Full Length Research Paper

Quinolone resistance in *Escherichia coli* and *Salmonella* spp. isolates from diseased chickens during 1993-2008 in China

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A total of 363 *Escherichia coli* and 224 *Salmonella* spp. were isolated from diseased chickens during 1993-2008 in China. The susceptibility to eight quinolones and prevalence of plasmid-mediated quinolone resistance (PMQR) determinants was investigated in these isolates. Among the *E. coli* isolates obtained during 1993-1999, 65.2% were resistant to nalidixic acid, while more than 50% of the *E. coli* isolates collected during 2000-2008 were resistant to 7 quinolones. All 101 *Salmonella* spp. isolates obtained during 1993-1999 were susceptible to quinolones, while more than 50% of the *Salmonella* spp. isolates collected during 2000- 2008 were resistant to only nalidixic acid (82.9%) . Among the 363 *E. coli* isolates, 4 (1.1%) were positive for *aac(6)-Ib-cr*, 3 (0.8%) for *qepA* and 1 (0.3%) for *qnrB10*. No PMQR gene was identified in 224 *Salmonella* spp. isolates. The resistance of *E. coli* and *Salmonella* spp. to quinolones has been increasing in the past twenty years and the resistance of *Salmonella* spp. was much lower than that of *E. coli*, although they were separated in the same period. There is a rising trend of avian isolates harboring PMQR genes in China.

Key words: Quinolone resistance, *qnr*; *aac(6)-Ib-cr*; *qepA*, chicken.

INTRODUCTION

E. coli and *Salmonella* are important pathogens that cause gastrointestinal infections and septicemia in human and animals as well as a range of secondary conditions, including respiratory tract infection in animals. Quinolones/ fluoroquinolones are broad-spectrum antibacterial agents used in human and veterinary medicine. The development of resistance to quinolone by Gram-negative pathogens is an important step in bacterial evolution (Hopkins et al., 2005). Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid, which is a completely synthetic agent that in clinical concentrations has bactericidal effects on most members of *Enterobacteriaceae*. The extensive use of quinolones has been associated with an increasing level of quinolone resistance (Winokur et al., 2000; Jiang et al., 2008).

All quinolones have the same mechanism of action, namely, the inhibition of DNA replication, regardless of whether they are used in clinical or veterinary medicine. Until 1998, it was believed that quinolone resistance could be acquired through chromosomal mutation. Later, it became clear that quinolone resistance may be associated with plasmid-mediated resistance genes (Martinez-Martinez et al., 1998; Robicsek et al., 2006a). Thus far, the following mechanisms have been reported to be involved in plasmid-encoded quinolone resistance: a quinolone-protective mechanism encoded by the *qnr* genes (Martinez-Martinez et al., 1998), a double class antibiotic-modifying enzyme encoded by *aac(6)-Ib-cr* which involves in the acetylation of ciprofloxacin and norfloxacin (Robicsek et al., 2006b), and the *qepA* gene, which encodes an efflux pump belonging to the major facilitator subfamily (Yamane et al., 2007). In China, quinolones are commonly used for disease prevention and control in poultry production, and there were some surveys on the prevalence of antimicrobial resistance among

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E. coli and *Salmonella* spp. from food-producing animals (Yang et al., 2004; Dai et al., 2008; Yue et al., 2008; Huang et al., 2009; Pan et al., 2009; Wu et al., 2009). However, a longitudinal survey is still lacking on quinolone resistance determinants in *E. coli* and *Salmonella* spp. isolated from diseased chickens.

This study was conducted in order to compare the quinolone resistance and prevalence of plasmid-mediated quinolone resistance (PMQR) genes among *E. coli* and *Salmonella* spp. strains, which were isolated from ill chickens during 1993-2008 from 14 provinces of China.

MATERIALS AND METHODS

Bacterial isolates and serogroup determination

A total of 363 *E. coli* and 224 *Salmonella* spp. field isolates were derived from clinically affected chickens in 14 provinces (Jiangsu, Shanghai, Shandong, Shanxi, Henan, Guangdong, Zhejiang, Hubei, Beijing, Anhui, Shanxi, Sichuan, Guizhou, and Guangxi) of China between 1993 and 2008. All *E. coli* and *Salmonella* spp. strains were isolated from diseased chickens for which sufficient amounts of bacteria could be obtained from systemic lesions (heart, liver, spleen, brain, and blood) for culturing. The samples were cultured directly on MacConkey agar (Difco) and were identified as *E. coli* and *Salmonella* spp. using biochemical procedures (Chen et al., 2004; Pan et al., 2009). Only one isolate from each diseased bird was examined. After isolation, organisms were stored at -70°C in Luria-Bertani (LB) broth after the addition of 15% glycerol. *E. coli* ATCC 25922 was used as a quality control strain for susceptibility testing.

E. coli serogroup typing was performed by using the standard methods followed at the China Institute of Veterinary Drug Control (Chen et al., 2004). *Salmonella* spp. strains were grown overnight in LB broth, and plate agglutination test was performed for monoclonal antibodies (Pan et al., 2009).

Antimicrobial susceptibility testing

The MICs of 363 *E. coli* and 224 *Salmonella* spp. isolates were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute recommendations (CLSI, 2008; 2009). The following antimicrobial agents were tested: nalidixic acid, enoxacin, fleroxacin, norfloxacin, lomefloxacin, ciprofloxacin, ofloxacin, and gatifloxacin (National Institute for the Control of Pharmaceutical and Biological Products, China). The method was controlled by parallel testing using quality control reference strain recommended by the CLSI.

Screening for PMQR genes in *E. coli* and *Salmonella* strains

The isolates were investigated for the presence of *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6)-Ib* and *qepA* genes by PCR amplification with the primer sets described previously (Park et al., 2006; Cattoir et al., 2007; Yamane et al., 2008; Cavaco et al., 2009; Wang et al., 2009). The primers used for *qnrA* were 5´-AGAGGA TTTCTC ACGCCA GG and 5´-GCAGCA CTATKA CTCCCA AGG, giving a 619bp product. Both strands of the purified PCR products were sequenced and *qnr* alleles were assigned by referring to the *qnr* gene nomenclature (Jacoby et al., 2008) . All positive PCR products of *aac(6)-Ib* were further analyzed by digestion with *FokI*(TaKaRa Biotechnology, Dalian, China) and/or direct sequencing to identify *aac(6)-Ib-cr*, which lacks the *FokI* restriction site present in the

wild-type gene.

Conjugation assays

Conjugation experiments were attempted between the PMQR gene-positive *E. coli* donors and azide-resistant recipient strain *E. coli* J53AzR (Wang et al., 2003). Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 mg/l) and tetracycline (20 mg/l), chloramphenicol (50 mg/l), gentamicin (8 mg/l) or amoxicillin (100 mg/l) to select for plasmid-encoded resistance. MICs for the donor, recipient, and transconjugant strains were measured by the broth microdilution method according to CLSI guidelines (CLSI, 2009).

RESULTS

Serogroups

A total of 363 *E. coli* and 224 *Salmonella* spp. isolates were derived from clinically affected chickens in 14 provinces of China between 1993 and 2008, which 178 *E. coli* and 101 *Salmonella* spp. isolates were collected between 1993 and 1999, and 185 *E. coli* and 123 *Salmonella* spp. isolates between 2000 and 2008. Among the 363 avian *E. coli* isolates, 357 could be classified into serogroups, while 6 could not be serogrouped. These isolates were classified into 45 serogroups, and 65.6% of them (238/363) belonged to the following 4 O-serogroups: O1 (21/363), O2 (46/363), O18 (22/363), and O78 (149/363). 224 *Salmonella* spp. isolates were classified as *Salmonella pullorum*.

Antimicrobial susceptibility of avian *E. coli* isolates

Among the 363 avian *E. coli* isolates, resistance to quinolones from 1993-1999 was much lower than that of the isolates obtained during 2000-2008. More than 60% of the isolates obtained during 1993- 1999 exhibited resistance to only 1 quinolone, and more than 60% of the isolates collected during 2000-2008 demonstrated resistance to 6 quinolones. The results of susceptibility tests of avian *E. coli* of different serogroups are presented in Table 1. Among the 4 common serogroups, quinolone resistance in the O1 and O78 serogroup isolates (more than 50% of the strains were resistant to 6 and 5 quinolones, respectively) appeared to be higher than that of the O2 and O18 (more than 50% of the strains were resistant to 3 and 1 quinolones, respectively).

Resistance to quinolones in *Salmonella* spp. isolates

All the *Salmonella* spp. isolates obtained during 1993-1999 were susceptible to quinolones, and a nalidixic acid-resistant *Salmonella* spp. isolate was first observed in the year 2000. Resistance to various quinolones in *Salmonella* spp. isolates during 2000-2008 was presented

Table 1. Relationship between serogroups and quinolone resistance among 363 avian *Escherichia coli* isolates.

Quinolone	Percentage of resistance (No. of isolates)					
	O1 (n = 21)	O2 (n = 46)	O18 (n = 22)	O78 (n = 149)	Other* (n = 125)	Total (n = 363)
Nalidixic acid	81.0 (17)	80.4 (37)	68.2 (15)	82.5 (123)	79.2 (99)	80.2 (291)
Enoxacin	66.7 (14)	58.7 (27)	31.8 (7)	67.1 (100)	68.0 (85)	64.2 (233)
Fleroxacin	57.1 (12)	47.8 (22)	13.6 (3)	59.1 (88)	55.2 (69)	53.4 (194)
Norfloxacin	61.9 (13)	39.1 (18)	9.1 (2)	51.7 (77)	46.4 (58)	46.3 (168)
Lomefloxacin	66.7 (14)	50.0 (23)	18.2 (4)	61.1 (91)	51.2 (64)	54.0 (196)
Ciprofloxacin	61.9 (13)	19.6 (9)	4.5 (1)	38.9 (58)	44.0 (55)	37.5 (136)
Ofloxacin	47.6 (10)	19.6 (9)	13.6 (3)	29.5 (44)	47.2 (59)	34.4 (125)
Gatifloxacin	4.8 (1)	2.2 (1)	4.5 (1)	1.3 (2)	16.0 (20)	6.9 (25)

in Table 2. Among the *Salmonella* spp. isolates obtained during 2000-2008, more than 50% of the strains were found to be resistant to only nalidixic acid (82.9%). Less than 10% strains were resistant to five antimicrobial agents: lomefloxacin, ciprofloxacin, norfloxacin, ofloxacin, and gatifloxacin.

Comparison of the quinolone resistance of *E. coli* and *Salmonella spp.* isolates

In this study, 101 *Salmonella* spp. isolates obtained before the year 2000 were susceptible to all the 8 quinolone antimicrobial agents. While in the same period more than 50% of the *E. coli* isolates exhibited resistance to nalidixic acid. More than 50% of the *Salmonella* spp. isolates obtained during 2000- 2008 were found to be resistant to nalidixic acid (82.9%), while more than 50% of the *E. coli* isolates collected during the period exhibited resistance to 7 quinolones (Table 2).

Screening for the *qnr*, *aac(6)-Ib-cr*, and *qepA* genes

Among the 363 avian *E. coli* isolates, PMQR determinants *qepA* and *qnrB10* were detected in 3 (0.8%) and 1 (0.3%) of the isolates, *aac(6)-Ib* was detected in 11 of which 4 were *aac(6)-Ib-cr* (1.1%). In this study, *aac(6)-Ib* was first observed in 2007. Four *aac(6)-Ib-cr* - positive isolates were detected in 2008. No PMQR gene was detected in 224 *Salmonella* spp. isolates.

Conjugation experiment and antimicrobial susceptibility testing

Quinolone resistance could be transferred by conjugation from three of the eight PMQR genes positive donors. Transfer from the other five donor strains were not successful despite conjugation experiments in broth and on filter surfaces and including separate selections with

each of the antibiotics to which the donor was resistant (except quinolones). The MIC of ciprofloxacin against the transconjugants was 0.06 mg/l, representing an increase of 8-fold relative to that of the recipient *E. coli* J53AzR (Table 3). Various resistance to other antimicrobial agents were also transferred with the plasmids, all were resistance to ampicillin and chloramphenicol. All trans-conjugants had remarkable elevated MICs for amikacin, kanamycin and tetracycline.

DISCUSSION

Resistance to quinolones has increased markedly in some parts of the world since the introduction of these agents. Eight quinolones belonging to the first to fourth generations were selected for testing in this study. It was found that the isolates had high resistance to the first-generation quinolone like nalidixic acid and low resistance to the fourth-generation quinolone such as gatifloxacin.

E. coli isolates occur as non-pathogenic commensals in human or as animal pathogens, but the emergence of strains showing resistance to several quinolone antimicrobial agents is a public health concern. Before the early 1990s, clinical isolates of *E. coli* rarely showed resistance to quinolones. However, since then the frequency of resistance has significantly increased worldwide (Hopkins et al., 2005). In this study, *E. coli* was found to have high resistance to some quinolones after 1990. Resistance to 7 quinolone antimicrobial agents was observed in more than 50% of the isolates during 2000-2008.

High-level quinolone resistance is relatively uncommon in *Salmonella* spp. (Hopkins et al., 2005). In the year 2000, the results of antimicrobial susceptibility tests for isolates of human salmonellosis in 10 European countries revealed that clinical resistance to ciprofloxacin was rare, with only 0.5% of the isolates exhibiting CIP MIC greater than 1 mg/l (Threlfall et al., 2003). Quinolone resistance to *Salmonella* spp. was first observed in the year 2000 in this

Table 2. Results of antimicrobial resistance of 363 *E. coli* and 123 *Salmonella* spp. Isolates.

| Agent and species | No. of isolates | MIC (mg/l) | | | | % of resistant isolates |
| | | Range | | MIC$_{50}$ | MIC$_{90}$ | |
		Minimum	Maximum			
Nalidixic acid						
E. coli (1993–1999)	178	<0.125	>512	256	>512	65.2
E. coli (2000–2008)	185	0.25	>512	>512	>512	94.6
Salmonella (2000–2008)	123	1	>512	>512	>512	82.9
Enoxacin						
E. coli (1993–1999)	178	<0.125	>512	2	256	40.4
E. coli (2000–2008)	185	<0.125	>512	128	512	87.0
Salmonella (2000–2008)	123	0.5	>512	4	32	34.1
Fleroxacin						
E. coli (1993–1999)	178	<0.125	256	2	64	29.8
E. coli (2000–2008)	185	<0.125	512	64	128	76.2
Salmonella (2000–2008)	123	0.5	256	4	16	30.9
Norfloxacin						
E. coli (1993–1999)	178	<0.125	512	1	32	25.3
E. coli (2000–2008)	185	<0.125	>512	64	256	66.5
Salmonella (2000–2008)	123	0.5	256	2	4	7.3
Lomefloxacin						
E. coli (1993–1999)	178	<0.125	>512	1	32	27.0
E. coli (2000–2008)	185	<0.125	>512	32	128	80.0
Salmonella (2000–2008)	123	0.5	128	2	2	7.3
Ciprofloxacin						
E. coli (1993–1999)	178	<0.125	>512	0.5	8	11.8
E. coli (2000–2008)	185	<0.125	>512	8	64	62.2
Salmonella (2000–2008)	123	0.5	128	0.5	1	7.3
Ofloxacin						
E. coli (1993–1999)	178	<0.125	256	1	32	14.6
E. coli (2000–2008)	185	<0.125	512	16	64	53.5
Salmonella (2000–2008)	123	0.5	64	1	4	7.3
Gatifloxacin						
E. coli (1993–1999)	178	<0.125	32	0.5	2	1.7
E. coli (2000–2008)	185	<0.125	64	2	16	11.4
Salmonella (2000–2008)	123	0.5	32	0.5	1	3.3

study. Of the *Salmonella* spp. isolates, 82.9% of those obtained during 2000-2008 were found to be resistant to nalidixic acid. The number of quinolones for which resistance was developed was much lower for *Salmonella* than for *E. coli*.

Plasmid-mediated quinolone resistance was first reported in 1998, the gene responsible for PMQR has been identified as *qnr* (Tran and Jacoby, 2002). The *qnr* gene has been identified in various bacterial species of the Enterobacteriaceae family in many countries. Only one *qnrB10*-positive isolate was detected among the 363 avian *E. coli* isolates in this study. A variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, *aac(6′)-Ib-cr*, seems

Table 3. Characteristics of donor strains and *E. coli* J53 transconjugants.

Strain [a]	Sero group	PMQR determinant	MIC (mg/l) [b]															
			NAL	FLX	ENX	LML	CIP	NOR	OFL	GAT	Amp	CTX	CAZ	Gen	AN	Km	Tet	Cm
E. coli J53			4	0.03	0.03	0.03	0.008	0.03	0.03	0.008	4	0.06	0.125	0.25	0.5	4	2	8
C265	O116	*aac(6')-Ib-cr*	>512	8	16	8	16	64	4	2	>512	0.25	0.125	8	2	>512	64	64
J53(pC265-J1)		*aac(6')-Ib-cr*	16	0.06	0.25	0.25	0.06	0.5	0.125	0.06	>512	0.125	0.125	0.5	1	16	4	>32
C324	O115	*aac(6')-Ib-cr*	>512	32	64	32	32	128	16	8	>512	32	16	8	4	>512	64	256
J53(pC324-J1)		*aac(6')-Ib-cr*	16	0.125	0.25	0.25	0.06	0.5	0.06	0.06	>512	0.125	0.125	0.5	2	16	32	>32
C327	O86	*aac(6')-Ib-cr*	>512	64	128	64	64	512	32	16	>512	>256	2	512	256	>512	256	512
J53(pC327-J6)		*aac(6')-Ib-cr*	16	0.06	0.25	0.25	0.06	0.5	0.06	0.06	256	0.125	0.125	1	8	32	64	>32

[a] J53(pC265-J1), J53(pC324-J1) and J53(pC327-J6), transconjugants of *E. coli* C265, C324 and C327, respectively.
[b] NAL, nalidixic acid; FLX, fleroxacin; ENX, enoxacin; LML, lomefloxacin; CIP, ciprofloxacin; NOR, norfloxacin; OFL, ofloxacin; GAT, gatifloxacin; Amp, ampicillin; CTX, cefotaxime; CAZ, Ceftazidime; Gen, gentamicin; AN, amikacin; Km, kanamycin; Tet, tetracycline; Cm, chloramphenicol.

to have emerged more recently, but might be more prevalent than *qnr* (Hopkins et al., 2005). Four (1.1%) *E. coli* isolates were positive for *aac(6)-Ib-cr*. These 4 isolates were obtained from different henneries and belonged to 4 different serogroups. In this study, 3 (0.8%) *E. coli* isolates were positive for *qepA*, all of which were obtained from the same hennery and exhibited resistance to all 8 quinolones.

Although these *E. coli* and *Salmonella* spp. strains were separated in the same period, the susceptibility to quinolones and the presence of the PMQR genes were different from each other. The exactly reasons for the differences are not known, but it possible because that *E. coli* strains are common both in intestinal tract and the environment. The pollution of natural ecosystems by antibiotics and resistance genes might have consequences for the evolution of the microbiosphere (Martinez, 2009). The opportunity for *E. coli* strains to acquire the resistance is much more than the *Salmonella* spp. strains, and the antimicrobial resistance of the commensal *E. coli*

strains may also play an important role, transmission of resistance genes from normally nonpathogenic species to more virulent organisms is an important mechanism for acquiring antimicrobial-resistant organisms (Winokur et al., 2001).

In conclusion, the rate of quinolone resistance of *E. coli* and *Salmonella* isolates has increased considerably over the past twenty years. PMQR determinants appeared in avian *E. coli* and its prevalence is increasing in China. The prudent use of antimicrobial agents is thus necessary in veterinary medicine as well as in human medicine to minimize the spread of these resistance genes.

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