**NOTE** *Bacteriology*

# Fluoroquinolone Resistance Mechanism of Clinical Isolates and Selected Mutants of *Pasteurella multocida* from Bovine Respiratory Disease in China

Ling-Cong KONG[1)], Duo GAO[1)], Yun-Hang GAO[1)], Shu-Ming LIU[1)] and Hong-Xia MA[1)]*

[1)]*College of Animal Science and Technology, Jilin Agricultural University, Xincheng Street No.#2888, Changchun 130118, P.R. China*

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ABSTRACT. The minimum inhibitory concentrations (MICs), mutation prevention concentrations (MPCs) and contribution of quinolone resistance-determining region (QRDR) mutations to fluoroquinolone (ciprofloxacin, enrofloxacin and orbifloxacin) susceptibility in 23 *Pasteurella multocida* (*Pm*) isolates were investigated. Fluoroquinolone-susceptible isolates (MICs ≤0.25 μg/m*l*, 9 isolates) had no QRDR mutations, and their respective MPCs were low. Fluoroquinolone-intermediate isolates (MICs=0.5 μg/m*l*, 14 isolates) had QRDR mutations (Asp87 to Asn or Ala84 to Pro in *gyr*A), and their respective MPCs were high (4–32 μg/m*l*). First-step mutants (n=5) and laboratory-derived highly resistant fluoroquinolone mutants (n=5) also had QRDR mutations. The MICs of fluoroquinolones for mutant-derived strains were decreased in the presence of efflux inhibitors. The results indicated that the fluoroquinolone resistance of *Pm* is mainly due to multiple target gene mutations in *gyr*A and *par*C and the overexpression of efflux pump genes.

KEY WORDS: efflux pump, fluoroquinolone resistance, *Pasteurella multocida*, plasmid, target mutation

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Recent studies have suggested that one of the important pathogens of bovine respiratory disease (BRD) that seriously harm cattle in China and other countries is *Pasteurella multocida*(*Pm*) of Serogroup A [4, 6, 10]. A *Pm* infection is treated mainly using antibiotics [5, 11]. In recent years, fluoroquinolone antibiotics including ciprofloxacin (CIP) and enrofloxacin (ENR) have been increasingly used to treat *Pm* infections in China. Fluoroquinolones at subinhibitory concentrations lead to emergence of resistant bacterial strains [13]. Hence, fluoroquinolones at subinhibitory antimicrobial concentrations pose a major risk in the treatment of BRD. Fluoroquinolone resistance has been described for *Mannheimia haemolytica* and *Mycoplasma bovis* isolates from BRD [7, 12, 14]. However, fluoroquinolones resistance of *Pm* isolates has very rarely been studied. In the present study, the potential risk of fluoroquinolone resistance of *Pm* isolates was evaluated by determining the mutation prevention concentrations (MPCs) of fluoroquinolones, mutation-mediated alterations in quinolone resistance-determining regions (QRDRs), fluoroquinolone resistance plasmid and efflux–mediated mutational changes in *Pm* isolates obtained from various cattle farms in China.

Twenty-three field isolates of *P*m (designated *Pm*1 to *Pm* 23) were obtained from cattle lungs from 23 farms located in different provinces of China during the period of 2011 to 2013. All isolates and capsule serotypes were identified as described previously [17]. Antimicrobial susceptibility testing with ENR, CIP and orbifloxacin (ORB) was performed using the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute guidelines in VET01-A4 [2]. The reference strain *Escherichia coli* ATCC 25922 served as an internal control. The method for measuring MPC values has been previously described [3]; the lowest drug concentration that prevented the emergence of mutants after a 5-day incubation period was recorded as the MPC, and the values for mutant selection windows (MSWs) were calculated. Each experiment was repeated two times. A mutant of each original strain (*Pm*1-*Pm*23) was randomly selected from plates with a concentration of ENR, CIP and ORB that was one dilution (i.e., twofold) lower than the MPC (sub-MPC). Each mutant was cultured on antimicrobial-free agar plates for 3 serial passages, and then, colonies were tested for fluoroquinolone susceptibility. The minimum inhibitory concentrations (MICs) of mutants were measured using the agar dilution method [1], and MICs of mutants higher than those of the parent isolates confirmed the existence of mutants. *In vitro*-derived highly resistant fluoroquinolone mutants of *Pm* were obtained from wild-type (*Pm*-8, *Pm*-9, *Pm*-16 and *Pm*-20) and Type II (*Pm*-3) isolates of parent strains through serial inoculations in brain heart infusion (BHI, Oxoid Ltd., Cambridge, U.K.) agar plates containing ENR at a subinhibitory concentration. The suspension of bacterial inoculums in BHI agar was adjusted to 0.5 McFarland standards. Then, 200 μ*l* of this suspension was added into a tube containing BHI medium (1,800 μ*l*/tube) with twofold serial dilutions of ENR (final concentration ranging from 0.06 to 128 μg/m*l*). The tube was visually examined for bacterial growth, and the respective MICs were noted. The bacteria with the highest MIC were harvested and dispersed (100 μ*l*/tube) in a new tube containing BHI medium (1,900 μ*l*/tube) with twofold serial

*CORRESPONDENCE TO: MA, H.-X., College of Animal Science and Technology, Jilin Agricultural University, Xincheng Street No.#2888, Changchun 130118, P.R. China.
e-mail: hongxia0731001@163.com

Table 1. QRDR mutation genotypes and fluoroquinolone MICs and MPCs for *Pm* field strains

| Mutation type | Number of strains | MIC range ($\mu$g/m*l*) | | | Substitution in | | MPC range [a] ($\mu$g/m*l*)/ MSW [b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CIP | ENR | ORB | *gyr*A | *par*C | CIP | ENR | ORB |
| Wild | 9 | 0.03–0.25 | 0.03–0.25 | 0.03–0.06 | None | None | 0.125–0.5 / 0.06–0.25 | 0.125–0.5 / 0.06–0.25 | 0.125–0.5 / 0.06–1.9 |
| I | 13 | 0.5 | 0.5 | 0.25–0.5 | Asp87 to Asn | None | 4–16 / 3.5–15.5 | 8–32 / 3.5–31.5 | 4–32 / 3.5–31.5 |
| II | 1 | 0.5 | 0.5 | 0.5 | Ala84 to Pro | None | 4/ 3.5 | 16/ 15.5 | 32/ 31.5 |

a) MPCs, mutant prevention concentrations were determined on Mueller-Hinton plates for ciprofloxacin (CIP), enrofloxacin (ENR) and orbifloxacin (ORB). b) MSW, mutant selection window (antibiotic concentration found between the minimum inhibitory concentration and MPC).

Table 2. QRDR mutation genotypes and fluoroquinolone MICs in *Pm*-derived strains

| Mutation type | Number of strains | MIC range ($\mu$g/m*l*) | | | | Substitution (s) in | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CIP | ENR | ORB | CIP+ CCCP | *gyr*A | *gyr*B | *par*C | *par*E |
| III[a] | 1 | 4 | 2 | 1 | 2 | Asp87 to Asn | Pro415 to Thr | Glu84 to Lys | None |
| IV[a] | 4 | 8–16 | 8–16 | 4–8 | 2–4 | Ser83 to Ile Asp87 to Asn | None | Ser80 to Leu | None |
| V[b] | 2 | 128 | 256 | 128 | 64 | Ser83 to Ile Asp87 to Asn | Asp426 to Asn | Glu84 to Lys | None |
| VI[b] | 3 | 128 | 64–256 | 64–128 | 16–64 | Ser83 to Ile Asp87 to Asn | None | Ser80 to Leu Glu84 to Lys | None |

a) First-step mutant strains. b) Highly-resistant mutant strains selected using ENR.

dilutions of ENR as described above (final concentration was 128 $\mu$g/m*l*). Then, the mutant's colonies were randomly picked up and subjected to MIC determination. The mutant colonies were then subcultured (seven subcultures) in antibiotic-free BHI medium to assess the stability of the mutant strains. The primers of QRDRs of *gyr*A (F, 5′-CCTTATCGTAAATCCGCTCGTA-3′; R, 5′-CGCAGGGACTTTGGTTGGGAG-3′), gyrB (F, 5′-GAAATGACCCGCCGTAA-3′; R, 5′-CTTGCCTTTCTTCACTTTGTA-3′), *par*C (F, 5′-TACGAAGGCATTGAACAAACCC-3′; R, 5′-CACTGTCCCTTGCCCTAACTC-3′) and *par*E (F, 5′-CTAAAAGATTTGGAGCCAGTG-3′; R, 5′-TTATACGGCTAAATCCACCTGT-3′) were designed according to the published GenBank deoxyribonucleic acid sequences (accession No. CP003022.1). Protocols for amplification of *qnr*A, *qnr*B, *qnr*C, *qnr*D, *qep*A, *oqx*A, *oqx*B and *aac*(6')-Ib genes have been previously described [16]. The MIC of CIP was determined in the presence of potent efflux inhibitors, carbonyl cyanide m-chlorophenyl hydrazones (CCCPs) at appropriate concentrations (MIC/2) using the broth microdilution method. The *Pm* strains with a fourfold or greater reduction in their MICs in the presence of inhibitors were considered positive for CCCP efflux [15]. Each experiment was repeated three times.

The QRDR mutation types and fluoroquinolone MICs and MPCs for *Pm* isolates are shown in Table 1. Nine (39.1%) isolates (wild type) were susceptible, and the remaining 14 (60.9%) isolates (Type I, Asp87 to Asn in *gyr*A; Type II, Ala84 to Pro in *gyr*A) were intermediate to fluoroquinolones. These may be the first-step mutations in the *Pm* isolates resistant to fluoroquinolones. The fluoroquinolone MICs for Type I and II isolates were 8- to 16-fold higher than those of the wild type, and fluoroquinolone MPCs for Type I and II isolates were 32- to 256-fold higher than those of the wild type. A previous study suggested that wild-type strains had lower mutation frequencies compared with single-mutation strains [8].

The first-step mutant colonies were randomly picked up after exposure to sub-MPC concentrations; however, only five stable mutant strains were obtained from parent strains of Type I. It was easier to get first-step mutants compared with isolates with no mutations. The results are shown in Table 2. These first-step mutants had two QRDR mutation types (Type III, Asp87 to Asn in *gyr*A, Pro415 to Thr in *gyr*B and Glu84 to Lys in *par*C; Type IV, Ser83 to Ile and Asp87 to Asn in *gyr*A and Ser80 to Leu in *par*C). Five highly fluoroquinolone-resistant mutants derived from wild-type and Type II isolates had two QRDR mutation types (Type V, Ser83 to Ile and Asp87 to Asn in *gyr*A and Glu84 to Lys in *par*C; Type VI, Ser83 to Ile and Asp87 to Asn in *gyr*A and Ser80 to Leu and Glu84 to Lys in *par*C). In short, amino acid substitutions at codon 83 or codon 87 in *gyr*A and codon 80 or codon 84 in *par*C play an important role in first-step mutant strains. Similarly, the present study results showed that the highly resistant *Pm* mutants *in vitro* had double mutations in *gyr*A (codon 83 and 87) and single mutations in *par*C (codon 84), which played a role in conferring a higher level of resistance to fluoroquinolones. These amino acid substitutions have been described already in previous reports, which showed amino acid substitutions in *gyr*A (codon 83, codon 84 and codon 87) and *par*C (codon 80 and codon 84) [15, 18]. In addition, although the mutants were obtained from parent strains of Type II (Ala84 to Pro), the mutants had a substitution of Asp87 to Asn in *gyr*A (Types III to VI). This amino acid substitution may be reversible; however, the molecular basis of reversible actions is poorly understood.

Although plasmid-mediated quinolone resistance genes

were not detected in any isolate, the *aac*(6')-Ib gene was detected. The results of a previous study suggested that strains harboring a *aac*(6')-Ib gene variant (*aac*6'-Ib-cr) showed higher quinolone resistance [9].

The mutant-derived strains were tested for CCCP-sensitive efflux. The MICs of CIP were determined using agar dilution in the presence and absence of CCCP (4 $\mu$g/ml, MIC/2). The results indicated that the MIC of CIP was decreased in the presence of CCCP in mutant-derived strains. These results indicated that efflux pump genes might have played a role in the quinolone resistance of *Pm* mutants selected *in vitro*. The results are shown in Table 2. Sequence analysis of genome sequences of *Pm*(accession No. CP003022.1) showed that some genes coded for an efflux protein. However, the role of these proteins is uncertain, and this should be further investigated in future studies.

In conclusion, there are risks associated with the use of fluoroquinolone for *Pm* infections in cattle in China. The present study results suggested that for infections involving *Pm* with high MPCs, especially those containing mutations in *gyr*A and *par*C genes, treatment with a combination of antimicrobials should be adopted.

*Nucleotide sequence accession numbers*: The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence databases with the following accession numbers:#KM111304-KM111336 (*gyr*A), #KM111337-KM111369 (*gyr*B), #KM111370-KM111402 (*par*C), #KM111403-KM111435 (*par*E) and #KM111436-KM111468 (*aac*6'-Ib).

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