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Multiple transmissible genes encoding fluoroquinolone and third-generation cephalosporin resistance co-located in non-typhoidal *Salmonella* isolated from food-producing animals in China

Hong-Xia Jiang[a,1], Li Song[b,1], Ji Liu[a], Xiao-Hua Zhang[a], Yan-Na Ren[a], Wen-Hui Zhang[a], Jing-Yuan Zhang[a], Ya-Hong Liu[a], Mark A. Webber[c], David O. Ogbolu[c], Zhen-Ling Zeng[a], Laura J.V. Piddock[c,*]

[a] *College of Veterinary Medicine, National Reference Laboratory of Veterinary Drug Residues, South China Agricultural University (SCAU), Guangzhou 510642, China*
[b] *China Institute of Veterinary Drug Control, Beijing 100081, China*
[c] *Antimicrobials Research Group, School of Immunity and Infection, Institute of Microbiology of Infection and College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK*

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ABSTRACT

The aim of this study was to identify genes conferring resistance to fluoroquinolones and extended-spectrum β-lactams in non-typhoidal *Salmonella* (NTS) from food-producing animals in China. In total, 31 non-duplicate NTS were obtained from food-producing animals that were sick. Isolates were identified and serotyped and the genetic relatedness of the isolates was determined by pulsed-field gel electrophoresis of *Xba*I-digested chromosomal DNA. Antimicrobial susceptibility was determined using Clinical and Laboratory Standards Institute methodology. The presence of extended-spectrum β-lactamase (ESBL) and fluoroquinolone resistance genes was established by PCR and sequencing. Genes encoded on transmissible elements were identified by conjugation and transformation. Plasmids were typed by PCR-based replicon typing. The occurrence and diversity of numerous different transmissible genes conferring fluoroquinolone resistance [*qnrA*, *qnrD*, *oqxA* and *aac(6′)-Ib-cr*] and ESBLs (CTX-M-27 and CTX-M-14), and which co-resided in different isolates and serovars of *Salmonella*, were much higher than in European countries. Furthermore, different plasmids encoded fluoroquinolone resistance (ca. 6 kb) and β-lactam resistance (ca. 63 kb) and these co-resided in isolates with mutations in topoisomerase genes (*gyrA* and *parC*) giving very resistant *Salmonella*. The presence of multidrug-resistant bacteria in food-producing animals in countries that export foodstuffs suggests that global transfer of antibiotic resistances from country to country on food is possible.

1. Introduction

Non-typhoidal *Salmonella* (NTS) are one of the most common causes of food- and water-borne infections [1]. The emergence and transmission of multidrug-resistant (MDR) strains that may pass from food animals to humans is a public health concern [2]. Fluoroquinolones and extended-spectrum β-lactams are often the antimicrobials of choice for treating salmonellosis, especially for invasive *Salmonella* infections both in adults and children [1]. However, MDR *Salmonella* spp. with reduced susceptibility to these drugs are often reported worldwide [3–5]. Such resistance makes antibiotic therapy, when required, problematic. Whilst the morbidity of NTS infections is estimated to be 60%, the fatality rate of NTS is dependent upon the country and serovar, but is usually 1–4%. For the elderly and very young this rate is increased, especially in the developing world, and for antibiotic-resistant strains this rate is further increased by more than twofold [6].

Until 1998 it was thought that two mechanisms were responsible for fluoroquinolone resistance. First, mutations were described within the quinolone resistance-determining regions (QRDRs) of

* Corresponding author. Tel.: +44 121 414 6966; fax: +44 121 414 6819.
E-mail address: l.j.v.piddock@bham.ac.uk (L.J.V. Piddock).
[1] These two authors contributed equally to this work.

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the genes encoding the target bacterial topoisomerase enzymes. Second, decreasing intracellular drug accumulation by upregulation of innate MDR efflux pumps, either alone or together with decreased expression of outer membrane porins, also confers resistance [7]. Over the last decade, plasmid-mediated quinolone resistance (PMQR) has been increasingly recognised. PMQR determinants fall into one of three groups [8,9]: (i) Qnr proteins (A, B, C, D and S); (ii) a variant of an aminoglycoside acetyltransferase designated AAC(6′)-Ib-cr, which acetylates ciprofloxacin and norfloxacin; and (iii) the plasmid-mediated quinolone efflux pumps *oqxAB* and *qepA*. Plasmid-mediated quinolone efflux genes can export multiple drugs including aminoglycosides, fluoroquinolones and β-lactams [8].

Although these PMQR determinants confer only low-level resistance to fluoroquinolones [ciprofloxacin minimum inhibitory concentrations (MICs) typically 0.125–2 μg/mL] [10], the presence of PMQR (particular *qnr* genes) may provide a selective advantage for bacteria exposed to fluoroquinolones and facilitate development of high-level chromosomal quinolone resistance [8,10]. Concern has been raised over the apparent correlation between the presence of PMQR genes [such as *qnr* and *aac(6′)-Ib-cr*] and plasmid-encoded extended-spectrum β-lactamase (ESBLs) genes or plasmidic *ampC* genes [4,5,11]. Upon exposure to a fluoroquinolone or a β-lactam, this association would favour the co-selection of the relevant resistance genes and the maintenance and dissemination of these genes when one or other (fluoroquinolone or β-lactam) is absent [10,12].

The endogenous faecal flora of food-producing animals is considered a reservoir for antibiotic resistance genes. Zoonotic pathogens containing such genes may spread and transiently colonise the human gut [13–15] and/or transfer the antibiotic resistance genes to other bacteria. The purpose of the present study was to identify the genes conferring antibiotic resistance in NTS from food-producing animals in China.

2. Materials and methods

2.1. Bacteria and growth conditions

In total, 31 geographically representative, non-duplicate *Salmonella enterica* isolates were obtained from sick animals at Foshan University veterinary teaching hospital (Foshan, Guangdong, China) during November 2009 to February 2010, comprising 20 isolates from ducks, 9 from pigs and 2 from geese. *Salmonella* spp. were isolated by plating cloacal swabs into 4 mL of buffered peptone water (Difco, Cockeysville, MD) and incubating at 37 °C for 18–24 h, followed by subculture in 10 mL of selenite cysteine broth (Haibo Biological Technology Co., Ltd., Qingdao, China) at 37 °C for 24 h. A loop of inoculum from the selenite cysteine broth was streaked onto bismuth sulphite agar and Hektoen enteric agar (both from Haibo Biological Technology Co., Ltd.) and was incubated for 24 h at 37 °C. A minimum of two presumptive *Salmonella* colonies were picked from each plate and stabbed into triple sugar iron and lysine–iron agar slants (both from Haibo Biological Technology Co., Ltd.), respectively, and incubated for 24 h at 36 °C. Isolates with positive slant reactions were submitted to the China Institute of Veterinary Drug Control (Beijing, China) for identification using an API 20E system (bioMérieux sa, Marcy l'Étoile, France) and serotyping using slide agglutination with hyperimmune sera (S&A Company, Bangkok, Thailand). Isolates were identified as serovars *Salmonella* Indiana ($n = 21$), *Salmonella* Nitra ($n = 4$), *Salmonella* Batonrouge ($n = 2$), *Salmonella* Neftenbach ($n = 1$), *Salmonella* Bergues ($n = 1$), *Salmonella* Charters ($n = 1$) and *Salmonella* Hisingen ($n = 1$).

2.2. Antimicrobial susceptibility testing

MICs of antibiotics were determined by the agar doubling dilution procedure according to the standards and guidelines described by the Clinical and Laboratory Standards Institute (CLSI) [16]. The following antimicrobials were assessed: ampicillin; cefotaxime; ceftazidime; ceftriaxone; streptomycin; gentamicin; kanamycin; chloramphenicol; florfenicol; tetracycline; doxycycline; nalidixic acid; ciprofloxacin; and olaquindox. *Escherichia coli* ATCC 25922 strain was used as the control strain.

2.3. Detection of plasmid-mediated quinolone resistance determinants and mutations in the quinolone resistance-determining regions of topoisomerase genes

Detection of *qnr*, *aac(6′)-Ib-cr*, *qepA* and *oqxA* genes was performed using PCR as described previously [17]. All isolates containing PMQR determinants were also analysed for mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes. PCR amplification and DNA sequencing of the QRDRs of *gyrA* and *parC* was carried out as described previously [18]. Both strands of the purified PCR amplimers were sequenced.

2.4. Detection of β-lactamase-encoding genes among isolates with plasmid-mediated quinolone resistance determinants

All isolates harbouring PMQR determinants were screened using PCR as described previously for $bla_{\text{CTX-M}}$ groups, bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CMY} [17]. All PCR products obtained by amplification were sequenced at BGI Co. Ltd. (Beijing, China) and were aligned using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Genetic relatedness of Salmonella isolates with plasmid-mediated quinolone resistance genes

All isolates containing a PMQR gene were analysed by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested chromosomal DNA using a CHEF Mapper® System (Bio-Rad Laboratories, Hercules, CA). Test conditions were as follows: agarose concentration, 1%; voltage gradient, 6 V/cm; angle, 1200°; run time, 22 h; and temperature, 14 °C. A DNA ladder (48.5 kb concatemer) (Bio-Rad Laboratories) with 21 fragments from 50 kb to 1000 kb served as the internal standard. Bands of each isolate were assessed with reference to the fragments of the ladder. Resulting PFGE patterns were interpreted according to the method of Tenover et al. [19].

2.6. Plasmid transfer and analysis

Conjugation experiments were carried out with isolates carrying both PMQR and ESBL genes by the liquid mating-out assay using streptomycin-resistant *E. coli* C600 as the recipient strain, as described elsewhere [20]. Transconjugants were selected on tryptic soy agar plates (Haibo Biological Technology Co., Ltd.) containing streptomycin (1000 μg/mL) and cefotaxime (2 μg/mL) (both from Guangzhou Institute for Drug Control, Guangzhou, China). PCR-based replicon typing (PBRT) was performed on conjugative plasmids as described by Carattoli et al. [21]. Plasmid DNA was isolated from transconjugants using a QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Düsseldorf, Germany) according to the manufacturer's instructions. Plasmid size was estimated by comparison against plasmids isolated from *E. coli* V517 (Biotech, Dalian, China) after gel electrophoresis in a 0.8% agarose gel. Isolated plasmid DNA was also then electroporated into *E. coli* J53 and transformants were selected on either 0.25 μg/mL ciprofloxacin or 2 μg/mL cefotaxime