Fluoroquinolone Resistance Mechanism of Clinical Isolates and Selected Mutants of *Pasteurella multocida* from Bovine Respiratory Disease in 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/ml, 9 isolates) had no QRDR mutations, and their respective MPCs were low. Fluoroquinolone-intermediate isolates (MICs=0.5 µg/ml, 14 isolates) had QRDR mutations (Asp87 to Asn or Ala84 to Pro in gyrA), and their respective MPCs were high (4–32 µg/ml). 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 gyrA and parC and the overexpression of efflux pump genes.

**KEYWORDS:** 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, mutagen-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 *Pm* (designated Pm1 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 (Pm1-Pm23) 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 µl of this suspension was added into a tube containing BHI medium (1,800 µl/tube) with twofold serial dilutions of ENR (final concentration ranging from 0.06 to 128 µg/ml). 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 µl/tube) in a new tube containing BHI medium (1,900 µl/tube) with twofold serial
dilutions of ENR as described above (final concentration was 128 μg/ml). 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 fluoroquinolone-resistant mutants derived from wild-type 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 gyrA, 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 the wild type. A previous study suggested that wild-type strains had lower mutation frequencies compared with single-mutation strains [8].

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

| Mutation type | Number of strains | MIC range (μg/ml) | Substitution in gyra | MPC range (μg/ml) |
|---------------|------------------|-------------------|---------------------|-------------------|
|               |                  | CIP | ENR | ORB | CIP | ENR | ORB |
| Wild          | 9                | 0.03–0.25 | 0.03–0.25 | 0.03–0.06 | None | None | 0.125–0.5 |
| I             | 13               | 0.5  | 0.5  | 0.25–0.5 | Asp87 to Asn | None | 4–16 |
| II            | 1                | 0.5  | 0.5  | 0.5   | Ala84 to Pro | None | 4/ |

- 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 (μg/ml) | Substitution in | gyra | gyrB | parC | parE |
|---------------|------------------|-------------------|----------------|-----|-----|-----|-----|
|               |                  | CIP | ENR | ORB | CIP | ENR | ORB | CIP | ENR | ORB | CIP | ENR | ORB | CIP | ENR | ORB |
| III a)        | 1                | 4   | 2   | 1   | 2   | None | None | 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 | Asp262 to Asn | Glu84 to Lys | None |
| VII 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.
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 (aac6′-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 µg/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 gyrA and parC 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 (gyrA), #KM111337-KM111369 (gyrB), #KM111370-KM111402 (parC), #KM111403-KM111435 (parE) and #KM111436-KM111468 (aac6′-Ib).

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