Characteristics of quinolone-resistant *Escherichia coli* isolated from bovine mastitis in China

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ABSTRACT

Escherichia coli is the leading causative agent of bovine mastitis worldwide. Quinolone-resistant *E. coli* is becoming a potential threat to veterinary and public health. The aim of this study was to investigate the characteristics of quinolone-resistant *E. coli* isolated from bovine mastitis cases in China. Antimicrobial susceptibility of the isolates against 15 antimicrobial agents was determined by disc diffusion method. Phylogenetic grouping was detected by PCR. Extended-spectrum β -lactamase-producing isolates were determined by double-disc synergy test. In addition, the plasmid-mediated quinolone resistance (PMQR) and β -lactamase-encoding genes, as well as mutations of quinolone resistance-determining regions in GyrA, GyrB, ParC, and ParE, were measured by PCR and DNA sequencing. Overall, 75 (22.9%) out of 328 *E. coli* isolates were confirmed as ciprofloxacin-resistant from 2,954 mastitic milk samples. Phylogenetic group analysis showed that the majority of these strains belonged to phylogenetic group A (57.3%) and group B1 (24.0%). All the resistant isolates were identified as multidrug resistant, showing high resistance to cephalosporins and non- β -lactams. Forty-nine (65.3%) of the quinolone-resistant isolates were positive for PMQR genes; *aac*-(6')-Ib-cr was the most common PMQR determinant detected in 33 (44.0%) isolates. Eighteen (24.0%), 4 (5.3%), 3 (4.0%), and 1 (1.3%) of the quinolone-resistant isolates were harboring *oqx*A/B, *qep*A4, *qnr*S, and *qnr*B2, respectively. Additionally, 55 (73.3%) of the quinolone-resistant *E. coli* isolates were found to be extended-spectrum β -lactamase producers. The preponderant β -lactamase-encoding gene, *bla*_{TEM}, was detected in 44 (58.7%) isolates; *bla*_{CTX-M}, *bla*_{CMY}, and *bla*_{SHV} were found in 35 (46.7%), 22 (29.3%), and 2 (2.7%) isolates, respectively. Moreover, the most

frequently identified substitutions were S83L/D87N or S83L in GyrA, detected in all of the quinolone-resistant isolates. Meanwhile, 74 (98.7%), 33 (44.0%), and 6 (8.0%) of the isolates were carrying substitutions S80I in ParC, S458A in ParE, and S492N in GyrB, respectively. All 58 (77.3%) isolates with a high level of ciprofloxacin resistance ($>32 \mu\text{g/mL}$) carried single or double mutations in GyrA combined with single mutation in ParC. To the best of our knowledge, this is the first report on the high occurrence of PMQR determinants and quinolone-determining resistant regions mutations in quinolone-resistant *E. coli* isolated from bovine mastitis in China.

Key words: *Escherichia coli*, plasmid-mediated quinolone resistance, quinolone resistance-determining regions, bovine mastitis

INTRODUCTION

Bovine mastitis is the most prevalent and costly disease of dairy industry worldwide (Yang et al., 2016). Although mastitis can be caused by a variety of bacterial pathogens, *Escherichia coli* is one of the major causes (Zhang et al., 2016). Antibiotic therapy is the mainstay of treatment for this disease; however, the therapeutic effectiveness is limited due to the increasing incidence of antibiotic-resistant infections (Dias et al., 2013). Quinolones are potent antimicrobial agents for treatment of various infections caused by *E. coli* in veterinary medicine. Unfortunately, quinolone-resistant *E. coli* in food-producing animals have increased in China because of extensive usage of quinolones (Wang et al., 2008; Xiao et al., 2011; Zhao et al., 2014).

Quinolone target enzymes include DNA gyrase (GyrA and GyrB) and DNA topoisomerase IV (ParC and ParE). Both enzymes are necessary for bacterial DNA replication. Mutations in the chromosomal genes that encode quinolone target enzymes DNA gyrase or topoisomerase IV make the enzymes less sensitive to inhibition by quinolones. Multiple mutations causing AA changes in the quinolone resistance-determining regions (QRDR) of the target enzymes is the most common

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mechanism of high-level quinolone resistance in clinical strains (Redgrave et al., 2014; Correia et al., 2017).

Quinolone resistance increased by harboring transferable plasmid-mediated quinolone resistance (PMQR) determinants is low compared with mutations in chromosomal genes. However, PMQR genes can transfer between bacterial interspecies at a higher frequency than chromosomal mutations, making their spread much faster than the occurrence of other types of antimicrobial resistance (Kao et al., 2016). In addition, co-resistance to β -lactams and quinolones is a common phenomenon due to the presence of β -lactamases and substitutions in QRDR, with or without PMQR genes (Bajaj et al., 2016). To date, 3 types of PMQR genes have been described: the *qnr* genes coding for pentapeptide to protect target enzymes DNA gyrase and topoisomerase IV from quinolone inhibition; genes *qepA* and *oqxAB* coding for transporters to remove antibiotics from bacterial cells; and the gene *aac-(6')-Ib-cr* coding for aminoglycoside acetyltransferase to acetylate several fluoroquinolones (Strahilevitz et al., 2009).

Although quinolone-resistant *E. coli* was reported worldwide, isolates from diseased food-producing ani-

mals in China were limited, especially from bovine mastitis cases. The aim of our study was to characterize the quinolone-resistant *E. coli* isolated from bovine mastitis cases in China.

MATERIALS AND METHODS

Sample Collection and Selective Cultivation of *E. coli*

Mastitic milk samples of cows ($n = 2954$) were obtained from 99 commercial dairy herds located in 19 provinces of China during August 2013 to September 2017 (Figure 1). After delivery to the laboratory, milk samples were inoculated onto MacConkey agar (Oxoid, Thermo Fisher Scientific, Waltham, MA) and cultivated at 37°C for 18 to 24 h. Colonies with the dark pink to red colors were further confirmed by the Vitek system (BioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations (<http://www.biomerieux-industry.com/veterinary-diagnostics/vitek-2-technology>). Ciprofloxacin susceptibility of *E. coli* isolates were determined by E-test (BioMérieux) method (0.002 to 32 μ g/mL) on Mueller-Hinton agar

Figure 1. Sampling areas (provinces in China) involved in this study.

Table 1. Antimicrobial susceptibility patterns of 75 quinolone-resistant *Escherichia coli* isolates from bovine mastitis cases

Antibiotic	Concentration (μg)	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	20	2.7	0.0	97.3
Amoxicillin/clavulanic acid	20/10	48.0	37.3	14.7
Cefazolin	30	14.7	8.0	77.3
Cefepime	30	70.7	4.0	25.3
Cefuroxime	30	28.0	2.7	69.3
Ceftazidime	30	64.0	6.7	29.3
Ceftizoxime	30	52.0	14.7	33.3
Cefpodoxime	10	28.0	2.7	69.3
Meropenem	30	100.0	0.0	0.0
Gentamicin	10	13.3	0.0	86.7
Tetracycline	30	5.3	0.0	94.7
Ofloxacin	5	0.0	0.0	100.0
Levofloxacin	10	0.0	0.0	100.0
Trimethoprim/sulfamethoxazole	1.25/23.75	12.0	0.0	88.0
Chloramphenicol	30	24.0	1.3	74.7

(Oxoid). A total of 75 ciprofloxacin-resistant isolates were identified according to the CLSI (2013) criteria.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility to 15 antimicrobial agents were determined by the disc diffusion method according to the recommendations of CLSI (2013). The panel of antimicrobial agents were list in Table 1. *Escherichia coli* ATCC 25922 and *E. coli* ATCC 35218 were used as quality control strains. Multidrug resistant (MDR) *E. coli* was defined as isolates that were resistant to at least 3 classes of the tested antimicrobial agents (Magiorakos et al., 2012).

Detection of the Phylogenetic Group of *E. coli* Isolates

Phylogenetic grouping of *E. coli* isolates was performed by PCR, as previously described (Clermont et al., 2013). Specific primers used were described in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-14156>). The PCR-amplified products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Strains were assigned to 1 of 7 phylogenetic groups A, B1, B2, C, D, E, and F, on the basis of presence and absence of corresponding DNA fragments.

Detection of ESBL-Producing *E. coli*

The ESBL-producing *E. coli* isolates were first screened by ChromID ESBL agar (BioMerieux) according to the manufacturer's recommendation (<http://www.biomerieux-diagnostics.com/chromidr-esbl>). Each

E. coli isolate was aerobically incubated on ChromID ESBL agar at 37°C for 18 to 24 h. Bacterial colonies showing pink to burgundy color on the agar were interpreted as ESBL-suspected strains. The presumptive ESBL-producing strains were further confirmed by double-disc synergy test with cefotaxime (30 μg) and ceftazidime (30 μg) alone and in combination with clavulanic acid (10 μg) as recommended by CLSI (2013).

Characterization of Antimicrobial Resistance Genes

The DNA of all 75 quinolone-resistant *E. coli* were extracted by the Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's recommendation (<http://omegabiotek.com/store/product/bacterial-dna-kit/>). The PMQR genes [*aac(6')-Ib-cr*, *oqx*A, *oqx*B, *qep*A, *qnr*A, *qnr*B, *qnr*C, *qnr*D, and *qnr*S] and β-lactamase -encoding genes (*bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{DHA}, and *bla*_{CMY}) were detected by PCR amplification with specific primers (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14156>). Subsequently, DNA sequencing was carried out on PMQR and β-lactamase genes, and the DNA sequences were compared with genes in the GenBank database using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the subtypes of antimicrobial resistance genes.

Detection of QRDR Mutations in Quinolone-Resistant *E. coli* Isolates

The QRDR mutations in GyrA, GyrB, ParC, and ParE genes were examined by PCR and sequencing using primers listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-14156>). The PCR-amplified

Table 2. Distribution of plasmid-mediated quinolone resistance (PMQR) genes and β -lactamase genes in 75 quinolone-resistant *Escherichia coli* isolates from bovine mastitis cases

Resistant determinant	No. of isolates
PMQR genes	
<i>aac(6')-Ib-cr</i>	33
<i>oqxA/B</i>	18
<i>qepA</i>	4 (<i>qepA4</i>)
<i>qnrB</i>	1 (<i>qnrB2</i>)
<i>qnrS</i>	3 (<i>qnrS1</i>)
<i>oqxA</i> + <i>oqxB</i> + <i>qepA</i>	1
<i>aac(6')-Ib-cr</i> + <i>oqxA</i> + <i>oqxB</i> + <i>qnrS</i>	1
<i>aac(6')-Ib-cr</i> + <i>oqxA</i> + <i>oqxB</i> + <i>qnrB</i> + <i>qnrS</i>	1
β -lactamase genes	
<i>bla</i> _{TEM}	44 (<i>bla</i> _{TEM-1})
<i>bla</i> _{CTX-M}	35 (<i>bla</i> _{CTX-M-15} , 21; <i>bla</i> _{CTX-M-55} , 14)
<i>bla</i> _{SHV}	2 (<i>bla</i> _{SHV-12})
<i>bla</i> _{CMY}	22
<i>bla</i> _{TEM} + <i>bla</i> _{CMY}	16
<i>bla</i> _{CTX-M} + <i>bla</i> _{CMY}	13
<i>bla</i> _{CTX-M} + <i>bla</i> _{SHV}	1
<i>bla</i> _{TEM} + <i>bla</i> _{CTX-M} + <i>bla</i> _{CMY}	10
<i>bla</i> _{TEM} + <i>bla</i> _{CTX-M} + <i>bla</i> _{SHV}	1

products were sequenced and AA mutations were evaluated using the control strain *E. coli* MG1655 (NC_000913.2) as a reference.

Analysis of Correlation

The correlation between antimicrobial resistance genes was assessed by one-tailed Pearson correlation (SPSS 17.0, SPSS Inc., Chicago, IL). *P*-value smaller than 0.05 was considered statistically significant.

RESULTS

Antimicrobial Susceptibility of Quinolone-Resistant *E. coli*

Overall, 328 (11.1%) *E. coli* isolates were obtained from 2,954 mastitic milk samples from 19 provinces of China during August 2013 to September 2017, of which 75 isolates (22.9%) were identified as ciprofloxacin-resistant by the E-test method. The antimicrobial susceptibility of the 75 resistant isolates against 15 antimicrobial agents was shown in Table 1. All isolates were resistant to ofloxacin and levofloxacin. The majority of isolates were resistant to ampicillin (97.3%), tetracycline (94.7%), trimethoprim-sulfamethoxazole (88.0%), gentamicin (86.7%), cefazolin (77.3%), chloramphenicol (74.7%), cefuroxime (69.3%), and cefpodoxime (69.3%). However, we observed high susceptibility to meropenem (100.0%), cefepime (70.7%), ceftazidime (64.0%), and ceftizoxime (52.0%). Moreover, all the isolates were found to be MDR.

Phylogenetic Group of Quinolone-Resistant *E. coli*

The phylogenetic group analysis showed 4 groups in 75 quinolone-resistant *E. coli* isolates. 57.3% of the quinolone-resistant isolates belonged to phylogenetic group A, 24.0% to group B1, 9.3% to group B2, and 9.3% belonged to group D.

Characterization of Antimicrobial Resistance Genes

The frequency of PMQR genes [*aac(6')-Ib-cr*, *qepA*, *oqxA*, *oqxB*, and *qnr* alleles] were determined by PCR and sequencing (Table 2). The results showed that 49 quinolone-resistant isolates (65.3%) carried at least 1 PMQR gene. Genes *aac(6')-Ib-cr* and *oqxA/B* were found in 44.0 and 24.0% of the isolates, respectively; *qepA4*, *qnrB2*, and *qnrS1* were identified in 5.3, 1.3, and 4.0% of the isolates, respectively. Notably, 1 isolate harbored 5 PMQR genes [*aac(6')-Ib-cr*, *oqxA*, *oqxB*, *qnrB2*, and *qnrS1*] in combination, 1 harbored 4 PMQR genes [*aac(6')-Ib-cr*, *oqxA*, *oqxB*, and *qnrS1*], and 1 harbored 3 PMQR genes (*oqxA*, *oqxB*, and *qepA4*). The *qnr* alleles, including *qnrA*, *qnrC*, *qnrD*, and *qnrVC*, were not found in any of the isolates.

The double-disc synergy test revealed that 73.3% of the *E. coli* isolates were ESBL producers. The frequency of β -lactamase-encoding genes among the 75 quinolone-resistant isolates are shown in Table 2. The dominant β -lactamase gene was *bla*_{TEM} (58.7%), followed by *bla*_{CTX-M} (46.7%), *bla*_{CMY-2} (29.3%), and *bla*_{SHV} (2.7%). Sequence analysis revealed that all 44 *bla*_{TEM} type β -lactamase were identified as *bla*_{TEM-1}, and 21 *bla*_{CTX-M-15} and 14 *bla*_{CTX-M-55} were confirmed among the

35 *bla*_{CTX-M} type β-lactamase, respectively. The 2 *bla*_{SHV} genes were identified as *bla*_{SHV-12}. Moreover, the coexistence of *bla*_{TEM} and *bla*_{CMY} (21.3%) were most frequently observed, followed by *bla*_{CTX-M} and *bla*_{CMY} (17.3%) and *bla*_{CTX-M} and *bla*_{SHV} (1.3%). Notably, 14.7% of the isolates carried 3 β-lactamase genes (*bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{CMY}, 13.3%; *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{SHV}, 1.3%) in combination.

The most abundant antimicrobial resistance genes found in our study were PMQR genes *aac*(6′)-*Ib-cr*, *oqx*A, and *oqx*B, as well as the β-lactam resistance genes *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{CMY}. The correlation between these genes showed that PMQR genes *oqx*A and *oqx*B were significantly (*P* < 0.05) correlated. Moreover, significant correlations (*P* < 0.05) were found between *aac*(6′)-*Ib-cr* and *bla*_{CMY} (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-14156>).

QRDR Mutations in Quinolone-Resistant *E. coli* Isolates

Mutations of QRDR in GyrA, GyrB, ParC, and ParE of the 75 quinolone-resistant *E. coli* isolates were determined by PCR and sequencing (Table 3). The results showed that no isolate with wild-type GyrA was observed. The most common point mutations in the strains were S83L/D87N (88.0%) and S83L (12.0%) in GyrA, followed by S80I (98.7%) in ParC, S458A (44.0%) in ParE, and S492N (8.0%) in GyrB. All the isolates (77.3%) with high-level ciprofloxacin resistance (>32 μg/mL) carried single or double mutations in GyrA combined with a single mutation in ParC.

DISCUSSION

Quinolones are important antimicrobial agents widely used in veterinary and human medicine. In the past few years, quinolone resistance in gram-negative bacteria has been on the rise worldwide, particularly in *E. coli* (Robicsek et al., 2006; Xiao et al., 2011; Machuca et al., 2017). We reported a similar occurrence (22.9%) of quinolone-resistant *E. coli* among 328 *E. coli* from bovine mastitis cases, as compared with previous studies about dairy cattle suffering mastitis (22.4%) and endometritis (25.0%) in China (Wang et al., 2008; Zhao et al., 2014). However, our result was much higher than data from developed countries, where the reported prevalence of quinolone-resistant *E. coli* in bovine mastitis cases were 0.7% for Finland and 0.0% for both Canada and the United States (Suojala et al., 2011; Saini et al., 2012; Metzger and Hogan, 2013). Xiao et al. (2011) presumed that widespread addition of quinolones to animal feed was associated with the high rates of quinolone resistance in China. In China, quinolones such as ciprofloxacin are permitted to be used for antimicrobial therapy in veterinary medicine (MOA 2013); however, use of quinolones in livestock has already been limited or banned in the developed countries (Pol and Ruegg, 2007; Swinkels et al., 2015; Kuipers et al., 2016). Thus, the disparity in quinolone resistance between isolates from China as compared with other countries may indicate that long-term and widespread use of this class of antimicrobials in livestock has contributed to a greater prevalence of antimicrobial resistance, at least among *E. coli* isolates. Moreover, our results found that all quinolone-resistant

Table 3. Minimum inhibitory concentrations, plasmid-mediated quinolone resistance (PMQR) genes, and quinolone resistance-determining regions (QRDR) mutations of 75 quinolone-resistant *Escherichia coli* isolates

Isolate	Ciprofloxacin MIC (μg/mL)	PMQR genes	QRDR mutations ¹			
			GyrA	GyrB	ParC	ParE
CR1	6	<i>aac</i> (6′) <i>Ib-cr</i>	S83L,D87N	—	S80I	—
CR 2	6	<i>aac</i> (6′) <i>Ib-cr</i>	S83L,D87N	—	S80I	—
CR 3	>32	<i>aac</i> (6′) <i>Ib-cr</i> , <i>oqx</i> A/B	S83L,D87N	—	S80I	—
CR 4	>32	— ²	S83L,D87N	—	S80I	S458A
CR 5	>32	<i>qep</i> A ₄	S83L,D87N	—	S80I	S458A
CR 6	8	<i>oqx</i> A/B	S83L,D87N	—	S80I	—
CR 7	>32	<i>oqx</i> A/B	S83L	—	S80I	—
CR 8	>32	<i>aac</i> (6′) <i>Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 9	>32	—	S83L,D87N	S492N	S80I	—
CR 10	6	—	S83L,D87N	—	S80I	—
CR 11	>32	—	S83L,D87N	S492N	S80I	—
CR 12	>32	<i>aac</i> (6′) <i>Ib-cr</i>	S83L,D87N	—	S80I	—
CR 13	>32	<i>oqx</i> A/B, <i>qep</i> A ₄	S83L,D87N	—	S80I	—
CR 14	>32	<i>oqx</i> A/B	S83L,D87N	—	S80I	—

Continued

Table 3 (Continued). Minimum inhibitory concentrations, plasmid-mediated quinolone resistance (PMQR) genes, and quinolone resistance-determining regions (QRDR) mutations of 75 quinolone-resistant *Escherichia coli* isolates

Isolate	Ciprofloxacin MIC (µg/mL)	PMQR genes	QRDR mutations ¹			
			GyrA	GyrB	ParC	ParE
CR 15	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	—
CR 16	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	—
CR 17	>32	—	S83L,D87N	—	S80I	—
CR 18	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 19	8	—	S83L,D87N	—	S80I	—
CR 20	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	S458A
CR 21	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	S458A
CR 22	>32	—	S83L,D87N	—	S80I	S458A
CR 23	4	—	S83L,D87N	—	S80I	—
CR 24	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 25	>32	—	S83L,D87N	—	S80I	S458A
CR 26	6	—	S83L	S492N	S80I	—
CR 27	>32	—	S83L,D87N	—	S80I	S458A
CR 28	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	—
CR 29	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	—
CR 30	>32	—	S83L,D87N	—	S80I	—
CR 31	4	—	S83L	—	S80I	—
CR 32	>32	—	S83L,D87N	—	S80I	—
CR 33	>32	—	S83L	—	S80I	S458A
CR 34	>32	—	S83L,D87N	—	S80I	—
CR 35	>32	—	S83L	—	S80I	S458A
CR 36	>32	—	S83L	—	S80I	S458A
CR 37	>32	—	S83L,D87N	—	S80I	S458A
CR 38	>32	—	S83L,D87N	—	S80I	S458A
CR 39	>32	<i>qepA4</i>	S83L,D87N	—	S80I	S458A
CR 40	>32	<i>aac(6')Ib-cr</i>	S83L	—	S80I	S458A
CR 41	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 42	>32	<i>aac(6')Ib-cr, oqxA/B</i>	S83L,D87N	—	S80I	—
CR 43	>32	<i>oqxA/B</i>	S83L,D87N	—	S80I	S458A
CR 44	>32	—	S83L,D87N	—	S80I	S458A
CR 45	>32	—	S83L,D87N	—	S80I	S458A
CR 46	6	<i>oqxA/B</i>	S83L	S492N	—	—
CR 47	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 48	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 49	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 50	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 51	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 52	>32	—	S83L,D87N	—	S80I	—
CR 53	>32	—	S83L,D87N	—	S80I	—
CR 54	>32	—	S83L,D87N	—	S80I	—
CR 55	8	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 56	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 57	8	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 58	12	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 59	6	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 60	>32	<i>aac(6')Ib-cr, oqxA/B</i>	S83L,D87N	—	S80I	—
CR 61	>32	<i>aac(6')Ib-cr, oqxA/B</i>	S83L,D87N	—	S80I	S458A
CR 62	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 63	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 64	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	S492N	S80I	—
CR 65	8	<i>aac(6')Ib-cr, oqxA/B, qnrB2, qnrS1</i>	S83L	—	S80I	S458A
CR 66	>32	<i>aac(6')Ib-cr, oqxA/B, qnrS1</i>	S83L,D87N	—	S80I	—
CR 67	6	<i>aac(6')Ib-cr, oqxA/B</i>	S83L,D87N	—	S80I	—
CR 68	>32	<i>aac(6')Ib-cr, oqxA/B</i>	S83L,D87N	—	S80I	S458A
CR 69	>32	<i>qepA4</i>	S83L,D87N	—	S80I	—
CR 70	>32	<i>qnrS1</i>	S83L,D87N	—	S80I	—
CR 71	6	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	—
CR 72	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 73	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 74	>32	<i>aac(6')Ib-cr</i>	S83L,D87N	—	S80I	S458A
CR 75	8	—	S83L,D87N	S492N	S80I	—

¹QRDR of *E. coli* MG1655 (NC_000913.2) as a wild-type reference.²Isolates with no PMQR genes or QRDR mutations.

E. coli isolates were MDR, which is in accordance to the previous studies in China (Zhang et al., 2012; Ali et al., 2016). The *E. coli* isolates showed high resistance to ampicillin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, cefazolin, chloramphenicol, cefuroxime, and cefpodoxime, agreeing with previous studies in China and other countries (Lei et al., 2010; Jones-Dias et al., 2013; Begum et al., 2016). The high-level resistance among the *E. coli* isolates may reflect the general use of antimicrobials in dairy cows in China (Swinkels et al., 2015). Thus, governmental agencies should pay more attention to critically supervise antibiotics usage, as the frequent occurrence of resistant isolates pose a serious threat to public health through the food chain (Kilani et al., 2015).

The phylogenetic group B2 and group D were the most common virulent extraintestinal *E. coli* in many countries, whereas most commensal strains belong to groups A and B1 (Clermont et al., 2000). Our results showed that most of the quinolone-resistant *E. coli* isolates belonged to groups A (57.3%) and B1 (24.0%). Similar results were reported that *E. coli* isolates associated with mastitis mainly belonged to groups B1 and A in China (Liu et al., 2014; Ali et al., 2016); these studies have given us a better understanding of the importance of environmental *E. coli* in bovine mastitis cases.

We found that 65.3% of quinolone-resistant *E. coli* isolates contained PMQR genes. The dominant PMQR genes among these strains were *aac(6')-Ib-cr* (44.0%) and *oqxA/B* (24.0%), whereas *qepA*, *qnrB* and *qnrS* were detected with lower frequencies. Similar reports regarding PMQR genes have been published in previous studies (Zurfluh et al., 2014; Zhao et al., 2015; Osei Sekyere and Amoako, 2017). These findings suggest that *aac(6')-Ib-cr* and *oqxA/B* play a major role in plasmid-mediated quinolone resistance in *E. coli* from bovine mastitis in China.

β -Lactamase genes are frequently found together with quinolone resistance genes (Schultsz and Geerlings 2012). Our results showed that 73.3% of the quinolone-resistant *E. coli* isolates were identified as ESBL producers. In addition, the predominant β -lactamase gene in the quinolone-resistant isolates was *bla*_{TEM} (58.7%), followed by *bla*_{CTX-M} (46.7%), *bla*_{CMY} (29.3%), and *bla*_{SHV} (2.7%). These findings agree with other contemporary studies that also reported these β -lactamase genes were frequently observed in quinolone-resistant *E. coli* isolates (Ma et al., 2009; Park et al., 2012; Yan et al., 2017). Moreover, our results were in accord with previous studies and confirmed that CTX-M-15 was the most common CTX-M β -lactams in *E. coli* from bovine mastitis (Ohnishi et al., 2013; Ali et al., 2016; Tark et al., 2017). The correlation analysis between PMQR genes and β -lactamase genes showed only *aac(6')-Ib-cr*

and *bla*_{CMY} were significant correlated, which suggest that the co-existence of resistance to quinolones and β -lactams is not yet epidemic in bovine mastitis cases in China.

Strains of food-producing animals with mutations in DNA gyrase (GyrA and GyrB) and DNA topoisomerase IV (ParC and ParE) have been reported worldwide (Röderova et al., 2017). Quinolone resistance arises initially through mutations of GyrA, and additional mutation of ParC leads to highly resistant *E. coli*. Topoisomerase IV acts as a secondary target (Pan and Fisher, 1996). Our results were in line with previous studies that reported point mutations in quinolone-resistant *E. coli* happened more frequently in S83L/D87N (88.0%) and S83L (12.0%) in GyrA and S80I (98.7%) in ParC (Yue et al., 2008; Morgan-Linnell et al., 2009; Bansal and Tandon, 2011; Hu et al., 2017). Moreover, in our study, all quinolone-resistant isolates with high-level ciprofloxacin resistance (>32 mg/L) carried single or double mutations in GyrA combined with single mutation in ParC, as described previously (Röderova et al., 2017). These findings indicate that mutations in GyrA and ParC may contribute to the high-level of quinolone resistance in *E. coli* isolated from bovine mastitis cases in China, and the specific mechanisms should be studied further.

Zhao et al. (2014) reported that P385A substitution of GyrB was found in all resistant isolates from dairy cattle in China. However, in our study, only 8.0% of the *E. coli* strains carried substitutions of GyrB at position S492N. Nevertheless, similar substitution of GyrB was observed in ESBL- or AmpC-producing *E. coli* from India, although mutations in GyrB subunit of DNA gyrase in clinical *E. coli* had a less significant association with quinolone resistance (Bajaj et al., 2016). Similar to our study, lines of investigations had reported the substitutions of ParE at position S458A in clinical *E. coli* isolates (Liu et al., 2012; Zhao et al., 2014; Zurfluh et al., 2014; Del Rio-Avila et al., 2016). Likewise, we found all of the substitutions of ParE at position S485A (44.0%) to be present in high levels of ciprofloxacin-resistant strains combined with substitutions in GyrA and ParC. These findings suggest that the substitution at position 458 of ParE appears to confer high-level quinolone resistance. However, the contribution of this mutation needs to be investigated in greater details because the position lies outside of the QRDR of ParE (Bajaj et al., 2016).

CONCLUSIONS

In summary, in response to frequent resistance against most antimicrobials in *E. coli* isolates from bovine mastitis, more comprehensive surveillance of antimicrobial

agent usage in livestock in China is required. Although the coordinated expression of major PMQR genes and β -lactam resistance genes is not yet a widespread phenomenon, the PMQR- and β -lactamase-encoding genes are highly prevalent in quinolone-resistant *E. coli*. In addition, high occurrence of QRDR mutations in DNA gyrase GyrA and DNA topoisomerase IV ParC suggests that both of the enzymes may play a key role as a quinolone target in *E. coli* recovered from bovine mastitis in China. To the best of our knowledge, this is the first report on the high occurrence of PMQR determinants and QRDR mutations in quinolone-resistant *E. coli* isolates from bovine mastitis in China.

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