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High-levels of resistance to quinolone and cephalosporin antibiotics in MDR-ACSSuT *Salmonella enterica* serovar *Enteritidis* mainly isolated from patients and foods in Shanghai, China

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ABSTRACT

In this study, 2887 *Salmonella* strains were mainly obtained from patients and foods in Shanghai from 2006 to 2014 in order to assess the susceptibility to 16 antibiotics. Among them, 3.8% (110/2887) *S. Enteritidis* isolates were shown to have an ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) resistance pattern. The resistance genes of ACSSuT included sul2 (74.55%), flo (67.27%), tetA (49.09%), and aph (3)-IIa (46.36%). In addition, class 1 integron profiles were detected in 9 isolates, and 55.6% (5/9) were shown to carry resistant genes against aminoglycosides and sulfonamides. Moreover, these isolates had a high rate of resistance to nalidixic acid (95.29%), cefotaxime (70.64%), cefepime (58.72%), and ceftazidime (48.62%). Detection of quinolone genes showed that 93.64% (103/110) of the strains had gyrA single mutations (D87G, D87Y, D87N, S83Y, and S83F), where D87G was the dominant mutation in 55.45% isolates. 19.1% (21/110) isolates carried plasmid-mediated quinolone resistance (PMQR) genes (qnrB and aac(6′)-Ib-cr), and the most prevalent was qnrB. Furthermore, we also detected ESBLS genes. The most common were $bla_{CTX\text{-}M\text{-}55}$ (57.27%) followed by bla_{TEM} (23.6%) and bla_{OXY} (4.55%). *Mart*, *prot6E*, *steB*, *fimA*, and *sopE2* genes (100%) were the most in these isolates. The strains in the dominant PFGE profiles of G1 were all co-resistant to quinolones, cephalosporins, and ACSSuT, and were isolated from different sources. This suggests that existence of these genes lead to the emergence of high-levels of resistance to quinolone and cephalosporin in these ACSSuT resistance pattern isolates. And these isolates are transmitted between humans and food.

1. Introduction

Non-typhoidal salmonellosis (NTS) is an important public health problem worldwide. It is estimated that over 90 million global cases of gastroenteritis due to NTS occur each year, with 85% of those cases being linked to food (Hung et al., 2017). More than 2600 serotypes of *Salmonella* have been identified (Stevens et al., 2009). *Salmonella enterica serovar Enteritidis* (*S. Enteritidis*) is one of the most important serotypes globally. In the UK, 46,623 human *Salmonella* infections cases, 16.8% of these were due to *S. Enteritidis* which accounts for the largest proportion (Centers for Disease Control and Prevention, 2018). Moreover, *S. Enteritidis* is the most prevalent serotype that causes human infections in China (Zhang et al., 2014).

Multidrug-resistant (MDR) *Salmonella enterica* have become a significant global public health concern. Among MDR isolates, the ACSSuT resistance pattern (defined as resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) has attracted significant attention. The ACSSuT resistant phenotypes were first identified in *S. Typhimurium* DT104 in England in 1984 (Lauderdale et al., 2006; Threlfall et al., 1993; Yu et al., 2008). MDR-ACSSuT *S. Typhimurium* have been widely reported worldwide. The data from American NARMS, Taiwan, and China showed that the isolation rate has reached

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14.5%, 72.7%, and 28.8%, respectively (Centers for Disease Control and Prevention, 2010; Lauderdale et al., 2006; Wang et al., 2017). MDR-ACSSuT *S. Enteritidis* also have been found (Lauderdale et al., 2006). However, there is no specific report on MDR-ACSSuT *S. Enteritidis* at the global level.

Due to the rise of MDR in *Salmonella*, especially ACSSuT resistance patterns, quinolones or cephalosporins are commonly chose for treating gastrointestinal infections (Gilbert, 2016). Yet, this practice will positively select resistant isolates. And MDR-ACSSuT *Salmonella* co-resistant to quinolones and cephalosporins make treatment even more difficult. Moreover, quinolone, third generation cephalosporin, and ACSSuT resistant *S. Typhimurium* have been found in Taiwan and Spain but *S. Enteritidis* was not identified in these two regions (Campos et al., 2013; Lauderdale et al., 2006). Indeed, there is no report of high-levels of co-resistance to quinolones, cephalosporins, and ACSSuT *S. Enteritidis*. In this study, we analyzed the molecular characterization of MDR-ACSSuT *S. Enteritidis* isolates from 2006 to 2014 in Shanghai. We further assessed the susceptibility to quinolone and cephalosporin in these isolates.

2. Materials and methods

2.1. Specimen collection and isolate identification

Salmonella isolates were recovered from samples of clinically suspected patients, animals, foods and other sources in Shanghai from 2006 to 2014. *Salmonella* was isolated according to the U.S. FDA Bacteriological Analytical Manual (Andrews et al., 2014). Isolates with typical *Salmonella* phenotypes were further confirmed using API identification kits (bioMérieux, France) and O and H antigens were characterized using slide agglutination with salmonella diagnostic serum (S &A Reagents Lab, Bangkok, Thailand). The serological determination of *Salmonella* serotypes was performed in accordance with the Kauffmann-White scheme (Popoff et al., 2004).

2.2. Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was used in this study to examine the antimicrobial susceptibility of *Salmonella*. Disk diffusion assays were performed on Muller-Hinton agar with antibiotic impregnated disks (Oxoid, UK). The panel of the 16 antimicrobials tested was ampicillin 10 μg (AMP), amoxicillin/clavulanic acid 20/10 μg (AMC), cefotaxime 30 μg (CTX), ceftazidime 30 μg (CAZ), cefepime 30 μg (FEP), streptomycin 10 μg (S), sulfamethoxazole/trimethoprim 23.75/1.25 μg (SXT), sulfonamides 300 μg (S3), nalidixic acid 30 μg (NA), ofloxacin 5 μg (OFX), ciprofloxacin 5 μg (CIP), chloramphenicol 30 μg (C), imipenem 10 μg (IMP), gentamicin 10 μg (CN), methoxybenzyl aminopyrimidine 5 μg (W) and tetracycline 30 μg (TET). *E. coli* strain ATCC 25922 and ATCC 35218 were used as quality control strains. Results were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012).

2.3. Detection of ACSSuT resistance genes, integron encoding genes, and DNA sequencing

Salmonella isolates exhibiting the antimicrobial resistance profile of ACSSuT were analyzed by PCR assays. PCR screening resistance genes included catI, flo, tetA, tetB, tetC, aadA1, aadA2, aac(3)-IIa, ant(3)-Ia, aph(3)-IIa, sulI, and sulII (Chen et al., 2004). We detected integron encoding genes including class1 integron, intI1, and intI2 (Cui et al., 2015). Integron PCR products were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing. Sequence data were then analyzed by DNAstar (DNAstar Inc., Madison, WI, USA) and the sequences were aligned using GenBank online BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Detection of quinolone resistance genes and DNA sequencing

Quinolone resistance-determining regions (QRDRs) of gyrA and plasmid-mediated quinolone resistance (PMQR) determinants [qnrA, qnrB, qnrS, aac(6′)-Ib-cr, and qepA] were analyzed by PCR assays (Cui et al., 2015). We also performed DNA sequencing as described previously.

2.5. ESBL screening, detection of β-lactamase genes and DNA sequencing

Extended spectrum β-lactamase (ESBL) phenotype was assessed by using cefotaxime and cefotaxime (30 μg)/clavulanic acid (10 μg) disks and ceftazidime (30 μg)/clavulanic acid (10 μg) disks (Oxoid, UK) according to the double-disk synergy test method (DDST) (Pitout et al., 2003). The results were interpreted according to the standards of the CLSI (CLSI, 2012). PCR screening for the ESBL genes of MDR-ACSSuT *S. Enteritidis* isolates included bla_{CTX-M} groups, bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{OXY}, and bla_{CMY} (Hasman et al., 2005). DNA sequencing of bla_{CTX-M} was performed as described previously.

2.6. Detection of virulence genes

In order to study the virulence gene carrying of MDR-ACSSuT *Salmonella* isolates. We use PCR to analyze the virulence gene of these isolates. These genes refer to previous study, including *avrA*, *hil A*, *sifA*, *Mart*, *siiE*, *pipA*, *spvC*, *spvR*, *pef A*, *prot6E*, *rck*, *steB*, *tcfA*, *fimA*, *lpfD*, *cdtB*, *sopE2*, *stkc*, *sseI*, *irsA* (Kuang et al., 2015).

2.7. Pulse field gel electrophoresis (PFGE)

MDR-ACSSuT *Salmonella* isolates were subjected to molecular typing by pulsed-field gel electrophoresis (PFGE), which clonally evaluates isolates. PFGE was performed according to the PulseNet protocol (Ribot et al., 2006). PFGE was performed after digestion of genomic DNA with the restriction enzyme *Xba*I as described previously (Xu et al., 2017). *Salmonella* H9812 was used as a standard control strain. PFGE gel pictures were visualized using the molecular Imager Gel Doc XR System Universal Hood II (Bio-Rad Laboratories, CA, USA). The results were analyzed using Bionumerics software (Version 5.1; Applied-Maths, Sint-Martens-Latem, Belgium).

2.8. Statistical analysis

Comparison of frequencies was calculated by the Chi-squared test using SAS 9.2 (SAS Institute, Cary, N.C.). A P-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. S. Enteritidis isolation and identification

A total of 2887 *Salmonella* isolates were obtained from a variety of sample sources including clinically suspected patients, animals, foods and other sources in Shanghai from 2006 to 2014. 88.26% (2548/2887) *S. Enteritidis* isolates were isolated from patients where the age of patients ranged from 17 days to 96 years. The percentage of children under 5 years was 40.27% (1026/2548, $P < 0.01$) of all study patients. In addition, 52.59% (1340/2548) patients were male, with the ratio of males to females being 1.1:1 (Fig. 1). 11.74% (339/2887) of non-human isolates were mainly isolated from chickens (65.78% [223/339]), duck meat (13.86% [47/339]) and pork (13.86% [47/339]) (Table 3). Fig. 1B shows that all *S. Enteritidis* isolates were mainly cultured at the end of spring and summer, with a peak value being reached in August. However, the peak value of MDR-ACSSuT *S. Enteritidis* isolates was observed in May, and mainly cultured between May and September.