultry flocks with suspected chronic respiratory disease were recruited for the mutation detection of four target genes. The evolution of resistance in *M. gallisepticum* was more readily and more quickly with enrofloxacin selection than with ciprofloxacin selection. Selection with enrofloxacin gave mutations at GyrA83 (Ser-Ille) firstly and subsequent additional mutation at GyrA82 or GyrA103, combining substitution at ParC80 in association with high-level resistance to FQs. Selection with ciprofloxacin gave primary change at Gyr87 and subsequent additional alteration at ParC80, elevating the MICS for FQs significantly. The presence of alterations of both the GyrA and ParC in 7 clinical isolates of *M. gallisepticum* is in good agreement with the results from mutants selected in vitro. No mutations in GyrB and ParE were found in mutants selected both in vitro and in vivo.

**Key words:** *Mycoplasma gallisepticum*, enrofloxacin, ciprofloxacin, quinolone resistance-determining regions, poultry flocks

**INTRODUCTION**

*Mycoplasma gallisepticum*, characterized as cell wall less prokaryote (Razin, 1992; Razin et al., 1980) is an important pathogen in Chronic Respiratory Disease (CRD) and resulting in reduced weight gain and egg production in infected birds (Ley, 2003). Infected birds, despite of remain asymptomatic become life-long carriers with the means for horizontal and vertical transmission (Ley, 2003). Control of *M. gallisepticum* infection in China by vaccination is limited because of lack of effective vaccine, chemotherapeutically control is necessary and a long-term strategy.

Fluoroquinolones (FQs) are broad-spectrum antibiotics used extensively in controlling CRD and minimizing its transmission in case of an outbreak (Hamann et al., 1997). Among these antimicrobial agents, enrofloxacin, ciprofloxacin, danofloxacin and sarafloxacin are approved for use in poultry farms in China however, their resistance presents ascending trend due to extensive application.

Chromosomal mechanisms involved in FQs resistance has been well reviewed (Hernandez et al., 2011; Jacoby, 2005) and well studied in human mollicutes (Bebear et al., 1998, 1999; Kenny et al., 1999). Emergence of resistance to FQs is mainly due to chromosomal mutations in genes encoding the subunits of the drug’s target enzymes, DNA gyrase and topoisomerase IV which are essential for DNA replication (Gellert et al., 1976; Khodursky et al., 1995). DNA gyrase (GyrA and GyrB subunits) is thought to be the primary target in gram-negative bacteria such as *Escherichia coli*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae* and *Haemophilus influenzae* whilst topoisomerase IV in gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (Jacoby, 2005). Several studies on quinolone-resistant
Mycobacterium hominis, S. aureus and S. pneumoniae obtained in vitro showed selective targeting of GyrA or ParC for different quinolone drugs (Bebear et al., 1998a; Jacoby, 2005). To date, several studies focused on the acquisition and the mechanisms of resistance in M. gallisepticum obtained in vitro (Gautier-Bouchardon et al., 2002; Gerchman et al., 2008; Reinhardt et al., 2002a, b), however only one reported that in clinical isolates (Lysnyansky et al., 2008). In this study, researchers determined the selective target for ENR and CIP in M. gallisepticum obtained by stepwise selection and compared the in vitro and in vivo emergence of resistance and mechanism to FQs.

MATERIALS AND METHODS

Bacterial and growth: Mycobacterium gallisepticum reference strain S6 used for selection of fluoroquinolone resistant mutants was purchased from the Institute of Animal Drug Control of China (Beijing, China). Seven fluoroquinolone-resistant clinical isolates of M. gallisepticum from chickens with suspected CRD infection described before (Jiang et al., 2009) were recruited in this study for the detection of mutation in the QRDR of topoisomerase genes. As previously described (Jiang et al., 2009), M. gallisepticum strains were grown at 37°C in FM-4 medium supplemented with 15% heat-inactivated swine serum.

Antimicrobial agents and determination of MICs: MICs of different FQs and other antibiotics were determined by a serial broth dilution method in FM-4 medium (Wang et al., 2001). The MIC was determined as the lowest concentration of antimicrobial agent that prevent a color change in the medium at the time when the drug free growth control first showed a color change after about 5 days of incubation at 37°C. Antibiotics including Enrofloxacin (ENR), Ciprofloxacin (CIP), Danofloxacin (DAN), Sarafloxacin (SARA), Ofloxacin (OFL), Erythromycin (ERY), Tylosin (TYL) and Gentamycin (GEN) were purchased from the Institute of Animal Drug Control of China (Beijing, China). A strain was considered susceptible to enrofloxacin when the MIC was ≤0.5 mg L⁻¹ and resistant when the MIC was >2 mg L⁻¹ according to the breakpoints given for these antibiotics (Hamman, 2000).

Selection of fluoroquinolone-resistant mutants: According to previous study described with slight modification, two selection methods with stepwise selection of mutants in FM-4 broth containing either double increasing concentration or 1/2×MIC concentration of ENR or CIP were used for M. gallisepticum S6. For method with double increasing drug concentration, the concentrations used for during the first passage were 1/2×MIC of ENR or CIP for the wild-type organism. Once the MICs for mutants reached 128 μg mL⁻¹ after a serial transfer, passages were completed. For the other method, 1/2×MIC of ENR or CIP was always used for the wild-type organism for 10 sequential passages. Mutants from passages 5 (Se5, Sc5) and 10 (Se10, Sc10) were studied. The stability of the acquired resistance for all selected mutants was tested by subculture of the organisms to drug-free FM-4 broth for 5 consecutive then determining the MICs for the selected resistant strains and confirming that the susceptibilities were not restored.

PCR amplification and DNA sequence analysis: Chromosomal DNA of each resistant mutant was used as a template in a PCR to amplify the Quinolone Resistance Determining Regions (QRDRs) of the gyrA, gyrB, parC and parE genes. The primers selected for gyrA, gyrB, parC and parE were 5'-TATGTTGCTCTACCACCTACG-3' and 5'-CTACGGCAATACCACCTGG-3', 5'-TGACCGTAA GATTAGCAAA-3' and 5'-ACATCAGCATGC TGA-3', 5'-AAGGATAGGATAAGAAA-3' and 5'- CTCTTTGTATATTTCCCA-3', 5'-GTATACAAAT TACAGGAAAAAC-3' and 5'-CATCCATCTCTG GATCGA-3', respectively which yielded a respective 370, 580, 415 and 440 bp fragment. PCR was carried out with 1 μM each primer and 5 μL of template DNA for the QRDR amplification of the four topoisomerase genes for M. gallisepticum. PCR products were directly sequenced on both strands (Invitorgen, Shanghai, China). The DNASIS analysis programs were used for DNA and amino acid sequence alignments.

RESULTS AND DISCUSSION

Development of resistance in M. gallisepticum with ENR or CIP selection: A serial of resistant mutants of which with greatest MIC of ≥128 μg mL⁻¹ for all tested FQs (Table 1) were selected after sequential passages in 1/2×MIC or escalating concentrations of ENR or CIP in this study. Whatever the method used for selection, the evolution of resistance in M. gallisepticum was more readily and more quickly with ENR selection than with CIP selection. The emergence of resistance (MIC≥2 μg mL⁻¹) selected with increased ENR concentrations occurred at passages 2 whilst occurred at passages 6 with CIP selection. MICs of 5 FQs for mutants (Se8M) from increased ENR selection at passages 5 ranged 16-64 μg mL⁻¹, significantly higher than those (Se8M)
Table 1: MICs and mutations observed in fluorquinolone-resistant mutants of Mycoplasma gallisepticum selected in vitro and in vivo

<table>
<thead>
<tr>
<th>Strains</th>
<th>ENR</th>
<th>CIP</th>
<th>SARA</th>
<th>DAN</th>
<th>OFL</th>
<th>82</th>
<th>83</th>
<th>87</th>
<th>103</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.06</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc5</td>
<td>16.000</td>
<td>32.000</td>
<td>8.000</td>
<td>16.000</td>
<td>32.000</td>
<td>64.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc10</td>
<td>128.000</td>
<td>128.000</td>
<td>&gt;128.000</td>
<td>&gt;128.000</td>
<td>64.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc5</td>
<td>4.000</td>
<td>2.000</td>
<td>2.000</td>
<td>4.00</td>
<td>8.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc10</td>
<td>128.000</td>
<td>128.000</td>
<td>64.00</td>
<td>64.00</td>
<td>64.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc8M</td>
<td>32.000</td>
<td>32.000</td>
<td>16.000</td>
<td>64.00</td>
<td>64.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc32M</td>
<td>&gt;128.000</td>
<td>128.000</td>
<td>64.00</td>
<td>&gt;128.000</td>
<td>64.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sc5 and Sc10 correspond to mutants selected from S6 with 1/2 MIC enrofloxacin for 5 and 10 consecutive passages, respectively; similarly, Sc5 and Sc10 mutants obtained with 1/2 MIC ciprolfloxacin for the same passages. Sc8M, Sc32M, Sc8M and Sc16M corresponds to mutants selected from S6 with 8-MIC, 16-MIC and 32-MIC enrofloxacin or ciprofloxacin, respectively. ENR: Enrofloxacin; CIP: Ciprofloxacin; SARA: Sarafloxacin; Dano: Danofloxacin; OFL: Ofloxacin; *No change in the amino acid sequences

from increased CIP selection at the same passages, ranging 1–4 μg mL⁻¹. When drug concentration used for selection escalated to 32–MIC, mutants only selected (Sc32M) with ENR selection and were able to grow in high concentration (128 μg mL⁻¹ or greater) of FQs. Mutants could not be able to grow in broth containing 32–MIC concentration of CIP. After 5 consecutive passages in 1/2-MIC concentration, MICs of 5 FQs for mutants (Sc5) induced by ENR ranged from 8–32 μg mL⁻¹ whilst 2–8 μg mL⁻¹ for mutants (Sc5) by CIP. While MICs reached 128 μg mL⁻¹ or greater with either ENR or CIP selection after 10 consecutive passages.

Results from the above showed that the induction of antibiotic resistance was influenced by the nature of the antibiotics used in the selection assay. Resistance to FQs in M. gallisepticum induced by ENR whatever the drug concentration used for selection, developed readily and quickly in vitro within 2–3 passages. The results are in accordance with those of Zanella et al. (1998) and Gautier-Bouchard et al. (2002) who obtained a gradual increase in the concentration of enrofloxacin used for the selection of M. gallisepticum resistant mutants. It is of note that M. gallisepticum can acquire high level fluoroquinolone resistance when exposed to even 1/2MIC of ENR or CIP for 10 consecutive passages, suggesting administration of ENR or CIP under the repetitive dosing (for prophylactic purpose) in poultry farms promoting the development of resistance to FQs in M. gallisepticum. Despite the slower development of resistance in M. gallisepticum exposing to CIP, high level resistance to FQs could be obtained after multiple passages. Whatever the drug used for selection, all mutants presented cross-resistance to 5 tested Fqs but still susceptibility to erythromycin, tylosin and gentamycin which same as results from Gautier-Bouchard et al. (2002). MIC values for FQs of 7 clinical isolates of M. gallisepticum ranged from 2–32 μg mL⁻¹ which were marked higher than the MICs of those isolated from meat type turkeys reported by Gerchman et al. (2008). Resistance level to CIP were much higher than to ENR in most clinical isolates in this study, this phenotype could be raised from higher lipid solubility for CIP than ENR leading to more drug access into the cell (Riviere and Papich, 2009). Clinical isolates were cross-resistant to all tested FQs and other antibiotics except for gentamicin.

Mutations of the gyrase and topoisomerase IV genes in M. gallisepticum selected in vitro: The QRDR of GyrA, GyrB, ParC and ParE in all selected mutants in vitro and in vivo were characterized. As shown in Table 1, common mutant site in GyrA were localized at position 83 with Ser→Ile substitution and at position 87 with Gln→Gly/Gln alteration. Besides two changes, Asp82→Gly and Ile103→Val substitution were found respectively in two mutants selected in vitro. For most mutants, a Ser80→Leu change in the ParC QRDR was detected, combined with mutations in GyrA. No mutations were found in GyrB and ParE QRDR.

The study showed that the preferential target of ENR and CIP in M. gallisepticum is DNA gyrase which in accordance with Reinhardt et al. (2002a) results unlike mycoplasma hominis in which the primary target of CIP is topoisomerase IV (Bebear et al., 1998b). Differently the first and major mutation occurred at position 83 and 87 in GyrA for ENR and CIP, respectively. In the study, just one
mutation pattern (Ser83–Ile) in GyrA occurred in the selected mutants, unlike observation of Reinhardt et al. (2002b) in which 3 various alterations (Ser83–Ile/Asn/Arg) existing in M. gallisepticum mutants. Ser–Arg mutation led to greater increases in resistance than the other two substitution (Ser–Ile and Ser–Asn) due to structural differences between these three amino acids (Kim et al., 1998). In the study, substitution Ser83–Ile in GyrA associated with great increases in resistance and the linkage effect of subsequent mutation at position 80 in ParC. These two substitutions in GyrA and ParC led to 256 fold increase in the MIC of ENR and CIP for mutants Se5 and Se8M, compared with the parent strain. Additional mutation at position 82 (Asp–Gly) and 103 (Ile–Val) were responsible for high-level resistant (>1024 fold increase) for mutant Se32M and Se10, respectively. Truong et al. (1997) reported a novel mutation Asp82–Gly contributed high resistant to nalidixic acid and FQs in addition to Gly81–Asp in the QRDR of GyrA in E. coli. To the knowledge, there is no literatures on Ile103–Val mutation which involving in high level resistance to FQs in M. gallisepticum.

Mutation at position 87 in GyrA just found in mutants from CIP selection which contributed comparatively small increases in resistance (16 fold) unlike the mutation Ser–Ile at position 83 and its linkage effect. Agree with Reinhardt et al. (2002b), mutation of Ser80–Leu in the ParC QRDR plays a role in ENR resistance. In present study, substitution Gla87–Gln in GyrA combining Ser80–Leu in the ParC resulted in 64 fold increase in the MIC of ENR and CIP for mutant Se10. A additional mutation at site 83 (Ser–Ile) in GyrA seems to play a slight influence on resistance for mutant Sc16M.

Unlike plasmid-mediated bacterial resistance in which resistance may disappear after selective antibiotic pressure is removed, chromosomal (mutational) resistance exhibited by fluoroquinolone-resistant bacteria can be maintained in bacteria after drug administration is discontinued (Strahilevitz et al., 2009). From this study, use of ENR or CIP in poultry farms either for therapeutic or prophylactic purpose could readily and quickly selected fluoroquinolone-resistant M. gallisepticum and contribute to the resistance persistence in flocks.

Mutations of the gyrase and topoisomerase IV genes in M. gallisepticum selected in vivo: In 7 clinical isolates of M. gallisepticum, two mutations in GyrA and one mutation in ParC were detected which gave high-level resistance to FQs. Only Ser–Ile change at position 83 in GyrA was found which was in agreement with the mutants selected in vitro (this study) and in vivo described recently (Lysnyansky et al., 2008). In contrast, the Gla87–Gly substitution selected in two clinical isolates was found in none of the in vitro selected mutants in this study. Same as the alteration in ParC in mutants selected in vitro, only Ser–Leu alteration was detected at position 80 which combining mutation at GyrA and ParC resulted in the high-level resistance to FQs in clinical isolates of M. gallisepticum. As with the in vitro mutants of M. gallisepticum this study, none of the 7 clinical isolates was found to carry an alteration in the GyrB and ParE QRDR, unlike Lysnyansky et al. (2008) report in which two substitutions existing in GyrB. This clearly confirms the prevalence of mutation in gyrA and parC over those in gyrB and parE. The highest resistance was associated with three mutations in GyrA and ParC in one clinical isolates of M. gallisepticum in accordance with that in mutant Sc16M selected in vitro. Previous studies focused mainly on mutants selected in vitro. It is worthy to note that two isolates exhibited no mutations in any target genes or just one mutation in ParC but had high MIC profiles of the FQs tested. Additional mechanisms, like mutations elsewhere in the topoisomerase genes or modifications in drug efflux system only described in M. hominis (Raherison et al., 2002) may contribute to the resistant phenotype of these mutants.

CONCLUSION

This study shows the results generally characterized the alteration in DNA gyrase and Topoisomerase IV in fluoroquinolone-resistant mutants of M. gallisepticum obtained in vitro and in vivo. The presence of alterations of both the GyrA and ParC in clinical isolates of M. gallisepticum is in good agreement with the results from mutants selected in vitro, high-level resistance to FQs was associated with modification in both enzymes targets.

ACKNOWLEDGEMENTS

This research was supported by the The National Natural Science Foundation of China (Grant No. 30871890), the National Natural Science Foundation of China (Grant No. U1031004). Jian Li and Dian-Hong Lu contributed equally to this research.

REFERENCES


