First description of plasmid mediated quinolone resistance genes in salmonella isolates from Saudi hospitals

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ABSTRACT

The objective of this study was to detect resistance genes to fluoroquinolones and β-lactams in Salmonella isolates from a Saudi hospital.

Methods: From October 2015 to December 2016, a total of 149 Salmonella strains were collected from stool specimens from patients admitted to King Fahad Hospital of the University, AlKhobar, Saudi Arabia using CHROMagar Salmonella. The organism identification and antimicrobial susceptibility testing were performed using Vitek 2 System. Strain serogrouping was performed using Wellcolex color Salmonella kit. Fluoroquinolone resistance genes, extended-spectrum β-lactamases (ESBLs), and AmpC β-lactamase were determined using polymerase chain reaction (PCR). Enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) was used to determine clonal relatedness.

Results: The resistance rates to cefotaxime were 1.3% and ciprofloxacin 19.5%. Plasmid mediated quinolone resistance (PMQR) genes, qnrB and qnrS, were detected in 8 strains, qnrB (n=5) and qnrS (n=3), respectively. No ESBLs, AmpC, or mutations in the topoisomerases were detected. Salmonella isolates formed 7 clusters with similarity.

Conclusions: This study reveals the emergence of fluoroquinolone resistant Salmonella in the region imposing public health concerns.
Acute gastroenteritis caused by *Salmonella* species is a worldwide health issue for humans and animals.\(^1,^3\) Although most cases of salmonellosis are uncomplicated, it can be very serious and life-threatening in infants, elderly, and immuno-compromised patients and may require antibiotic treatment.\(^1,^4\) Fluoroquinolones and cefalosporins are the drugs of choice for invasive salmonellosis treatment.\(^1,^5\)

The prevalence of *Salmonella* strains with resistance to fluoroquinolones and beta lactams have increased globally with important impact on hospitalization, therapeutic failure, and mortality.\(^2,^3\) Antimicrobial resistance to fluoroquinolones and cefalosporins has developed under selective antibiotic pressure in *Salmonella* species especially in food producing animals and it became a major concern.\(^5,^5\) The main mechanism of fluoroquinolone resistance is consequence of mutations in the quinolone resistance determining regions (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). The mutations confer high-level resistance.\(^6,^9\) The overexpression of efflux pumps is another fluoroquinolone resistance mechanism.\(^6,^9\) Plasmid mediated quinolone resistance (PMQR) genes, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac (6’)-Ib-cr*, and *qepA* are associated with low level resistance to fluoroquinolones with minimum inhibitory concentrations (MIC) around 0.12-4 μg/ml.\(^3,^7,^9,^10\) Plasmid mediated quinolone resistance genes are causing treatment failure and are an infection control concern since they are encoded on plasmids, mobile DNA elements, and can be easily acquired via horizontal transfer by different *Enterobacteriaceae species*.\(^3,^8,^10\) The major β-lactam resistance mechanism in *Salmonellae* is the production of β-lactamases such as extended spectrum β-lactamases (ESBLs) and plasmid mediated AmpC β-lactamases (pAmpC).\(^11-13\) According to literature, these β-lactamases can be co-harbored on plasmids along with PMQR genes.\(^1,^4\) There are few reports of *Salmonella* infections and antibiotic resistance in Saudi Arabia.\(^14,^15\) In addition, there are no reports delineating the molecular mechanisms of fluoroquinolones and/ or β-lactam resistance in *Salmonella* strains from Saudi hospitals. The aim of this study was to determine the prevalence of resistance to fluoroquinolones and β-lactams in *Salmonella* isolates from patients admitted to a Saudi hospital. In addition, this study also aimed to characterize the contribution of mutations in QRDRs (*gyrA*, *gyrB*, *parC*, and *parE*) and/or PMQR genes, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac (6’)-Ib-cr*, and *qepA* to fluoroquinolone resistance as well as the involvement of ESBLs and/or pAmpC β-lactamases in third generation cephalosporin resistance.

**Methods.** Salmonella identification and antimicrobial susceptibility testing. This study was conducted at King Fahad Hospital of the University (KFHU), Alkhobar, Saudi Arabia from October 2015 to December 2016. Non-duplicate specimens were collected from patients admitted to the hospital during the study period. The ethical committee reviewed and approved the study at Imam Abdulrahman Bin Faisal University (IRB 2017-01- 203). Screening for *Salmonella* was performed on stool specimens using Chromagar *Salmonella* (Chromagar, Paris, France) as instructed by the manufacturer. Pink colonies on Chromagar were confirmed to be *Salmonella* using Vitek 2 system (BioMe’rieux, Marcy l’Etoile, France). In addition, Vitek 2 system was used to identify *Salmonella* from specimens other than stool such as blood, wounds, and urine. Wellcolex color *Salmonella* kit (Remel Europe, London, UK) was used for *Salmonella* serotyping as instructed by the manufacturer. Vitek 2 AST-N291 card was used to detect antimicrobial susceptibility testing for trimethoprim-sulfamethoxazole, ampicillin, cefoxitin, imipenem, meropenem, cefotaxime, and cefepime. Susceptibility testing for ciprofloxacin was performed using E test strips (Epsilonometer assay; BioMe’rieux, Marcy l’Etoile, France). *Escherichia coli* (E. coli) ATCC 25922 strain and *Pseudomonas aeruginosa* ATCC 27853 strain were used as quality control strains. *Salmonella* strains not susceptible to ciprofloxacin MIC ≥0.12 μg/ml and/or resistant to cefotaxim MIC ≥4 μg/ml were included in this study. Minimum inhibitory concentrations results were interpreted using the criteria of Clinical and Laboratory Standards Institute (CLSI).

**Molecular analysis.** Polymerase chain reaction (PCR) method was used to detect genes responsible for resistance to fluoroquinolones and β-lactams using specific primers and conditions previously described (Table 1).\(^11,^12,^16\) Positive controls were used in each PCR. For fluoroquinolone resistant *Salmonella* strains, PMQR genes were tested using primers amplifying *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac (6’)-Ib-cr*, and *qepA*. In addition, *gyrA*, *gyrB*, *parC*, and *parE* were examined for QRDR mutations. *Salmonella* isolates resistant to third generation cephalosporins were tested for ESBL production using TEM, SHV, and CTX-M primers. Screening for pAmpC genes in strains resistant

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to third generation cephalosporins and/or cefoxitin was carried out using Philisa AmpC ID kit (Streck Company, Omaha, NE, USA) as recommended by the manufacturers. The kit detects the following pAmpC genes DHA, CMY, EBC, FOX, ACC, and MOX. ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) was used for amplicon sequencing.

Enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) was used to determine the clonal relatedness among Salmonella strains as previously described. Gel compar II software version 6 (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the DNA fingerprint patterns.

The variables investigated in the current study were non-continuous variables. Thus, the statistical methods used in the manuscript were only descriptive that included assessment of prevalence and percentage of Salmonella resistance to antimicrobial agents tested in this study.

**Results.** From October 2015 to December 2016, a total of 149 Salmonella species were isolated from patients admitted to KFHU. They were isolated from 76 females and 73 males with ages from 1 to 90 years and average of 25.3 years old. Children younger than 5 years old were most affected with 44 cases (29.5%). The most common source of these isolates was stool (n=140) strains followed by blood (n=6), urine (n=2), and wounds (n=1). The most common Salmonella serogroups detected were B (n=42), D (n=36), and C

| Target gene | Primer sequence (5’-3’) | Amplicon size (bp) | Reference |
|-------------|-------------------------|-------------------|-----------|
| qnrAF       | ATT TCT CAC GCC AGG ATT TG | 627              | 16        |
| qnrAR       | GAT CGG CAA AGG TTA GGT CA |                |           |
| qnrBF       | GAT CGT GAA AGC CAG AAA GG | 469              |           |
| qnrBR       | ACG ATG CCT GGT AGT TGT CC |                |           |
| qnrCF       | GGG TTT TAC AT TAT TTA ATC G | 307              |           |
| qnrCR       | CAC TTA CCC ATT TAT TTA CA |                |           |
| qnrDF       | CGA GAT CAA TTTA CGG GGA ATA | 533              |           |
| qnrDR       | AAC AAG CTG AAG CGC CTG |                |           |
| qnrSF       | ACG ACA TTC GTC AAC TCG AA | 417              |           |
| qnrSR       | TAA ATT GGC ACC CTG TAG GC |                |           |
| qepAF       | AAC TGC TTT AGC CCG TAG AT | 596              |           |
| qepAR       | GTC TAC GCC ATG GAC CTC AC |                |           |
| aac(6’)-Ib-crF | TTT CGA TGC TCT ATG AGT GGCTA | 482              |           |
| aac(6’)-Ib-crR | CTC GAA TGG CTC CGG TGG TT |                |           |
| gyrAF       | CGA CCT TGC GAG AGA AAF | 626              |           |
| gyrAR       | GAT CCA TCA GCC CCT CTT CAA |                |           |
| gyrBF       | CGG CTG TCG GAA CTG TAC CT | 181              |           |
| gyrBR       | TGA TCA GGC TGC CCA CIT CCA |                |           |
| parCF       | TAC GTC ATG GAC AGG | 460              |           |
| parCR       | GCC ACT TCA CTC AGG ATG TT |                |           |
| parEF       | TCT CTT CCG ATG AAG TCG TG | 240              |           |
| parER       | ATA CGG TAT AGC GGC GTG AG |                |           |
| CTX-M   grp1F | AAA AAT CAC TGC GGC AGT TC | 415              | 11        |
| CTX-M   grp1R | AGC TTA TTC ATC GGC AGC TT |                |           |
| CTX-M   grp2F | CGA CGC TAC CCC TGC TAT T' | 552              |           |
| CTX-M   grp2R | CCA GCG TCA GAT TTT TTA GG |                |           |
| CTX-M   grp9F | CAA AGA GAG TGC AAC GGA TG | 205              |           |
| CTX-M   grp9R | ATT GGA AAG CGT TCA TCA CC |                |           |
| CTX-M   grp8F | TCG CGT TAA GCG GAT GAT GC | 666              |           |
| CTX-M   grp8R | AAC CCA CGA TGT GGG TAG C |                |           |
| CTX-M   grp25F | GCA CGA TGA CAT TCG GG | 327              |           |
| SHVFSMU    | GCA AAA CGC CCG GTT ATT C | 940              | 12        |
| SHVRSMU    | GGT TAG CGT TGC CAG TGC T |                |           |
| TEMFSMU    | ATG AGT ATT CAA CAT TGC CG | 851              |           |
| TEMRSMU    | TTA ATC AGT GAG GCA CCT AT |                |           |

Table 1 - Primers used in this study.

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(n=21) isolates. Serogroups E (n=10), A (n=4), and G (n=3) were also detected. There were 33 untypable *Salmonella* strains.

All strains were susceptible to imipenem, meropenem, and cefepime. A total of 35 strains were resistant to ampicillin while 6 strains were resistant to cefoxitin which makes the rate of resistance to ampicillin (23.5%; 35/149) and cefoxitin (4%; 4/149). For the third generation cephalosporins, 2 strains were resistant to cefotaxime with a resistance rate of 1.3% (2/149). The resistance rate to trimethoprim-sulfamethoxazole was 17.4% (26/149). Based on Etest results, 29 out of 149 strains were resistant to ciprofloxacin with a resistance rate of 19.5% (29/149).

Using PCR, *qnrB* and *qnrS* were detected in 5 and 3 isolates, respectively (Table 2). No *qnrA*, *qnrC*, *qnrD*, *aac (6')-Ib-cr*, and *gcpA* genes were detected. In addition, no mutations in the QRDR region of *gyrA*, *gyrB*, *parC*, and *parE* were identified in these isolates. No ESBLs or pAmpC genes were detected in these isolates.

Using ERIC-PCR, *Salmonella* strains grouped into 7 clusters with clonal relatedness scores ranging from 89% to 98% (data not shown). Environmental samples collected from different hospital wards did not grow any *Salmonella* strains and no outbreaks were reported during the study duration.

### Discussion

Hospitalization, clinical therapeutic failure, and mortality due to fluoroquinolone resistant *Salmonella* have increased worldwide. It is interesting to know that most prevalent cases were identified in children younger than 5 years. Our data correlates with data published from China, Thailand, and USA. It is not clear why *Salmonella* is associated most with children younger than 5 years. Further epidemiological and immunological studies are needed to explain this association.

The prevalence of fluoroquinolone resistance in this study is 19.5% which is significant increase in the Eastern province of Saudi Arabia compared to 3% fluoroquinolone resistance rate in studies conducted in the same region from 2008-2011. Multiple factors can contribute to this increase of resistance including over the counter use of antibiotics and misuse of the antibiotics empirically when patients do not necessarily need antibiotic treatment. The fluoroquinolone resistance rate in this study is comparable to rates in other countries such as Palestine (15%), Philippines (14.9%), and Finland (21.3%) while it is higher than that in USA (2.4%), Hong Kong (7.1%), Sri Lanka (8%), and Ghana (6.6%). In addition, it is lower than fluoroquinolone resistance rates in other countries such as Taiwan (48.1%), Thailand (46.2%), Romania (60%), and Korea (36.5%). Plasmid mediated quinolone resistance genes, *qnrB* and *qnrS*, were detected in 8 strains while fluoroquinolone resistance mechanisms could not be identified in 21 strains. It is possible that resistance in these 21 strains may be due to the over-expression of efflux pumps. In addition, efflux pumps may contribute partly to resistance is the 8 strains encoding PMQR genes. However, we did not test for efflux pump overexpression in this study.

Plasmid mediated quinolone resistance genes confer low level fluoroquinolone resistance. However, their detection is crucial since they facilitate the selection of QRDR mutations which results in higher fluoroquinolone resistance. In addition, they are encoded on plasmids which can transfer between organisms creating a major infection control and public health concern. Plasmid mediated quinolone resistance genes can also be encoded with other antimicrobial resistance determinants such as ESBL limiting the therapeutic options.

The resistance to the third generation cephalosporins was 1.3% which is comparable to that in USA (4.1%) while it is lower than that in China (11%). Cefepime was fully susceptible in our study compared to 10% resistance in studies recently published from China. No ESBLs or pAmpC were detected in these isolates suggesting that resistance to cefotaxime was due to other mechanisms not examined such as permeability changes or other β-lactamases.

*Salmonella* strains were distributed in 7 clusters showing different susceptibility profiles. The strains were isolated from patients admitted to the hospital from different geographical locations in Saudi Arabia. In addition, no outbreaks were reported during the study period. Taken together, these data suggest that these cases are sporadic and not related to any outbreak.

### Table 2 - Characteristics of *Salmonella* strains harboring PMQR genes.

| Isolate # | Source | Serogroup | Ciprofloxacin (MIC interpretation) | PMQR Gene |
|-----------|--------|-----------|----------------------------------|-----------|
| 1         | Stool  | C         | (1.0, R)                          | *qnrB*    |
| 2         | Stool  | B         | (1.0, R)                          | *qnrB*    |
| 3         | Stool  | D         | (1.0, R)                          | *qnrB*    |
| 4         | Stool  | C         | (0.75, I)                         | *qnrS*    |
| 5         | Stool  | D         | (1.0, R)                          | *qnrS*    |
| 6         | Urine  | C         | (1.0, R)                          | *qnrB*    |
| 7         | Stool  | C         | (1.5, R)                          | *qnrB*    |
| 8         | Stool  | C         | (1.0, R)                          | *qnrS*    |

MIC - minimum inhibitory concentration (µg/ml), PMQR - plasmid mediated quinolone resistance, R - resistant, I - intermediate.
The performance of this study in a single center is a limitation because having multiple centers involved will present more comprehensive data regarding characterization of *Salmonella* strains and their susceptibility profiles phenotypically and genotypically. The overexpression of efflux pumps and changes of permeability were not tested which is another limitation factor.

In conclusion, this study showed the prevalence of fluoroquinolone resistance in Saudi Arabia. It also detected for the first time *Salmonella* isolates harboring plasmid mediated quinolone resistant determinants, *qnrB* and *qnrS*. Additionally, this article revealed the significance of conducting nationwide surveillance and epidemiological studies to determine the prevalence and antibiotic resistance mechanisms of an important organism such as *Salmonella*.

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