Plasmid-mediated quinolone resistance in *Salmonella* serotypes isolated from chicken carcasses in Turkey

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Abstract

Quinolones have been extensively used for treatment of a variety of invasive and systemic infections of salmonellosis. Widespread use of these agents has been associated with the emergence and dissemination of quinolone-resistant pathogens. The quinolone resistance and plasmid-mediated quinolone resistance determinants (*qnr*A, *qnr*B, *qnr*S and *aac(6')-Ib-cr*) of 85 *Salmonella* isolates from chicken carcasses were investigated in this study. Isolates were serotyped according to the Kauffman-White-Le Minor scheme, and broth microdilution method was used to determine quinolone resistance. Plasmid-mediated quinolone resistance genes were investigated by real-time PCR and positive results were confirmed by sequencing. Among the *Salmonella* isolates, 30/85 (35%) and 18/85 (21%) were found to be resistant to enrofloxacin (MIC ≥ 2 mg/ml), and danofloxacin (MIC ≥ 2 mg/ml), respectively. All the isolates were negative for *qnr*A, *qnr*B and *aac(6')-Ib-cr* genes, nevertheless 2% (*S.* Brandenburg and *S.* Dabou) were positive for *qnr*S (*qnr*S1 determinant). This study is the first and unique investigating the plasmid-mediated quinolone resistance determinants of *Salmonella* isolated from chicken carcasses in Turkey.

Quinolone susceptibility, qnr, aac(6')-Ib-cr, real-time PCR, minimal inhibition concentration

Salmonellae are among the most common Gram negative pathogens implicated in foodborne illnesses and also a major public health concern worldwide (Guner et al. 2012). Chicken meat, fresh and processed meat and eggs have been the most commonly incriminated foodstuffs in several outbreaks of salmonellosis (Tauxe 1991; Benenson and Chin 1995; Mead et al. 1999). Quinolone resistant *Salmonella* isolates have been documented in numerous locations with variable prevalence including Japan (Taguchi et al. 2009) and Brasil (Ferrari et al. 2011). Molbak et al. (2002) reported that quinolone resistance in *Salmonella* spp. isolated in Denmark had increased almost 10 fold in five years. Unfortunately, these results indicate that widespread fluoroquinolone use for the treatment of infections both in human and in veterinary medicine has led to increasing numbers of quinolone resistant *Salmonella* isolates.

Several reports in Turkey have evaluated the plasmid-mediated quinolone resistant (PMQR) in animal isolates of *Escherichia coli* strains (Cengiz et al. 2012; Mustak et al. 2012). However, this study is the first and unique one investigating the PMQR determinants of *Salmonella* isolated from chicken carcasses in Turkey. The aim of this study was to investigate the presence of PMQR determinants of *qnr*A, *qnr*B, *qnr*S, *aac(b)-1b-cr* in *Salmonella* strains isolated from chicken carcasses between 2005 and 2009 in the Bursa and Ankara provinces of Turkey.

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Materials and Methods

Bacterial strains

A total of 85 *Salmonella* spp. strains which were previously isolated from chicken carcasses in Bursa and Ankara provinces of Turkey between 2005 and 2009 were obtained from Uludag University, Faculty of Veterinary Medicine Department of Food Hygiene and Technology laboratory. All presumptive *Salmonella*-positive isolates were confirmed by biochemical tests according to ISO 6579:2002 and serotyped according to the Kauffman-White-Le Minor scheme (ISO 2002; Grimont and Weill 2007).

Antimicrobial susceptibility testing

Enrofloxacin and danofloxacin MICs were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012a). Enrofloxacin and danofloxacin analytical standards were obtained from Fluka (Sleinheim, Germany) and dissolved in 20:80 methanol/phosphate buffered saline (v/v). *Salmonella* cultures were prepared in Mueller-Hinton Broth at 37 °C for 16–20 h. Freshly prepared stock solution was sterilized using 0.20 µm single-use filter units (Minisart®, Sartorius Stendim Biotech, Göttingen, Germany). Enrofloxacin and danofloxacin dilutions ranging from 0.008 to 256 mg/l were prepared in Mueller-Hinton Broth. Inoculants with a density equivalent to 0.5 McFarland turbidity standard were prepared and each of enrofloxacin and danofloxacin dilutions were added into 96 well plates (Thermo Scientific, Epsom, UK). After incubating at 37 °C for 16–20 h, the MICs were defined as the minimum concentration of enrofloxacin and danofloxacin inhibiting growth of bacteria. The optical densities (ODs) of the cultures were measured at a wave length of 595 nm (iMark™, Bio-Rad, Hercules, CA, USA). *Escherichia coli* ATCC 25922 was used as control for antimicrobial susceptibility testing. Susceptibility to enrofloxacin and danofloxacin were interpreted according to CLSI (CLSI 2012b).

Detection of PMQR genes

Plasmids were extracted from *Salmonella* isolates with a DNA-spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Seoul, Republic of Korea) according to manufacturer's recommendations. Presence of PMQR was investigated with *qnr*A, *qnr*B, *qnr*S, *aac(6')-Ib-cr* specific primers designed for this study by real-time PCR using Melting Curve Analysis. The PMQR gene sequences were acquired from NCBI Data Bank (NCBI Data Bank) and sequence analyses were made by ClustalW programme (ClustalW2). Primers aiming secured sequences were designed manually and tested with Primer-Blast (NCBI/Primer-BLAST).

A total of 85 isolates were screened by *qnr* determinants using specific primers for *qnr*A (5′-CCG AGT TTG GCC AGA TAG AC-3′ [forward] and 5′ CTG CTC CAG CAA ATC CTG TT-3′ [reverse]), *qnr*B (5′-CAG TAC ACC GGC CAG AAA GT-3′ [forward] and 5′-TTG AAA TGG CAC ATG CTG AT-3′ [reverse]), and *qnr*S (5′-ACG ACA TTC GTC AAC TGC AA-3′ [forward] and 5′-CGA AGA TCT GCG ACA TCA AA-3′ [reverse]) and for *aac(6')-Ib-cr* (5′-TGA CCT TGC GAT GCT CTA TG-3′ [forward] and 5′ TGG TCT ATT CCG CGT ACT CC-3′ [reverse]). Amplification was carried out in a 50 µl volume containing 0.2 µM each of dNTP and the primer pairs at concentrations each of 2.5 pM and 2.5 U of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 2 µM $MgCl_2$ and 1 × EvaGreen. Isolated DNA (3 µl) was used as a template. The PCR was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation for 10 min at 95 °C; 35 cycles of denaturation for 10 s at 94 °C, annealing for 15 s at 55 °C and extension for 10 s at 72 °C; and final extension for 5 min at 72 °C. The temperature had been raised to 95 °C from 50 °C by the speed of 0.5 °C/s in the melting curve analysis, meanwhile the specification of PCR products had been tested by reading the fluorescent constantly.

PCR products were purified and then sequenced with 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) by Sanger method. The sequences were compared and confirmed with the GenBank nucleotide database using the BLAST (Basic Local Alignment Search Tool) program (NCBI/BLAST Home).

Results

Serotyping results showed that majority of the *Salmonella* isolates were *S.* Enteritidis serotype (n = 42). MICs of *Salmonella* isolates ranged from 0.016 mg/l to higher than 256 mg/l for enrofloxacin and danofloxacin. Among the 85 *Salmonella* isolates, 11 (13%) and 8 (9%) were resistant to enrofloxacin and danofloxacin (MIC ≥ 4 mg/ml), respectively. Fifty-five isolates (65%) and 19 isolates (22%) were exhibited as susceptible and at intermediate level of susceptibility, respectively, to enrofloxacin. Variable level of susceptibility was also noted for danofloxacin. A total of 67 isolates (79%) were determined as susceptible, and 10 isolates (12%) were intermediate susceptible (Tables 1 and 2). Quinolone resistance reached MIC in *Salmonella* serotypes of ≥ 256 mg/ml for enrofloxacin (2%) and danofloxacin (5%). The MICs of enrofloxacin and danofloxacin for *qnr*-containing *Salmonella* isolates ranged from 16 mg/ml to 64 mg/ml.

The *qnr*S gene was identified in two isolates (2%) of *Salmonella* strains (*S.* Brandenburg and *S.* Dabou) isolated from chicken carcasses, whereas *qnr*A, *qnr*B and *aac(6')-Ib-cr* were not detected (Table 3).

Table 1. Determined MIC values for *Salmonella* serotypes to enrofloxacin and danofloxacin.

Salmonella serotypes	Number of serotypes	Range of MIC values (mg/ml)	
		Enrofloxacin	Danofloxacin
S. Agona	1	0.128	0.064
S. Brandenburg	4	4-64	2-64
S. Chincol	1	0.064	0.064
S. Corvallis	7	0.032-2	0.032-2
S. Dabou	4	0.064-32	0.016-16
S. Emek	1	0.064	1
S. Enteritidis	42	0.032-4	0.016-4
S. Essen	1	0.512	0.256
S. Hadar	5	0.032-128	0.032-256
S. Infantis	1	2	2
S. Kentucky	6	0.064->256	0.032-256
S. Kingston	1	0.064	0.032
S. Senftenberg	1	0.064	0.064
S. Typhimurium	9	0.064->256	0.016-256
S. Virchow	1	1	0.256

Table 2. Number of *Salmonella* strains susceptible, intermediate susceptible, and/or resistant to enrofloxacin and danofloxacin.

Antimicrobials	S/I/R[a]	Concentration (mg/ml)	Number (%)
Enrofloxacin	S	≤1	55 (65)
	I	2	19 (22)
	R	≥ 4	11 (13)
Danofloxacin	S	≤1	67 (79)
	I	2	10 (12)
	R	≥ 4	8 (9)

[a] S/I/R, susceptible/intermediate susceptible/resistant

Table 3. Resistance profile of *qnr*-positive *Salmonella* serotypes.

Salmonella serotypes	MIC (mg/ml)		Plasmid mediated quinolone resistance determinants			
	Enrofloxacin	Danofloxacin	*qnr*S	*qnr*A	*qnr*B	*aac(6')-Ib-cr*
S. Brandenburg	64	64	Positive	ND	ND	ND
S. Dabou	32	16	Positive	ND	ND	ND

ND = not detected

Discussion

Quinolone resistance in *Salmonella* has been observed all over the world (Angulo et al. 2000; Helmuth 2000). In the present study, 11 (13%) and 8 (9%) *Salmonella* isolates were found to be resistant to enrofloxacin and danofloxacin, respectively. The results have also shown that *Salmonella* strains were highly resistant to quinolones. High resistances (≥ 256 mg/ml) to enrofloxacin and danofloxacin in two *S.* Hadar*,* one *S.* Kentucky and one *S.* Typhimurium may be due to mutations in quinolone resistance determining regions. Resistance to quinolones in *Salmonella* spp. have been reported by previous studies in chickens (Wang et al. 2006; De Jong et al. 2014).

The PMQR determinants are distributed among *Enterobacteriaceae*, including *Salmonella*, worldwide (Cattoir and Nordmann 2009). The *qnr*A, *qnr*B, *qnr*S, and *aac(6')-Ib-cr* genes are the most commonly identified resistance determinants in Turkey (Nazik et al. 2009; Ozgumus et al. 2009). The prevalence of *qnr* genes among the *E. coli* isolates obtained from animals in the EU and Turkey ranged between 3% and 15% (Veldman et al. 2011; Cengiz et al. 2012). In another study from Turkey, Nazik et al. (2009) reported *qnr* genes in *Enterobacter* spp.*, E. coli* and *K. pneumoniae.* In agreement with previous studies (Kehrenberg et al. 2006; Avsaroglu et al. 2007), our results confirmed the presence of *qnr*S gene associated with fluoroquinolone resistance in two different *Salmonella* isolates from Turkey. In contrast, Yu et al. (2011), reported that all *Salmonella* isolates were negative for *qnr* genes, whereas a high prevalence (37.1%) of *aac(6')-Ib-cr* was found.

The presence of PMQR determinants has been reported in clinical isolates of *S.* Enteritidis (Cheung et al. 2005). In contrast, PMQR genes were not detected in *Salmonella* strains isolated from ready to eat foods and chicken meat as reported by Kim et al. (2011). Low prevalence (2%) of *qnr* genes in *Salmonella* isolates in comparison with other studies was observed. These genes were located on plasmids which are frequently associated with *Enterobacteriaceae* isolated from various foods and humans (Hopkins et al. 2007; Ferrari et al. 2011).

Even though the topic is widely investigated by several researchers, to our knowledge, there are only limited numbers of studies handling the subject in Turkey. By the present study, PMQR determinants in *Salmonella* isolated from chicken carcasses were investigated for the first time. This finding has indicated that chicken may be an important vector for the spread of quinolone-resistant *Salmonella* among animals, as well as from animals to humans through the food chain.

The PMQR determinants in *Salmonella* isolated from food-producing animals such as chickens are an important public health issue. Preventing the spread of quinolone-resistant *Salmonella* strains will require the establishment of continuous surveillance program for all resistance determinants at a national level. Furthermore, our results can provide useful information prompting further studies on the prevalence of quinolone-resistant *Salmonella* spp.

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References

Angulo FJ, Johnson KR, Tauxe RV, Cohen ML 2000: Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: implications for the use of fluoroquinolones in food animals. Microb Drug Resist **6**: 77-83

Avsaroglu MD, Helmuth R, Junker E, Hertwig S, Schroeter A, Akcelik M, Bozoglu F, Guerra B 2007: Plasmid-mediated quinolone resistance conferred by *qnrS1* in *Salmonella enterica* serovar Virchow isolated from Turkish food of avian origin. J Antimicrob Chemother **60**: 1146-1150

Benenson AS, Chin J 1995: Control of Communicable Diseases Manual. American Public Health Association, Washington, DC, 577 p.

Cattoir V, Nordmann P 2009: Plasmid-mediated quinolone resistance in Gram negative bacterial species: an update. Curr Med Chem **16**: 1028-1046

Cengiz M, Buyukcangaz E, Arslan E, Mat B, Sahinturk P, Sonal S, Gocmen H, Sen A 2012: Molecular characterisation of quinolone resistance in *Escherichia coli* from animals in Turkey. Vet Rec **171**: 155, 1-4

Cheung TK, Chu YW, Chu MY, Ma CH, Yung RW, Kam KM 2005: Plasmid-mediated resistance to ciprofloxacin and cefotaxime in clinical isolates of *Salmonella enterica* serotype Enteritidis in Hong Kong. J Antimicrob Chemother **56**: 586-589

Clinical and Laboratory Standards Institute (CLSI) 2012a: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 9th edn. Approved standard, Wayne, PA, USA, 88 p.

Clinical and Laboratory Standards Institute (CLSI) 2012b: Performance Standards for Antimicrobial Susceptibility Testing; 22nd informational supplement. Wayne, PA, 188 p.

ClustalW2. Available at: www.ebi.ac.uk/Tools/msa/clustalw2/. Last modified September 10, 2012. Accessed September 10, 2012.

De Jong A, Smet A, Ludwig C, Stephan B, De Graef E, Vanrobaeys M, Haesebrouck F 2014: Antimicrobial susceptibility of *Salmonella* isolates from healthy pigs and chickens (2008–2011). Vet Microbiol **171**: 298-306

Ferrari R, Galiana A, Cremades R, Rodriguez JC, Magnani M, Tognim MC, Oliveira TC, Royo G 2011: Plasmid-mediated quinolone resistance by genes *qnrA1* and *qnrB19* in *Salmonella* strains isolated in Brazil. J Infect Dev Ctries **5**: 496-498

Grimont PAD, Weill FX 2007: Antigenic Formulas of *Salmonella* Serovars. 9th edn, WHO Collaborating Centre for Reference and Research on *Salmonella,* 167 p.

Guner A, Atasever M, Atasever AM 2012: New emerging and re-emerging bacterial foodborne pathogens (in Turkish). Kafkas Univ Vet Fak Derg **18**: 889-898

Helmuth R 2000: Antibiotic resistance in *Salmonella*. In: Wray C, Wray A (Eds): *Salmonella* in Domestic Animals. CAB International, Wallingford, Oxfordshire, UK, pp: 89-103

Hopkins KL, Wootton L, Day MR, Threlfall EJ 2007: Plasmid-mediated quinolone resistance determinant *qnrS1* found in *Salmonella enterica* strains isolated in the UK. J Antimicrob Chemother **59**: 1071-1075

International Standards Organization (ISO) 2002: Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for the Detection of *Salmonella* spp. ISO 6579:2002 (E). Geneva, Switzerland, 27 p.

Kehrenberg C, Friederichs S, De Jong A, Michael GB, Schwarz S 2006: Identification of the plasmid-borne quinolone resistance gene *qnrS* in *Salmonella enterica* serovar Infantis. J Antimicrob Chemother **58**: 18-22

Kim KY, Park JH, Kwak HS, Woo GJ 2011: Characterization of the quinolone resistance mechanism in foodborne *Salmonella* isolates with high nalidixic acid resistance. Int J Food Microb **146**: 52-56

Mead PS, Slutsker L, Dietz V, Mccaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV 1999: Food-related illness and death in the United States. Emerg Infect Dis **5**: 607-625

Molbak K, Gerner-Smidt P, Wegener HC 2002: Increasing quinolone resistance in *Salmonella enterica* serotype Enteritidis. Emerg Infect Dis **8**: 514-515

Mustak HK, Ica T, Ciftci A, Diker KS 2012: Plasmid-mediated quinolone resistance in *Escherichia coli* strains isolated from animals in Turkey. Vet J Ankara Univ **59**: 255-258

Nazik H, Ilktac M, Ongen B 2009: Prevalence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr* (in *qnr*-positive isolates) among the ESBL-positive and/or ciprofloxacin-resistant isolates in Turkey. J Chemother **21**: 219-221

NCBI/BLAST Home. Available at: http://blast.ncbi.nlm.nih.gov/Blast. Last modified March 20, 2013. Accessed March 20, 2013

NCBI Data Bank. Available at: http://www.ncbi.nlm.nih.gov/. Last modified September 07, 2012. Accessed September 07, 2012

NCBI/Primer-BLAST. Available at: www.ncbi.nlm.nih.gov/tools/primer-blast/. Last modified September 12, 2012. Accessed September 12, 2012

Ozgumus OB, Sandalli C, Sevim A, Celik-Sevim E, Sivri N 2009: Class 1 and class 2 integrons and plasmid-mediated antibiotic resistance in coliforms isolated from ten rivers in Northern Turkey. J Microbiol **47**: 19-27

Taguchi M, Kawahara R, Seto K, Inoue K, Hayashi A, Yamagata N, Kamakura K, Kashiwagi E 2009: Plasmid-mediated quinolone resistance in *Salmonella* isolated from patients with overseas travelers' diarrhea in Japan. Jpn J Infect Dis **62**: 312-314

Tauxe RV 1991: *Salmonella*: a postmodern pathogen. J Food Protect **54**: 563-568

Veldman, K, Cavaco, LM, Mevius D, Battisti A, Franco A, Botteldorn N, Bruneau M, Perrin-Guyomard A, Cerny T, De Frutos Escobar C, Guerra B, Schroeter A, Gutierrez M, Hopkins K, Myllyniemi AL, Sunde M, Wasyl D, Aarestrup FM 2011: International collaborative study on the occurrence of plasmid-mediated quinolone resistance in *Salmonella enterica* and *Escherichia coli* isolated from animals, humans, food and environment in European countries. J Antimicrob Chemother **66**: 1278-1286

Wang YC, Yeh KS, Chang CC, Hsuan SL, Chen TH 2006: Fluoroquinolone-resistant *Salmonella* sp. in carcasses. Emerg Infect Dis **12**: 351-352

Yu F, Chen Q, Yu X, Pan J, Li Q, Yang L, Chen C, Zhuo C, Li X, Zhang X, Huang J, Wang L 2011: High prevalence of plasmid-mediated quinolone resistance determinant *aac(6')-Ib-cr* amongst *Salmonella enterica* serotype Typhimurium isolates from hospitalised paediatric patients with diarrhoea in China. Int J Antimicrob Agents **37**: 152-155