Evaluation of Plasmid-Mediated Quinolone Resistance associated with the Qnr Genes in Clinical Isolates of Shigella Spp. in Baghdad

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Abstract

Background Although quinolone resistance results mostly from chromosomal mutations in Enterobacteriaceae, it may also be mediated by plasmid-encoded qnr determinants. Shigella harboring the novel qnr plasmid-mediated mechanism of quinolone resistance has been described worldwide.

Objective To understand the distribution of serogroup of Shigella spp, as well as antimicrobial susceptibility and to investigate the plasmid mediated quinolone-resistant qnr genes in clinical isolates of Shigella spp. resistant to quinolone.

Methods Fifty nine clinical isolates of Shigella spp. were collected from two hospitals in Baghdad. Antimicrobial susceptibility tests were performed using disk diffusion test and minimum inhibitory concentration. The isolates were screened for the plasmid-mediated qnr genes of qnrA, qnrB, and qnrS by Multiplex polymerase chain reaction.

Results The isolation rate of Shigella spp. was 14% and observed to be high among children < 10 years and low in teenagers and adults. The highest percentage was Sh. flexneri (54.2%) followed by Sh. sonnei (37.3%) then Sh. dysenteriae (8.5%), while no Sh. boydii was found in this study. Antimicrobial susceptibility tests revealed that 54.23% and 49.2% of both Sh. flexneri and Sh. sonnei were resistant to nalidixic acid and ciprofloxacin, respectively, while Sh. dysenteriae isolates were fully susceptible to these antibiotics. The minimum inhibitory concentration value of resistant isolates of Sh. flexneri and Sh. sonnei ranged between 2-64 μg/ml and 32-512 μg/ml for ciprofloxacin and nalidixic acid, respectively. Multiplex polymerase chain reaction amplification of plasmid-borne qnrA, qnrB, qnrS genes revealed that the overall percentage of qnr-genes were (52.9%) distributed as (29.4%) qnrA, (20.6%) qnrS and (2.94%) qnrB detected alone or in combination. The genes were identified in (44.1%, 15/34) of quinolone resistance Shigella isolates.

Conclusion To our knowledge, this is the first report detected fluoroquinolone resistance due to the qnr gene among Shigella isolates in Iraq which is indicated that plasmid-mediated quinolone resistance has emerged in Iraqi pediatric patients.

Keywords Drug resistance, Shigella spp., Plasmid; Quinolone, qnr

List of abbreviation: FQ = Fluoroquinolone, QR = quinolone resistance, Cip = ciprofloxacin, DNA = Deoxyribonucleic acid, PMR = plasmid-mediated resistance, PMQD = plasmid-mediated quinolone determinants, PCR = polymerase chain reaction, NA = nalidixic acid, MIC = minimum inhibitory concentration.

Introduction Shigellosis, an acute diarrhoeal disease, remains a major public-health problem especially in children in developing countries. Worldwide, an estimated 165 million cases of Shigellosis, of which 163 million are in developing countries. Two-thirds of them concern less than 10 years-old children. There are four species of Shigella: Sh. dysenteriae, Sh. flexneri, Sh. boydii and Sh. sonnei. Appropriate antimicrobial therapy shortens the duration of symptoms and can prevent life-threatening complications. Fluoroquinolones (FQ) are broad-spectrum antibiotics.
agents that have excellent activity against most enteric pathogens particularly against gram-negative bacteria. Ciprofloxacin (Cip) perhaps the most important as well as the most used FQ. According to the World Health Organization (WHO) revised guidelines for the control of Shigellosis, Cip is now the drug of choice for all patients with bloody diarrhoea. However, the increased use of FQ has led to increasing resistance to these antimicrobials.

Quinolone resistance (QR) in Enterobacteriaceae results mainly from mutations in type II Deoxyribonucleic acid (DNA) topoisomerase genes or changes in the expression of outer membrane and efflux pumps. Studies have shown that plasmid-mediated resistance mechanisms also play a significant role in QR, and its prevalence is increasing worldwide. The plasmid-mediated resistance (PMR) gene qnr is a member of the pentapeptide repeat family of proteins and has been shown to block the action of Cip on purified DNA gyrase and topoisomerase IV. Several members of qnr determinants were identified and labeled as qnrA, qnrB, qnrS, qnrC and qnrD, while amino acid variations are indicated in numbering. These genes have a wide geographic distribution mainly in Enterobacteriaceae. qnrA is encoding a 218 amino acid protein of the pentapeptide family. The first qnrS gene was detected in 2003, in single clone of Shigella flexneri 2b was resistant to FQ, caused an outbreak of enterocolitis in Japan. The most heterogenous cluster of the qnr gene family is qnrB, having 47 different alleles. The mechanism of the qnr protective effect is not completely understood. It has been shown that qnrA can bind to the DNA gyrase holoenzyme as well as to its respective subunits, gyrA and gyrB. This binding occurred in the absence of relaxed DNA, Cip, or ATP, indicating that the binding of qnrA to gyrase did not require the presence of the ternary complex of enzyme, DNA, and quinolone. Similar findings were also reported for qnrA and topoisomerase IV. The direct effect of qnrA is the reverse of the inhibition of gyrase-mediated DNA supercoiling caused by Cip minimizing opportunities for these agents to stabilize the lethal gyrase-DNA-quinolone complex. This study is designed to determine the susceptibility of Shigella species isolated from two hospitals in Baghdad against quinolone group and to detect the prevalence of plasmid-mediated quinolone determinants (PMQD) like qnrA, qnrB, qnrS by polymerase chain reaction (PCR) in Shigella spp.

**Methods**

**Patients and microbial identification**

A total of 59 Shigella spp. were isolated from 420 fresh stool specimens were collected from patients presenting with acute diarrhea from two hospitals in Baghdad; Children Welfare Hospital (Al-Mansour) and Al-Imamain Al-Kadhmain Medical City hospital during a period between 1st Jun. 2010 and 31st May 2011. The patient’s age were ranging from 5 months to 62 years. All specimens transferred to the laboratories of Al-Nahrain Medical College and incubated overnight in Selenite F broth then plated onto MacConkey, XLD and Salmonella-Shigella agar and incubated at 37°C for 24 hr in aerobic environment. The colorless non-lactose-fermenting colonies suggestive of Shigella were sub-cultured on nutrient agar and broth and were biochemically identified. Api20E was used to confirm the diagnosis and further identification at a group level by slide agglutination test with specific antisera was done.

**Antimicrobial susceptibility tests**

A total of 59 Shigella isolates were tested for susceptibility to quinolone group [Nalidixic acid (NA) and Cip] by Disk diffusion method in accordance to Clinical and Laboratory Standard Institute using E. coli ATCC25922 as a standard strain and decided as susceptible (S) and resistant (R). The minimum inhibitory concentrations (MICs) of NA and Cip for the resistant Shigella isolates were performed using agar dilution method according to Clinical and Laboratory Standards Institute and Wiegand et al recommendations.
Plasmid DNA extraction
Plasmid DNA was isolated from *Shigella* spp. according to Heringa et al.\textsuperscript{(22)} using modified alkaline lysis method. The supernatant containing plasmid DNA subjected to electrophoresis and used as a template for PCR experiments to detect the presence of *qnr* genes. To estimate the size of plasmid DNA and PCR products, 1kb and 100bp DNA Marker (Lambda DNA cut with Hind-II) were used respectively.

Multiplex PCR-based screening for *qnr* genes
Multiplex PCR was done by modification of previously described PCR protocol\textsuperscript{(23)} for PCR amplification of PMQR *qnrA*, *qnrB* and *qnrS* genes. The amplification was performed using GoTaq Green Master Mix, specific primers sequences for *qnrA*, *qnrB* and for *qnrS* (1.5 forward and 1.5 reverse for each primer) and plasmid DNA of *Shigella* isolates as a template for PCR experiments (Table 1 and table 2).

| Table 1. Sequences and products of PMQR determinants (*qnrA*, *qnrB*, and *qnrS*) |
|-------------------|-------------------|-------------------|-------------------|
| **qnr genes**    | **Nucleotide Sequences** (5’ → 3’) | **Products bp** | **References** |
| *qnrA*           | F: GATAAAGTTTTTTCAGCAAAGG ATCCAGATCGGCAAGGTAT | 593             | Jacoby et al 2003 \textsuperscript{(24)} |
|                  | R: GATCCAGAAGGCAAGGG AAATGCCGATCGGCAAGGTAT |                  |                |
| *qnrB*           | F: GATCGTGAAGGCCAGAAAAGG ACGATGCCTGGTATGGTCC  | 469             | Robicsek et al 2006 \textsuperscript{(11)} |
|                  | R: TGGAAACCTACATACTACATATCG TTAGTCAAGGATAAAACAAATACCC  |                  |                |
| *qnrS*           | F: TGGAAACCTACATACTACATATCG TTAGTCAAGGATAAAACAAATACCC  | 656             | Pu et al., 2009 \textsuperscript{(25)} |
|                  | R: TGGAAACCTACATACTACATATCG TTAGTCAAGGATAAAACAAATACCC  |                  |                |

F = forward, R = reverse.

| Table 2. Concentrations of the components of PCR master mixture of different *qnr* genes used for multiplex PCR |
|---------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------------------|
| **Components** | **Volume/µl** | **Final concentration** |
| Green Master Mix, 2x                                       | 12.5 µl                               | 1x |
| *qnrA*          | Forward       | 1.5 µl               | 30pmol |
| *qnrB*          | Reverse       | 1.5 µl               | 30pmol |
| *qnrS*          | DNA template  | 2 µl                 | 30pmol |
|                 | ddH2O         | 1.5 µl               | 30pmol |
|                 | Total         | 25 µl                | 30pmol |

The cycling was performed using protocol showed in table 3. Multiplex PCR products were resolved by horizontal agarose gel electrophoresis and visualized under UV trans-illuminator using digital camera (Sony-Japan).

| Table 3. The conditions of PCR amplification steps for *qnr* genes |
|---------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------------------|
| **Steps**         | **Temperature** | **Time** | **Cycles** |
| Initial denaturation | 95 °C          | 10 min   |            |
| Denaturation       | 95 °C          | 45 sec   | 35         |
| Annealing          | 60 °C          | 45 sec   |            |
| Elongation         | 72 °C          | 1 min    |            |
| Final extension    | 72 °C          | 10 min   |            |
| Hold               | 4 °C           |          |            |
Statistical analysis
The significance of differences in proportions was analyzed by the Chi-square test using statistical package for social sciences (SPSS) version 15 and *P* values equal or less than 0.05 were considered statistically significant.

Results
*Shigella* spp. was isolated from 59 (14%) of 420 stool samples. Isolation rate of *shigella* spp. was observed to be high among children 5 m – 10 yr (93.2%, 55/59) and low in teenagers and adults (6.8%, 4/59). Statistically, the highest proportion of stool specimens infected with *Shigella* spp. was in the age group (5 month – 10 years) and there is significant association between this age group and *Shigella* infection. The highest percentage of *Shigella* isolates were *Sh. flexneri* (54.2%, 32/59) followed by *Sh. sonnei* (37.3%, 22/59) then *Sh. dysenteriae* (8.5%, 5/59), while no *Sh. boydii* was found in this study.

Antibiotic resistance of *Shigella* isolates
By the disc-diffusion method, 16 and 18 isolates of *Sh. flexneri* and 13 and 14 isolates of *Sh. sonnei* were resistant to Cip and NA in percentage reached to 49.2% and 54.23% (29 and 32 out of 59), respectively (Table 4).

Table 4. Number and percentage of resistant *Shigella* isolates to quinolone group

<table>
<thead>
<tr>
<th>AB</th>
<th><em>Sh. flexneri</em> (32)</th>
<th><em>Sh. sonnei</em> (22)</th>
<th><em>Sh. dysenteriae</em> (5)</th>
<th>Total (59)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>CIP</td>
<td>16</td>
<td>50.0</td>
<td>13</td>
<td>59.1</td>
<td>0</td>
</tr>
<tr>
<td>NA</td>
<td>18</td>
<td>56.3</td>
<td>14</td>
<td>63.6</td>
<td>0</td>
</tr>
</tbody>
</table>

Of these, 15 isolates of *Sh. flexneri* and 12 isolates of *Sh. sonnei* were resistant to both antibiotics. The MIC values of Cip and NA ranged from 2 to 64 μg/ml and from 32 to 512 μg/ml, respectively in all spp. except for *Sh. dysenteriae* which was susceptible to these antibiotics (Fig. 1 and Fig. 2).

Plasmid profile
In this study, analysis of plasmid DNA revealed that, all of the isolates contained multiple plasmids (2-8 plasmid bands), their molecular size ranged from (0.5 kb to more than 10 kb) forming a number of unique banding patterns. The total number of plasmid profiles was 14 of *Sh. flexneri* and 11 of *Sh. sonnei*. Mega Plasmids of the same size >10 kb which appeared before the chromosomal DNA were present in multiple strains (Fig. 3 & 4).

Fig. 1. MIC results of *Shigella* spp. resistant to Ciprofloxacin

Fig. 2. MIC results of *Shigella* spp. resistant to Nalidixic acid

Multiplex PCR screening for *qnr* A, B, S genes
Nineteen isolates of *Shigella flexneri* and 15 of *Shigella sonnei* resistant to quinolone group and contain multiple plasmids were screened for the presence of the PMQR genes *qnrA*, *qnrB*, and *qnrS* by multiplex PCR. In this study, the
prevalence of overall qnr-genes were 18/34 (52.9%), (Table 4).

The genes were identified in 15/34 (44.1%,) of Shigella isolates. As it is shown in table 5, the qnrA gene was the most common (29.4%) in Shigella spp. resistant to quinolone followed by qnrS (20.6%), whereas only one isolate of Sh. sonnei was positive for qnrB (2.94%) as shown on fig. 5 and fig. 6.

**Table 5. The prevalence of qnr genes in Shigella spp. resistant to quinolone**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sh.flexneri N= 19 N (%)</th>
<th>Sh.sonnei N = 15 N (%)</th>
<th>Total N = 34 N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnrA</td>
<td>6 (31.58)</td>
<td>4 (26.7)</td>
<td>10 (29.4)</td>
</tr>
<tr>
<td>qnrB</td>
<td>0 (0.0)</td>
<td>1 (6.7)</td>
<td>1 (2.94)</td>
</tr>
<tr>
<td>qnrS</td>
<td>5 (26.32)</td>
<td>2 (13.3)</td>
<td>7 (20.6)</td>
</tr>
<tr>
<td>Total</td>
<td>11 (57.9)</td>
<td>7 (46.7)</td>
<td>18 (52.94)</td>
</tr>
</tbody>
</table>

**Discussion**

Diarrheal disease is major cause of morbidity and mortality in the developing world \[^{26}\]. Like many other developing countries, diarrheal diseases are among the main health problems in Iraq. This is attributable to personal hygiene and sanitary conditions which promote spread of organisms like *Shigella* and other enteric pathogens \[^{27}\].

In the current study, the prevalence of Shigellosis among diarrheal patients was 14%, which is less than that of previous report from Iraq (26.1 %) \[^{28}\]. The predominant species of *Shigella* isolated in the present study was *Sh. Flexneri* (54.2%), followed by *Sh. Sonnei* (37.3%) then *Sh. dysenteriae* 5(8.5%), while no *Sh. boydii* was found. This distribution is very close to that seen in India by Bhattacharya et al \[^{29}\] when they found that *Sh. flexneri* was the dominant strain isolated followed by *Sh. sonnei* and *Sh. dysenteriae* but not *Sh. boydii*.

The variation according to the geographical area suggested that the factors involved in natural selection may have been the main reason for these discrepancies. The average age of patients with *Shigella* infection in our study was similar to Ranjbar et al \[^{30}\] who found that the highest frequency of isolation of *Shigella spp.* was seen among the patients with 1 to 5 years old and our results of other age groups are similar to other studies \[^{31}\]. Children within this age-group are most susceptible because of poor resistance,
lack of previous exposure, poor personal hygiene (32). The antibiotic resistance of *Shigella* spp. has been hindering the treatment of Shigellosis, particularly in children (1).

The current study showed that all *Shigella* spp. presented a similar resistance profile for quinolone except for *Sh. dysenteriae*, which was fully susceptible to Cip and NA. This result was far from the findings of Talukder et al (33) when they previously found that nine strains of *Sh. dysenteriae* between 2002 and 2003 from South Asia were resistant to NA and Cip, with high MIC values. On the other hand, in Bangladesh (34) reported results nearly similar to ours when they found that, about 51% of *Shigella* isolates were resistance to NA, and Srinivasa et al (35) reported that, the rate of FQR including Cip in 2004 was 5.9% and gradually increased to 48.5% in 2007.

In Iraq, Munim et al (28) found that 18.18% of *Shigella* isolates are resistant to Cip in 2008. Furthermore, Bhattacharya et al (29) found that 96% of *Sh. spp.* was resistant to NA and 82% of them to Cip.

The MIC values of NA and Cip, in the current study were (2-64 μg/ml) and (32-512 μg/ml), respectively in all spp. These results was included in the result of Bhattacharya et al (29) who found that MIC values were 0.5 to >256 μg/ml for NA and 1 to >256 μg/ml for Cip in different *Sh.* spp. These results may reflect the broad MIC values of quinolone group in *Sh.* spp. and these ranges of resistance are alarming. If *Sh.* spp. become resistant to such high levels of antibiotics, the treatment of disease with antibiotics would become quite difficult.
present at one or more copies per cell. This provides an additional survival mechanism for the bacteria. PMQR determinants (qnrA, qnrB and qnrS) were recently reported worldwide in many strains of Enterobacteriaceae, including Shigella. In this study, multiplex PCR technique showed that, PMQR determinants were present in (44.1% 15 of 34) of the isolates with qnr gene detected alone or in combination (3 isolates carried two types of qnr), and in a significant percentage (P = 0.01) reached to (52.9%, 18 gene) distributed as (29.4%, 10 qnrA), (20.6%, 7 qnrS) and (2.94 %, 1 qnrB). Similar findings were reported by Xiong et al. who found that PMQR determinants were present in (53.8%, 14 of 26) of Sh. flexneri isolates and qnrA1, qnrS1, qnrS2, were present in (30.8%, 11.5% and 3.8%), respectively. Hata et al in 2005 was the first to describe qnrS in Sh. flexneri isolates resistant to NA and Cip in Japan. The qnrS gene has ~60 and ~50% homology to the gene of qnrA and qnrB, respectively. The progenitor of qnr gene is bacteria widely distributed in aquatic environments and rarely involved in human infections. The genes of these determinants are plasmid localized and can be horizontally transferred. Although these PMQR determinants confer low-level resistance to quinolones and / or FQs, they are a favorable background for selection of additional chromosome - encoded QR mechanisms.

In conclusion: Sh. flexneri was the predominant spp. in Iraq. All spp. contains multiple plasmids and the prevalence of overall PMQR qnr-genes were (52.9%,18/ 34), these genes were identified in (44.1%, 15/34) of Shigella isolates.

Acknowledgements
The authors are greatly thankful to the staff of laboratories in Al-Imamein Al-Kadhimein Medical City, Al-Mansour Hospital and Central Public Health Laboratory for their support and participation in the research.

Author contribution
Dr. Abdulrahman prepare, perform and did the tests and interpret the results of the research; Jamal and Kadhim help in sampling and Dr. Belal supervise this paper as part from a thesis.

Conflict of Interest
No conflict of interest

Funding
Personal Funding

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Received: 18th Nov. 2014: Accepted 14th Jan. 2015.