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Emerging high-level ciprofloxacin resistance and molecular basis of resistance in *Salmonella enterica* from humans, food and animals

Dai Kuang[a,f], Jianmin Zhang[b], Xuebin Xu[c], Weimin Shi[d], Sheng Chen[e], Xiaowei Yang[a], Xudong Su[a], Xianming Shi[a,*], Jianghong Meng[a,f,*]

[a] Department of Food Science & Technology, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China
[b] National and Regional Joint Engineering Laboratory for Medicament of Zoonosis Prevention and Control, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China
[c] Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China
[d] Shanghai Municipal Ke-Ma-Jia Technology Center for Microbiology, Shanghai, China
[e] State Key Lab of Chirosciences, Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hong Kong, China
[f] Joint Institute for Food Safety & Applied Nutrition, Center for Food Safety & Security Systems, Department of Nutrition & Food Science, University of Maryland, MD, USA

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ABSTRACT

Disease caused by nontyphoidal serotypes of *Salmonella enterica* is the leading cause of foodborne illness worldwide. Many serotypes have also developed resistance to antimicrobials commonly used for the treatment of salmonellosis. Here we analyze 179 ciprofloxacin-resistant isolates identified among 3680 *Salmonella* isolated from humans, food, animals, and water collected in Shanghai, China. They were assessed for antimicrobial susceptibility, plasmid-mediated quinolone resistance (PMQR) determinants, and mutations in quinolone resistance determination regions (QRDRs); genetic relatedness was examined using PFGE. Ciprofloxacin resistance in *Salmonella* increased from 2.3% in 2006 to 5.9% in 2012. Multidrug resistance was common, and most carried mutations in QRDR (97.2%) and PMQR determinants (71.5%). Mutations frequently included changes in *gyrA:* Ser83Phe (53.6%) and Asp87Asn (35.8%), and in *parC:* Thr57Ser (53.1%) and Ser80Arg (44.1%). Mutations in *parC* and *parE* without changes in *gyrA* were identified in *S.* Derby and most *S.* Thompson. Among PMQR determinants, *aac(6′)-Ib-cr* (62.0%) and *oqxA/oqxB* (33.5%/33.0%) were most common, and conferred resistance without target mutations in five *S.* Typhimurium isolates. PFGE analysis revealed that *S.* Typhimurium, isolated from pork and aquatic products, and *S.* Indiana, isolated from chicken, were highly similar to isolates from humans, suggesting these products be the major source of ciprofloxacin-resistant infections. Our findings highlight the important role QRDRs and PMQR play in ciprofloxacin resistance of *Salmonella*, and reveal the potential sources of the pathogen associated with human infections.

1. Introduction

Salmonella is an important cause of foodborne bacterial infections in humans; globally, an estimated 78.7 million cases of foodborne illnesses caused by non-typhoidal *S. enterica* are reported each year, resulting in nearly 59,000 deaths (Havelaar et al., 2015). Although non-typhoid salmonellosis is usually self-limiting, effective antimicrobial therapy is essential for severe cases, particularly among immunocompromised individuals, children, and the elderly (Vugia et al., 2004). However, many countries have been reporting multidrug-resistant (MDR) *Salmonella.* Infections with such drug-resistant *Salmonella* are associated with increased morbidity and mortality (Majowicz et al., 2010).

The use of antimicrobial agents in humans and food animals contributes to the dissemination of antimicrobial resistance among *Salmonella*; as a result, the efficacy of older antimicrobials such as ampicillin, chloramphenicol, and tetracycline has become limited. Consequently, fluoroquinolones, such as ciprofloxacin, which is effective for treating a wide variety of human and animal infections, have become one of the first choices for treating invasive gastrointestinal infections (Hopkins et al., 2005). Yet, the extensive use of quinolones and fluoroquinolones is a cause for concern, as this practice will positively select resistant isolates and lead to the emergence of bacterial pathogens exhibiting novel resistance mechanisms. Increasing prevalence of resistance to this class of antimicrobials poses a great challenge for effective control and management of salmonellosis.

Ciprofloxacin resistance is attributed mainly to mutations in the

* Corresponding authors at: University of Maryland, 0112 Skinner Building, College Park, MD 20742, USA.
E-mail addresses: xmshi@sjtu.edu.cn (X. Shi), jmeng@umd.edu (J. Meng).

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quinolone resistance-determining regions (QRDRs) of the DNA gyrase and topoisomerase IV genes, which prevent antimicrobial drugs from binding to these targets (Hooper and Jacoby, 2015; Randall et al., 2005). However, the discovery of a series of plasmid-encoded resistance mechanisms has led to speculation about the origin of these mechanisms and about factors that may enhance the transfer of resistance. Multiple plasmid-mediated quinolone resistance (PMQR) genes, including *qnr* (*qnrA*, *qnrB*, *qnrS*, *qnr*C, and *qnrD*), *qepA*, *aac(6′)-Ib-cr*, and *oqxAB*, have been identified in *Salmonella* (Cavaco et al., 2009; Hansen et al., 2005; Kim et al., 2009a; Kim et al., 2009b; Park et al., 2006; Robicsek et al., 2006b; Wang et al., 2009). Although PMQR genes usually confer only low-level resistance to fluoroquinolones, bacteria carrying PMQR genes, particularly *qnr* genes, may have a selective advantage; having survived initial exposure to fluoroquinolones, these bacteria may consequently develop high-level chromosomal quinolone resistance (Hooper and Jacoby, 2015; Lin et al., 2015).

China is an excellent place to investigate antimicrobial resistance in bacteria. By producing 210,000 tons of antibiotics each year, China has become one of the primary antimicrobial sources in the world (Xu et al., 2014), and the use of antimicrobials is often permitted without prescription, for both human and veterinary medicine. Several studies have reported multidrug-resistant *Salmonella* in China (Lin et al., 2015; Yang et al., 2012; Zhang et al., 2014). Bacteria exhibiting resistance to fluoroquinolones as well as to third- and fourth-generation cephalosporins were also identified (Zhang et al., 2014). However, only limited data are available on the distribution and characteristics of ciprofloxacin-resistant *Salmonella enterica* (Bai et al., 2015; Cui et al., 2008; Yang et al., 2012). Therefore our study aims to investigate ciprofloxacin-resistant *S. enterica* isolates collected from humans, livestock, foods, and the environment, to determine the contribution of QRDRs and PMQR to the resistance, and to identify the potential sources of *Salmonella* isolates that cause multidrug-resistant infections.

2. Materials and methods

2.1. Bacterial strains

A total of 179 ciprofloxacin-resistant isolates were obtained and used in the study after screening 3680 *Salmonella enterica* isolates (2751 from humans and 929 from other sources) using disk diffusion method (CLSI, 2013) in Shanghai, China between 2006 and 2012. Isolation was done according to the Standard ISO-6579 method (ISO, 2002). The *Salmonella* isolates were serotyped with commercially available antisera (S&A Reagents Lab, Bangkok, Thailand). Serotypes were assigned according to the Kauffmann-White scheme (Popoff et al., 2004).

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined according to guidelines recommended by the Clinical and Laboratory Standard Institute (CLSI), using an agar dilution method (CLSI, 2003). Based on World Health Organization's categorization of antimicrobials of critical importance to human medicine (Collignon et al., 2016), six categories of antimicrobials (eighteen individual antibiotics) were tested — aminoglycosides: amikacin (AMK), gentamicin (GEN), kanamycin (KAN), streptomycin (STR); β-lactams: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), ceftiofur (EFT), cephalothin (CEP), ceftriaxone (CRO), cefoxitin (FOX); sulfomamides: sulfisoxazole (SUL) and trimethoprim/sulfamethoxazole (SXT); quinolone and fluoroquinolone: ciprofloxacin (CIP), ofloxacin (OFX), levofloxacin (LVX), nalidixic acid (NAL); and chloramphenicol (CHL), and tetracycline (TET). *Escherichia coli* ATCC25922 and ATCC35218 were used as quality control strains. The resistance breakpoints for each antimicrobial are shown in Table 1, adapted from the interpretive standards provided by CLSI (2013), with the exception of streptomycin, ceftiofur and sulfisoxazole whose breakpoints were based on the National Antimicrobial Resistance Monitoring System (NARMS) (CDC, 2016).

2.3. Molecular characterization of quinolone resistance genes

Plasmid mediated quinolone resistance (PMQR) genes *qnrA*, *qnrB*, *qnrS*, (Robicsek et al., 2006b) *qnrC*, (Wang et al., 2009) *qnrD*, (Cavaco et al., 2009) *aac(6′)-Ib-cr*, (Park et al., 2006) *qepA*, (Kim et al., 2009a) *oqxA*, and *oqxB* (Hansen et al., 2005) and genes encoding for DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) (Randall et al., 2005) in quinolone resistance determining regions (QRDRs) were tested as described previously.

We prepared DNA for PCR using thermal cell lysis in a dry bath. PCR products were analyzed using electrophoresis in agarose gel stained with GelRed and visualized with UV trans-illumination (Bio-Rad, Hercules, CA). The PCR products of *gyrA, gyrB, parC*, and *parE* were purified using a PCR purification kit (TaKaRa, Dailian, China), then sequenced by Sangon Biotech, Co., Ltd. (Shanghai, China).

We identified gene mutations in QRDRs by using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST) to compare sequences against those available in the GenBank nucleotide database at the US National Center for Biotechnology Information (NCBI).

2.4. Pulsed-field gel electrophoresis

PFGE was performed according to protocol developed by the US Centers for Disease Control and Prevention (Ribot et al., 2006). Briefly, genomic DNA was digested with 50 U of XbaI (TaKaRa, Dalian, China) for 1.5–2 h in a water bath at 37 °C. Restriction fragments were separated by electrophoresis in 0.5 × Tris-Borate-EDTA buffer at 14 °C for 18 h, using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16–63.8 s. *Salmonella enterica* serotype Braenderup H9812 was used as the molecular weight size standard. The gels were stained with GelRed, and DNA bands were visualized with UV trans-illumination. Gel images were scanned and analyzed using the BioNumerics Software (Applied-Maths, Kortrijk, Belgium). DNA patterns exhibiting approximately 80% similarity according to unweighted pair-group method with arithmetic means (UPGMA) were regarded as genetically very similar, and grouped into a cluster.

2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Version 22 (IBM Corporation, Armonk, NY, USA). Chi-square test was used to analyze categorical variables. Linear by linear association was used to determine the trend for resistance rate to ciprofloxacin during the study periods. A p value < 0.05 was considered significant.

3. Results

3.1. Ciprofloxacin-resistant S. enterica

A total of 179 isolates were resistant to ciprofloxacin, including 82 from humans, 43 from poultry, 30 from pigs, and 17 from seafood, 4 from river/waste water and 3 from vegetables (Table S1). There was a significant trend of increasing ciprofloxacin resistance among *Salmonella* in the Shanghai region between 2006 and 2012 ($p < 0.05$). With 2.3%, 1.6%, 3.3% and 2.4% resistance rate from 2006 to 2009, isolates collected during these first four years of our study, contained only 26 *Salmonella* isolates resistant to ciprofloxacin. Since then both the proportion and the absolute number of ciprofloxacin-resistant *Salmonella* has increased: 6.1% (n = 36) in 2010, 5.5% (n = 56) in 2011 and 5.9% (n = 61) in 2012.

Nineteen *Salmonella* serovars were identified among our set of ciprofloxacin-resistant isolates ($n = 179$). The two most common were Typhimurium (n = 64, 35.8%) and *S.* Indiana (n = 45, 25.1%). The