

## 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

**S**higellosis, an acute diarrhoeal disease, remains a major public-health problem especially in children in developing countries <sup>(1)</sup>. Worldwide, an estimated 165

million cases of Shigellosis, of which 163 million are in developing countries <sup>(2)</sup>. Two-thirds of them concern less than 10 years-old children <sup>(3)</sup>. There are four species of *Shigella*: *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei* <sup>(4)</sup>. Appropriate antimicrobial therapy shortens the duration of symptoms and can prevent life-threatening complications <sup>(5)</sup>. Fluoroquinolones (FQ) are broad-spectrum

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<sup>(6)</sup>. 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<sup>(7)</sup>. However, the increased use of FQ has led to increasing resistance to these antimicrobials<sup>(8)</sup>.

Quinolone resistance (QR) in *Enterobacteriaceae* results mainly from mutations in type II Deoxyribonucleic acid (DNA) topoisomerase genes<sup>(9)</sup> or changes in the expression of outer membrane and efflux pumps<sup>(10)</sup>. Studies have shown that plasmid-mediated resistance mechanisms also play a significant role in QR, and its prevalence is increasing worldwide<sup>(11-13)</sup>. 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<sup>(14)</sup>. Several members of *qnr* determinants were identified and labeled as *qnrA*, *qnrB*, *qnrS*, *qnrC* and *qnrD*, while amino acid variations are indicated in numbering<sup>(15)</sup>.

These genes have a wide geographic distribution mainly in *Enterobacteriaceae*<sup>(16)</sup>. *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<sup>(17)</sup>. The most heterogenous cluster of the *qnr* gene family is *qnrB*, having 47 different alleles<sup>(18)</sup>. 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<sup>(14)</sup>. Similar findings were also reported for *qnrA* and topoisomerase IV<sup>(19)</sup>. 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<sup>(11)</sup>. 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 1<sup>st</sup> Jun. 2010 and 31<sup>st</sup> 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<sup>(20)</sup> 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<sup>(20)</sup> and Wiegand *et al*<sup>(20)</sup> recommendations.

**Plasmid DNA extraction**

Plasmid DNA was isolated from *Shigella* spp. according to Heringa *et al* <sup>(22)</sup> 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-III) were used respectively.

**Multiplex PCR-based screening for *qnr* genes**

Multiplex PCR was done by modification of previously described PCR protocol <sup>(23)</sup> 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
<i>qnrA</i>	F	GATAAAGTTTTTCAGCAAGAGG	593	Jacoby <i>et al</i> 2003 <sup>(24)</sup>
	R	ATCCAGATCGGCAAAGGTTA		
<i>qnrB</i>	F	GATCGTGAAAGCCAGAAAGG	469	Robicsek <i>et al</i> 2006 <sup>(11)</sup>
	R	ACGATGCCTGGTAGTTGTCC		
<i>qnrS</i>	F	TGGAACCTACAATCATACATATCG	656	Pu <i>et al.</i> , 2009 <sup>(25)</sup>
	R	TTAGTCAGGATAAACAACAATACCC		

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
<i>qnrA</i> <i>qnrB</i> <i>qnrS</i>	Forward	1.5 µl	30pmol
	Reverse	1.5 µl	30pmol
DNA template		2 µl	
ddH2O		1.5 µl	
Total		25 µl	

The cycling was performed using protocol showed in table 3. Multiplex PCR products were resolved by horizontal agarose gel electro-

phoresis 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.

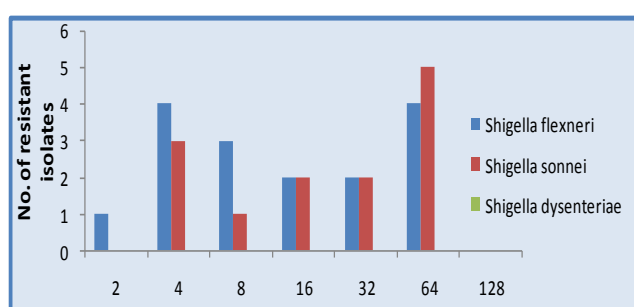
### Antibiotic resistance of *Shigella* isolates

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**

AB	<i>Sh. flexneri</i> (32)		<i>Sh. sonnei</i> (22)		<i>Sh. dysenteriae</i> (5)		Total (59)	%	
	No.	%	No.	%	No.	%		R	S
CIP	16	50.0	13	59.1	0	0	29	49.2	50.8
NA	18	56.3	14	63.6	0	0	32	54.23	45.8

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).

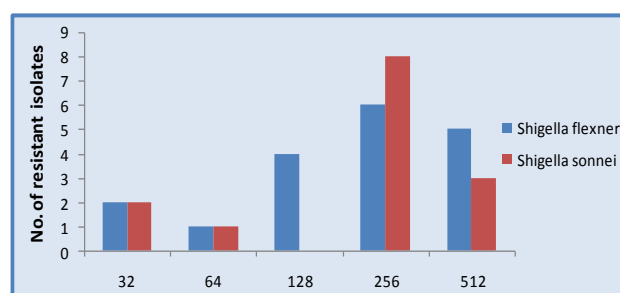


**Fig. 1. MIC results of *Shigella* spp. resistant to Ciprofloxacin**

### 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. 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).

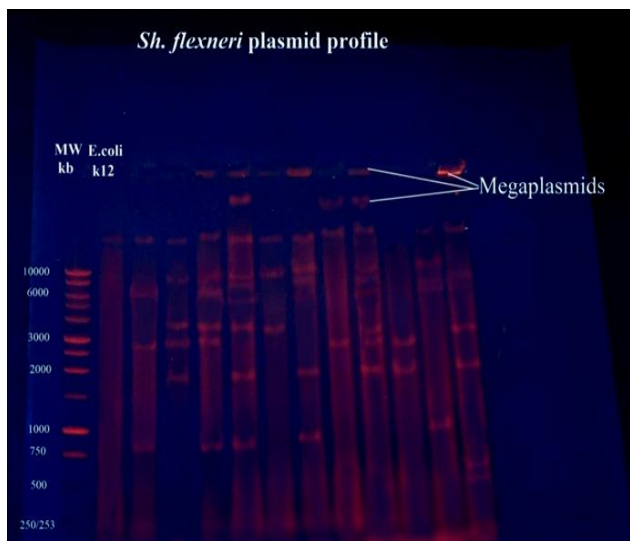


Fig. 3. Gel electrophoresis of *Shigella flexneri* isolates show plasmid profile (0.7% agarose, 7 v/cm, 2 hrs)

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.

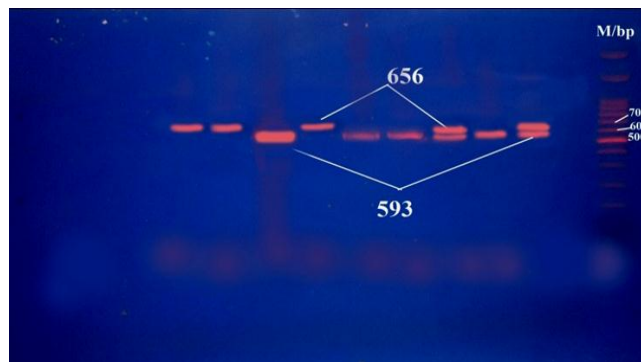


Fig. 4. Gel electrophoresis of *Shigella sonnei* show plasmid profile (0.7% agarose, 7 v/cm, 2 hrs)

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

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

### Discussion

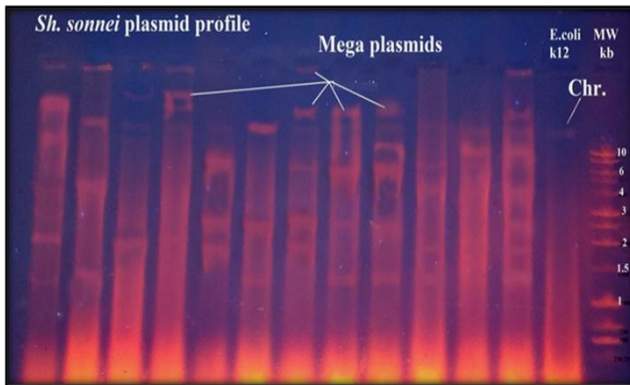
Diarrheal disease is major cause of morbidity and mortality in the developing world<sup>(26)</sup>. Like many other developing countries, diarrheal diseases are among the main health problems in Iraq. This is attributable to personal hygiene and sanitary conditions which promote spread of organisms like *Shigella* and other enteric pathogens<sup>(27)</sup>.

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 %) <sup>(28)</sup>. The predominant species of *Shigella* isolated in the present study was *Sh. Flexeneri* (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*<sup>(29)</sup> 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*<sup>(30)</sup> 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<sup>(31)</sup>. Children within this age-group are most susceptible because of poor resistance,

lack of previous exposure, poor personal hygiene<sup>(32)</sup>. The antibiotic resistance of *Shigella* spp. has been hindering the treatment of Shigellosis, particularly in children<sup>(1)</sup>.



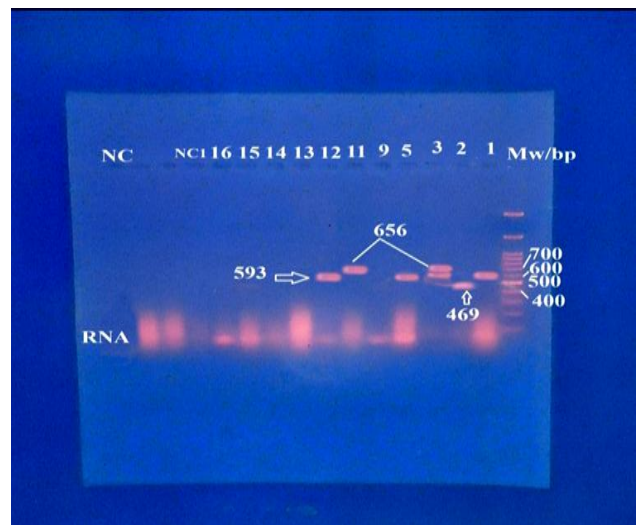
**Fig. 5. Gel electrophoresis of Multiplex PCR positive products for *qnrA* (593 bp) and *qnrS* (656 bp) of *Sh. flexneri* isolates. Lane 1 (M/bp): 100bp DNA ladder; lane 2: Negative control (DW); lane (3,4,5,6,7,9): *Sh. flexneri* positive isolates for *qnrA* gene; lane (3,5,8,10,11): *Sh. flexneri* positive isolates for *qnrS* gene; (1% agarose, 7 v/cm<sup>2</sup>, 1.5hrs)**

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*<sup>(33)</sup> 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<sup>(34)</sup> reported results nearly similar to ours when they found that, about 51% of *Shigella* isolates were resistance to NA, and Srinivasa *et al*<sup>(35)</sup> 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*<sup>(28)</sup> found that 18.18% of *Shigella* isolates are resistant to Cip in 2008. Furthermore, Bhattacharya *et al*<sup>(29)</sup> 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*<sup>(29)</sup> 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.



**Fig. 6. Gel electrophoresis of Multiplex PCR products for *qnrA*, *qnrB*, *qnrS* of *Sh. Sonnei* positive isolates. Lane M/bp: 100bp DNA ladder; lane (1,3,5,12): *qnrA* gene (593 bp) positive isolates; lane (2): *qnrB* (469bp) positive isolate; lane (3,11) *qnrS* (656 bp) positive isolates; NC1: Negative control (DW); NC: quinolone negative isolate; (1% agarose, 7 v/cm<sup>2</sup>,1.5hrs).**

The results showed that all *Shigella* 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 (14 of *Sh. flexneri*, 11 of *Sh. Sonnei*). This result was nearly similar to that of Ahmed *et al*<sup>(32)</sup> who found that (96.7%) of *Sh.* isolates harbored (1 to 9) plasmids and the number of plasmids were highly variable for all spp. This finding was also reported by Munim *et al*<sup>(28)</sup> who they identified numerous plasmid patterns in the 11 *Sh.* isolates in Iraq. Plasmids are

present at one or more copies per cell. This provides an additional survival mechanism for the bacteria<sup>(36)</sup>. PMQR determinants (*qnrA*, *qnrB* and *qnrS*) were recently reported worldwide in many strains of *Enterobacteriaceae*, including *Shigella*<sup>(37,38)</sup>.

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*<sup>(39)</sup> 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<sup>(17)</sup> 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<sup>(40)</sup>.

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.

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### 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

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