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

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ABSTRACT

الأهداف: الكشف عن الجينات المقاومة للفلوروكينولونات و اللاكتام في سلالات السالمونيلا من مستشفى سعودي.

الطريقة: من أكتوبر 2015م إلى ديسمبر 2016م، تم عزل ما مجموعه 149 سلالة من السالمونيلا من عينات البراز من المرضى المقبولين في جامعة الإمام عبد الرحمن بن فيصل ، الخبر ، المملكة العربية السعودية باستخدام أطباق السالمونيلا الكرومية. تم إجراء اختبار التعرف على الكائنات الحية واختبار الحساسية للمضادات الحيوية باستخدام نظام الفايتك 2 (Vitek 2).كما تم تحديد النوع المصلي لسلالات السالمونيلا باختبار ويلكوليكس .تم تحديد الجينات المقاومة للفلوروكينولون ، و الجينات المقاومة للاكتامات ذات الطيف الواسع (ESBLو (ampC) باستخدام تفاعل البلمرة المتسلسل. كما تم استخدام تفاعل البلمرة المتسلسل المترابط المتوالي الوراثي المعتمد على البكتريا لتحديد درجة صلة و تقارب سلالات السالمونيلا.

النتائج: كانت معدلات مقاومة السيفوتاكسيم وسيبروفلوكساسين 1.3٪ و 19.5٪ على التوالي. تم الكشف عن جينات مقاومة الكينولون المشفّرة على البلازميد ، qnrB و qnrS ، في 8 سلالات، 5(qnrB) و 3 (qnrS) . لم يتم اكتشاف أي مورث ESBLs أو ampC أو طفرات في topoisomerases. شكّلت سلالات السالمونيلا 7 مجموعات مع تشابه98%-89 .

الخاتمة: هذا هو أول تقرير للكشف عن الجينات المقاومة الكينولون المشفّرة على البلازميد في سلالات السالمونيلا في المملكة العربية السعودية. وتكشف هذه الدراسة عن ظهور السالمونيلا المقاومة للفلوروكينولون في المنطقة التي تفرض مخاوف على الصحة العامة.

Objectives: To detect resistance genes to fluoroquinolones and β-lactams in *Salmonella* strains 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.

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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 immuocompromised patients and may require antibiotic treatment.[1,4] Fluoroquinolones and cephalosporins 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 cephalosporins has developed under selective antibiotic pressure in *Salmonella* species especially in food producing animals and it became a major concern.[3,5] 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 *Salmonella* 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.

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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 (Epsilometer 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

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.[17] 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

Table 1 - Primers used in this study.

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 TTG TAC ATT TAT TGA ATC G	307	
qnrCR	CAC CTA CCC ATT TAT TTT CA		
qnrDF	CGA GAT CAA TTTA CGG GGA ATA	533	
qnrDR	AAC AAG CTG AAG CGC CTG		
qnrSF	ACG ACA TTC GTC AAC TGC AA	417	
qnrSR	TAA ATT GGC ACC CTG TAG GC		
qepAF	AAC TGC TTG AGC CCG TAG AT	596	
qepAR	GTC TAC GCC ATG GAC CTC AC		
aac(6')-Ib-crF	TTG CGA TGC TCT ATG AGT GGC TA	482	
aac(6')-Ib-crR	CTC GAA TGC CTG GCG TGT TT		
gyrAF	CGA CCT TGC GAG AGA AAT	626	
gyrAR	GTT CCA TCA GCC CTT CAA		
gyrBF	GCG CTG TCC GAA CTG TAC CT	181	
gyrBR	TGA TCA GCG TCG CCA CTT CC		
parCF	TAC GTC ATC ATG GAC AGG	460	
parCR	GCC ACT TCA CGC AGG TTG		
parEF	TCT CTT CCG ATG AAG TGC TG	240	
parER	ATA CGG TAT AGC GGC GGT AG		
CTX-M grp1F	AAA AAT CAC TGC GCC AGT TC	415	11
CTX-M grp1R	AGC TTA TTC ATC GCC ACG TT		
CTX-M grp2F	CGA CGC TAC CCC TGC TAT T	552	
CTX-M grp2R	CCA GCG TCA GAT TTT TCA 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	
CTX-M grp25R	AAC CCA CGA TGT GGG TAG C		
SHVFSMU	GCA AAA CGC CGG GTT ATT C	940	12
SHVRSMU	GGT TAG CGT TGC CAG TGC T		
TEMFSMU	ATG AGT ATT CAA CAT TTC CG	851	
TEMRSMU	TTA ATC AGT GAG GCA CCT AT		

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

Isolate #	Source	Salmonella 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

(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 *qepA* 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.[1,6] 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.[1,2,5,18] 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.[14] 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%).[1,4,5,19-21] 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%).[3,5,18] 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.[6-8,22] However, their detection is crucial since they facilitate the selection of QRDR mutations which results in higher fluoroquinolone resistance.[7,10,23] In addition, they are encoded on plasmids which can transfer between organisms creating a major infection control and public health concern.[10,23] Plasmid mediated quinolone resistance genes can also be encoded with other antimicrobial resistance determinants such as ESBL limiting the therapeutic options.[1,4]

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%).[1,2] Cefepime was fully susceptible in our study compared to 10% resistance in studies recently published from China.[2] 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.

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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