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

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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→Ile) 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

Mycoplasm 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 (Hannan *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

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Mycoplasma 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

Becterial and growth: *Mycoplasma 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 $\leq$0.5 mg L^{-1} and resistant when the MIC was >2 mg L^{-1} according to the breakpoints given for these antibiotics (Hannan, 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 duple increasing concentration or 1/2×MIC concentration of ENR or CIP were used for *M. gallisepticum* S6. For method with duple 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 consecutives 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'-TATGGTGCTTACACTTCAG -3' and 5'-CTACGGCAATACCACTTG-3', 5'- TGACGGTAA GATTAGCAAAG-3' and 5'-ACATCAGCATCG GTCA TGA-3', 5'-AAGAATAGATGGATAAGAAA-3' and 5'-TCTCTTTGTTAATATTCTCA-3', 5'-GGTATCAAAT TACAACGAAAAAC-3' and 5'-CCACCATCTTG GTA 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 (Invitrogen, 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 $\geq$128 μg mL^{-1} for all tested FQs (Table 1) were selected after sequential passages in 1/2×MIC or esclating 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 concentratrions occured at passages 2 whilst occured 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 (Sc8M)

Table 1: MICs and mutations observed in fluoroquinolone-resistant mutants of Mycoplasma gallisepticum selected *in vitro* and *in vivo*

	MIC[b]					Amino acid (codon) change in QRDR of[c] (GyrA)				ParC
Strains[a]	ENR	CIP	SARA	DAN	OFL	82	83	87	103	80
S6	0.125	0.125	0.125	0.06	0.25	Asp	Ser	Glu	Ile	Ser
Se5	16.000	32.000	8.000	16.00	32.00	-	Ile	-	-	Leu
Se10	>128.000	128.000	>128.000	>128.00	64.00	-	Ile	-	Val	Leu
Sc5	4.000	2.000	2.000	4.00	8.00	-	-	Gln	-	-
Sc10	128.000	128.000	64.000	64.00	64.00	-	-	Gln	-	Leu
Se8M	32.000	32.000	16.000	64.00	64.00	-	Ile	-	-	Leu
Se32M	>128.000	128.000	64.000	128.00	>128.00	Gly	Ile	-	-	Leu
Sc8M	1.000	2.000	4.000	2.00	1.00	-	-	Gln	-	-
Sc16M	64.000	16.000	4.000	32.00	32.00	-	Ile	Gln	-	Leu
RC1	4.000	16.000	8.000	16.00	32.00	-	Ile	-	-	Leu
YL4	4.000	16.000	8.000	8.00	16.00	-	Ile	-	-	Leu
JC6	2.000	8.000	4.000	8.00	16.00	-	Ile	-	-	Leu
CG5	2.000	8.000	4.000	8.00	8.00	-	Ile	-	-	Leu
FL	8.000	8.000	4.000	2.00	8.00	-	-	-	-	-
HS1	4.000	4.000	2.000	1.00	4.00	-	-	Gly	-	-
HS2	16.000	8.000	8.000	16.00	8.00	-	Ile	Gly	-	-

[a]Se5 and Se10 correspond to mutants selected from S6 with 1/2×MIC enrofloxacin for 5 and 10 consecutive passages, respectively; similarily, Sc5 and Sc10 mutants obtained with 1/2×MIC ciprofloxaxin for the same passages. Se8M, Se32M, Sc8M and Sc16M corresponds to mutants selected from S6 with 8×MIC, 16×MIC and 32×MIC enrofloxacin or ciprofloxaxin, respectively. [b]ENR: Enrofloxacin; CIP: Ciprofloxacin; Sara: Sarafloxacin; Dano: Danofloxacin; Oflo: Ofloxacin; [c]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 esclated to 32×MIC, mutants only selected (Se32M) with ENR selection and were able to grow in high concentration (128 μg mL^{-1} or great) of FQs. Mutants could not be able to grow in broth containing 32×MIC concentration of CIP. After 5 consecutive passaging in 1/2×MIC concentration, MICs of 5 FQs for mutants (Se5) 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 great with either ENR or CIP selection after 10 consecutive passaging.

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-Bouchardon *et al.* (2002) who botained a gradual increase in the concentration of enrofloxacin used for the selection of *M. gallisepticum* resistant mutants. It is of noteworth that *M. gallisepticum* can acquire high level fluoroquinolone resistance when exposured to even 1/2MIC of ENR or CIP for 10 consecutive passages, suggesting administration of ENR or CIP under the rapeutic dosing (for prophylastic purpose) in poultry farms promoting the development of resistance to FQs in *M. gallisepticum*. Despite the slower development of resistance in *M. gallisepticum* exposuring to CIP, high level resistance to Fqs could be obtained after mutiple 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-Bouchardon *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 arised from higher lipid solubility for CIP than ENR leading to more drug access into the cell (Riviere and Papich, 2009). Clinical isoltes were cross-resistant to all tested FQs and other antibiotics except for gentamicin.

Mutations of the gyrase and topoisomerase IV genes in *M. galliseptucim* 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 Glu→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 resistants (>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 Glu87→Gln in GyrA combining Ser80→Leu in the ParC resulted in 64 fold increase in the MIC of ENR and CIP for mutant Sc10. 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 theraputic or prophylatic 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. galliseptucim* 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 Glu87→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 GyrA83 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.

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