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Molecular characterization of quinolones resistant Salmonella typhi isolates from patients infected with Typhoid fever in Al-Najaf province, Iraq

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ABSTRACT

Background: The global spread of typhoid fever is still a major public health concern. Typhoid fever is a potentially fatal infection often treated with quinolones, especially fluoroquinolones. Resistance to quinolones antibiotics in Salmonella typhi has made it harder to treat and has led to more mortalities. In this study, we detected of the qnr genes (plasmid-mediated quinolone resistance PMQR) in S.typhi isolated from patients in two main hospitals in AL-Najaf province, Iraq and determined the clonal relatedness between S.typhi isolates carried qnr genes.

Materials and Methods: Antimicrobial susceptibility tests were performed using the disc diffusion method to investigate the ability of S. typhi to the resistance of 10 antibiotics. Qnr genes (qnrA,qnrB,qnrS) were detected by PCR amplification, and clonal relatedness between S.typhi isolates harbor qnr genes was analyzed following multilocus sequence typing (MLST).

Results: Out of 246 blood samples collected from patients with suspected typhoid fever, 32 (13%) cases of S. typhi were identified using culture methods and confirmed using an automated Vitek-2 system. The highest antibiotic resistance rates were for ampicillin (24/32; 75%) and levofloxacin (19/32; 59.3%). All isolated (100%) were susceptible to ceftriaxone, cefixime, imipenem, meropenem, and azithromycin. Among PMQR genes determinants, qnrA, qnrB, and qnrS were positive in (1/32; 3%), (4/32; 12.5%) and (3/32; 9.3%) of the isolates, respectively. ST19, ST34, and ST36 were identified in (5/8; 62.5%), (2/8; 25%), and 1 (1/8; 12.5%) in S. typhi isolates positive for qnr genes, respectively.

Conclusion: The first study from Iraq demonstrated the presence of qnr genes in S. typhi clinical isolates. In comparison to qnrS and qnrA, the qnrB gene was more common. The first study used multilocus sequence typing (MLST) to identify the sequence types of S. typhi isolates from hospitals in Al-Najaf. The Majority of ST19 sequence type of S. typhi was found.

Keywords: *Typhoid fever, PMQR, qnrB, MLST*

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INTRODUCTION

Salmonella typhi infections in humans are a big public health problem worldwide, especially in the Middle East and South Asia, where sanitation is inadequate in low- and middle-income nations(1,2). This bacterium is the causative agent of typhoid fever, a potentially fatal bloodstream infection. In many parts of the developing world, it has significantly contributed to morbidity and mortality rates(3). Over 21 million people are infected with typhoid fever every year, and regrettably, many of them will die(4).

Typhoid infections are becoming increasingly resistant to many antimicrobials because they have been repeatedly treated with the same antibiotic. As a result, antibiotic-resistant strains emerge and spread more often, worsening the situation(5,6). In recent years, S. typhi has developed greater resistance to various antibiotics, including chloramphenicol, ampicillin, ciprofloxacin, and levofloxacin, as well as another type of antimicrobials, and is therefore referred to as multidrug-resistant (MDR)(7). However, physicians have begun turning to quinolone antibiotics as an alternative treatment because of raised resistance to these first-line antimicrobial(8). Resistance to quinolones has been introduced, and since then, there has been no systematic surveillance in Iraq to detect resistant mutations. With few new antibiotics, research on resistant bacteria is crucial. The World Health Organization (WHO) has designated Salmonella spp. resistant to quinolone as a pathogen for which new treatments are urgently required(9). Studies have shown that people with S.typhi resistant to quinolones have longer times until their fever goes away and more treatment failures(10,11). Quinolone resistance is almost always caused by chromosomal mutations in the genes of resistant bacteria that code for targeted enzymes, such as DNA gyrase and topoisomerase IV(12). On the other hand, plasmid-encoded genes are another potential route to acquiring quinolone resistance. Groups of genes known as PMQR include the qnr families (qnrA, qnrB, qnrC, qnrD, qnrE, qnrS, and qnrVC), the genes for the efflux pumps used to remove antibiotics from the body, the gene for the enzyme responsible for modifying antibiotics, and a recently discovered phosphorylase gene(13,14). PMQR makes it easier for quinolone resistance to spread, ultimately developing high levels of quinolone resistance and making it more difficult to treat infections(15). Previous studies have shown that the qnr genes are often found in Asia. Because of this, we chose to look into these genes for the first time in this study in Iraq(16,17). The qnr, a pentapeptide repetition protein, preserves the activity of DNA gyrase and topoisomerase IV by impeding quinolone antimicrobial(16,18). Thus, the existence of qnr genes in S. typhi strains with less sensitivity to fluoroquinolones signifies that continued monitoring and clinical awareness are needed(19).

Molecular typing is crucial for characterizing bacteria because it shows how clones and strains are spread in different environments. Conventional microbial typing techniques are useful in epidemiological investigations, despite being imperfect, time-consuming, and labor-intensive. The analysis of microbes' DNA is the cornerstone of molecular detection and typing techniques(20,21). The most frequently employed techniques are pulsed-field gel electrophoresis (PFGE), whole gene single nucleotide polymorphism (wgSNP), and multilocus sequence typing (MLST) (22,23). MLST was created as one of these molecular typing methods to establish analytical microorganism typing and identify the evolutionary relationships between S.typhi strains(24,25). The MLST method uses allelic differences to classify strains into a hierarchy of seven housekeeping genes(26).

In Iraq, no research has been done to characterize the genetic relationships among S. typhi strains or look at the prevalence of quinolone resistance among S. typhi strains isolated from patients with typhoid fever. This study aimed to determine the prevalence of qnr genes (qnrA,qnrB, and qnrS) in S.typhi isolated from the blood of typhoid patients in two main hospitals in AL-Najaf province, Iraq, and also determined the genetic characteristics of these isolates using MLST.

MATERIALS AND METHODS

Sample collections

A cross-sectional study was performed on blood samples from clinically suspected typhoid fever patients attending Al-sader medical city and Al-zahra maternity and children hospital in Al-Najaf city from June to November 2022. Patients who had a fever for more than 15 days or were already on antibiotics were excluded from the study. The physician assessed the patient's clinical signs and symptoms. For microbial culture, approximately 5mL of blood was obtained from patients aged >5 years and 3mL from patients aged 5 years.

Blood culture, bacteria isolation, and identification

Culture bottles labeled BacT/ALERT®FA, and BacT/ALERT®PF PLUS were used to collect samples of the patient's blood (BIOMERIEUX, USA). The automated BacT/ALERT 3D system (BIOMERIEUX, USA) was utilized to incubate blood samples for three days. According to the manufacturer's recommendations, samples that failed to produce a signal after 72 hours of incubation were considered to lack growth (negative). The first indication of bacterial growth was picked up by the BacT/ALERT system and displayed on the 3D monitor along with the moment of detection. Specific identification of all culture positive samples was accomplished by sub-culture on Blood Agar, Xylose Lysine Deoxycholate Agar, and MacConkey Agar (HiMedia, India). All culture media were incubated aerobically at 37 C for 24 hours. It was determined that S. typhi was present based on the colony morphology on culture media and the biochemical assays' results and was confirmed by the automated Vitek-2 system (BioMérieux, France).

Antibiotic susceptibility testing

Mueller-Hinton Agar was tested for antibiotic susceptibility using the modified Kirby-Bauer disc diffusion method. The antibiotics cefotriaxone (30µg), cefixime (30µg), imipenem (10µg), meropenem (10µg), levofloxacin (5µg), chloramphenicol (30µg), ciprofloxacin (5µg), nalidixic acid (30µg), azithromycin (15µg) and ampicillin (15µg) (HiMedia, India) were tested. The diameter of the zone of inhibition was interpreted following the norms set out by the Clinical and Laboratory Standards Institutes (CLSI 2022). Antibiotic quality control tests were conducted using ATCC strains of Escherichia coli (25922), Staphylococcus aureus (29213), and Pseudomonas aeruginosa (27853).

Molecular detection of qnr genes

DNA extraction

We used a spherolyse DNA isolation kit to extract genomic DNA from S.typhi isolates following the manufacturer's instructions (HainLife Science, Nehren, Germany). qnr genes were identified using the extracted DNA as a template.

Amplification of qnr genes

All of the S. typhi isolates were subjected to PCR amplification to detect the qnr genes (qnrA, qnrB, qnrS) using the primers that had been used in the study earlier (26). After adding 25 µL of nuclease-free water, a reaction mixture with a total volume of 50 µL was created by adding 1 µL of template DNA, 1 µL of forward primers, 1 µL of reverse primers, and 22 µL of PCR master mix.

The PCR reactions for all three genes were run with the following cycling conditions: initial denaturation at 94 °C for the 45s, template denaturation at 94 °C for 35s, annealing at 52 °C for 55s, extension at 66 °C for the 60s, final extension at 65 °C for 5 minutes, and holding the reaction at 4 °C until amplicons were collected for agarose gel electrophoresis. The amplicons were resolved by agarose gel electrophoresis (1.5% agarose) at 120 V for an hour and band visualization done with the aid of a UV-transilluminator (Vilber Lourmat, Collegien, France).

Multilocus Sequence Typing

Multilocus sequence typing (ST) was performed on S.typhi isolates carrying qnr genes. The internal segments of seven housekeeping genes of S. typhi (aroC, dnaN, hemD, hisD, purE, sucA, and thrA) were amplified using particular primers by referring (27). The PCR cycled conditions at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. PCR results were sequenced by Macrogen in South Korea. The online MLST database was used to assign allele numbers and STs.

Statistical analysis

The information was recorded in Excel and then transferred to STATA 12 (Stata Corp., USA) for statistical analysis. Tables and graphs summarizing the distribution of the various variables were generated using descriptive statistics. Fisher's exact test examined the statistical significance between categorical variables.

RESULTS

Out of 246 blood specimens collected from a patient with suspected typhoid fever in Al-Sader Medical City and Al-Zahra Maternity and Children hospital, 13% (n= 32) were culture positive for S.typhi. The demographic information showed that 62.5% (n=20) of the S.typhi isolates were collected from female patients, whereas 37.5% (n=12) of this bacteria were obtained from male patients.

In an antibiotic susceptibility test, all S.typhi isolates were found to be susceptible to ceftriaxone, cefixime, imipenem, meropenem, and azithromycin. Resistance to ampicillin was the highest, followed by levofloxacin, chloramphenicol, and nalidixic acid, with ciprofloxacin and nalidixic acid having the lowest levels of resistance (Figure 1).

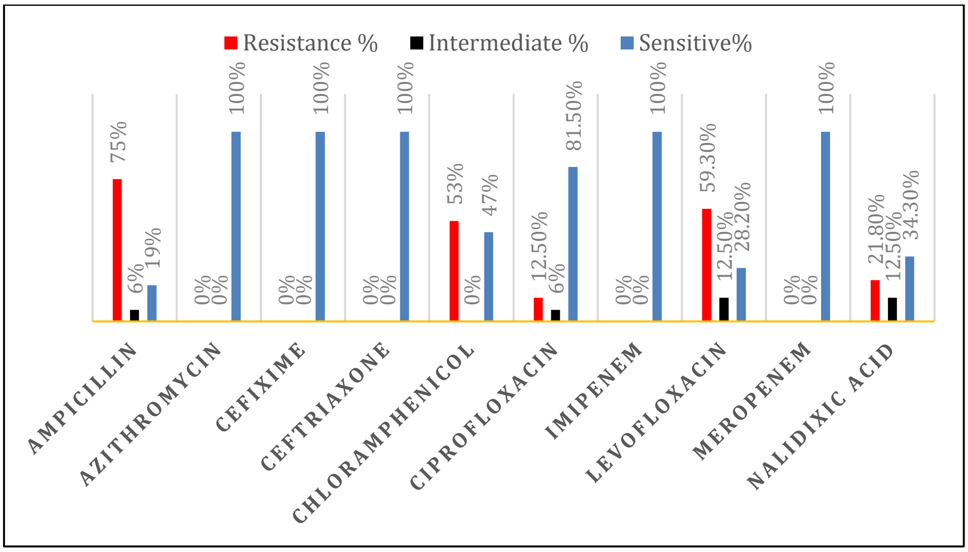

FIGURE 1: Rate (%) of antibiotic resistance in S.typhi isolated from two main hospitals in Najaf province

All 32 S.typhi isolates were examined by conventional PCR for detect presence of qnr genes. PCR analysis revealed that only one (3%) isolates harbored qnrA gene (Figure2) and four (12.5%) isolates harbored qnrB gene (Figure 3), while qnrS detected in three (9%) isolates (Figure 4). This results show no amplification of S.typhi isolates harbors more than one qnr genes.

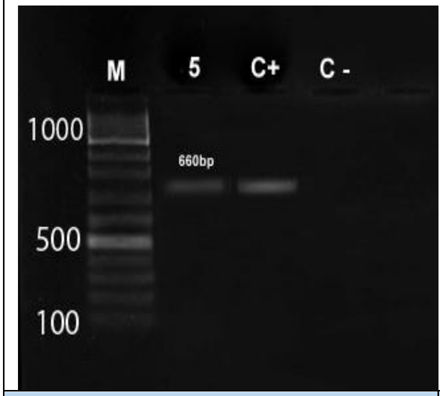

Figure 2: Amplification and detection of qnrA (550 bp) using PCR. qnrA is present in lane 5. +C, -C are positive and negative controls. M = 1000 bp molecular ladder.

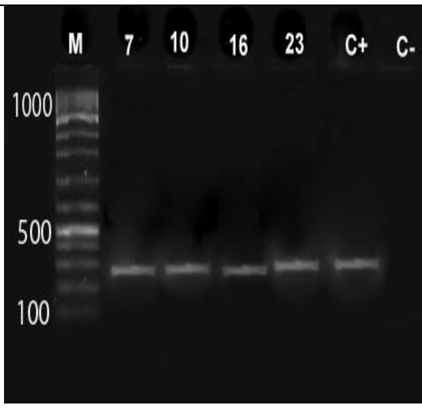

Figure 3: Amplification and detection of qnrB (264 bp) using PCR. Lanes 7,10,16,23 have qnrB.
+C, -C are positive and negative controls. M = 1000 bp molecular ladder.

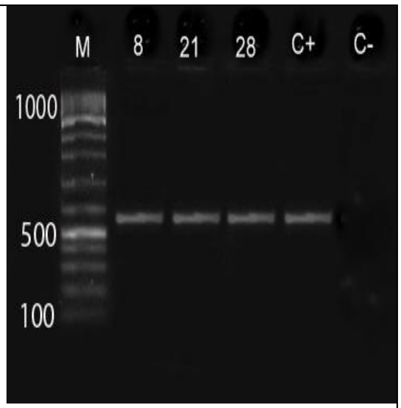

Figure 4: Amplification and detection of qnrS (550 bp) using PCR. Lanes 8,21,28 have qnrS.
+C, -C are positive and negative controls. M = 1000 bp molecular ladder.

A total of eight S.typhi isolates positive for qnr genes were model for genotyping analysis by MLST. Seven housekeeping genes aroC, dnaN, hemD, hisD, purE, sucA, and thrA were amplified with specific primers. The allelic profiles were matched in the database website (http://mlst.warwick.ac.uk/mlst/dbs/ S.enterica) to identify the sequence type (ST) of each isolate. By MLST, the eight S.typhi isolates positive for qnr genes were grouped into three distinct allelic profiles (ST clones): ST19 (n= 5 isolates), ST34 (n= 2 isolates), and ST36 (n= 1 isolate) as shown in (Table 1).

TABLE 1: Distribution of the 8 S.typhi positive qnr genes according to the gender of the patients, sequence type (ST), and antibiotic resistance profile.

Isolate	Sex	qnr genes	ST	Antibiotics resistance profile				
				AM	LEV	NA	CL	CIP
05	F	qnrA	19	R	S	R	S	R
07	M	qnrB	19	R	R	R	R	S
08	F	qnrS	34	R	S	I	S	S
10	M	qnrB	19	R	R	S	S	I
16	M	qnrB	36	R	S	R	S	S
21	M	qnrS	19	R	S	S	R	I
23	F	qnrB	19	R	R	S	S	S
28	F	qnrS	34	R	R	S	S	S

All the S.typhi positive qnr genes isolates were susceptible to ceftriaxone, cefixime, imipenem, meropenem, and azithromycin. AM: ampicillin, LEV: levofloxacin, NA: nalidixic acid, CL: chloramphenicol, CIP: ciprofloxacin.

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DISCUSSION

Typhoid fever is caused by S.typhi, which is notorious for being resistant to several antibiotics. One of the leading causes of death and illness in underdeveloped nations, it is also a frequent cause of bloodstream infections in the Middle East, particularly in Iraq(28).

The study used 246 blood samples collected from a patient with suspected typhoid fever to isolate and diagnose S.typhi. In the current study, only 32(13%) positive blood cultures identified as S.typhi depend on colony morphology on culture medium, biochemical testing, and the automated GN Vitek-2 system. Compared to our study, hospitals in Najaf, Iraq, reported a higher rate of culture-positive typhoid fever cases in 2017 and 2020(29,30). The high prevalence of the S.typhi strain in Iraq and other Middle Eastern countries is consistent with data stating that 80 % of S.typhi -infected patients originate from the Asian continent, while the remainder is primarily from Africa and Latin America(31). Typhoid fever is common in these countries, which do not have a lot of good sanitation or good administration of public health(32).

Antibiotic resistance is a big problem when it comes to treating typhoid fever. Multiple drug-resistant MDR strains of S.typhi have been identified all over the world(33). The majority of S.typhi isolates in our study exhibited ampicillin resistance. Most S.typhi isolates, including multidrug-resistant strains, were susceptible to ciprofloxacin and nalidixic acid but resistant to levofloxacin and chloramphenicol. Our results are the same as those of studies done in Samawa, Iraq, which found that clinical strains of S.typhi had high levels of resistance to chloramphenicol, ampicillin, and levofloxacin(34). Malehmir et al. found that paediatric cases of S.typhi in Tehran, Iran, were less sensitive to ciprofloxacin and nalidixic acid, more sensitive to chloramphenicol antibiotics, and had a very low proportion of MDR strains(35).

In the current study, cephalosporins (ceftriaxone and cefixime), a carbapenem (imipenem and meropenem), and azithromycin showed high levels of activity against S.typhi. As a result, these antibiotics can be presented as the foremost option for treating infections caused by this bacterium in our region. This result is similar to a study done in Baghdad, Iraq, where Salman HA et al. found that S.typhi isolated from suspected typhoid patients in 2020 were fully sensitive to carbapenems (imipenem and meropenem)(36). The overall rate of sensitivity to azithromycin that we found in our study is lower than the rate found in the study from Nepal(37). Azithromycin is increasingly used as some antimicrobial drugs have become almost worthless in treating MDR S.typhi; nonetheless, all isolates in our research were still within the azithromycin susceptibility range, suggesting that it may be a viable alternative for treating typhoid fever. According to our study and previous results(31,(38), showed no evidence of cephalosporins resistance (ceftriaxone and cefixime); hence, these antibiotics should be considered viable therapeutic s for treating MDR S.typhi-related typhoid complications, at least when administered parenterally. However, evidence of the development of resistance to ceftriaxone was identified in a study in Pakistan in 2014(39).

When classical MDR S.typhi first appeared, quinolones were the best antibiotics. However, S.typhi isolates resistant to quinolones have emerged because of the extensive use of quinolones to treat typhoid fever(40). The three most common PMQR genes (qnrA, qnrB, and qnrs) that have led to the persistent spread of quinolone-resistant S.typhi were chosen in this study.

8 (25%) of 32 S.typhi isolates harbour qnr genes, including one (3%) isolate positive for qnrA, four (12.5%) isolates positive for qnrB, and three (9.3%) isolates positive for qnrS (Figure2,3,4). A study on qnr genes in clinical S.typhi isolates from South Korea found that qnrB was the most common qnr gene(41). In India, the qnrB gene was amplified in 70% of S.typhi strains isolated from blood cultures, but neither the qnrA nor the qnrS gene was amplified(42). In Iran, qnrS was detected in 56.5% of the S.typhi strains, qnrA was found in 30.4%while qnrB was in 1.1%(34). In Brazil, qnrS was found in 53.3% of S.typhi strains, qnrB was found in 40%, and qnrA was not(43). Quinolones, particularly fluoroquinolones, are used broadly in poultry farms and to treat pets in Iraq. It makes it more likely that bacteria that are resistant to zoonotic agents will be distributed through the food chain(44). Quinolone resistance is common in Asia, which can be attributed partly to the extensive usage of antimicrobials in this class(45).

On 8 isolates carrying qnr genes, MLST was performed to evaluate genetic correlations. In the present study, five of the S.typhi clones belong to ST19; two were ST34, and one was ST36 (Table 2). Our MLST analysis revealed that S.typhi clones are closely related genetically. A dominant clone in the samples from both hospitals in Najaf indicated a common infection source or similar eating routines among the patients. Our studies found that ST19 was present in female and male patients and that the isolates had various antibiotic resistance patterns.

These findings showed no correlation between ST, antibiotic resistance profiles, and patients' sex. There have been no previous MLST studies on S.typhi isolates in Iraq. This study is the first MLST analysis of S.typhi isolates from typhoid fever patients. The findings of this study agree with a study from Iran, which revealed that most S.typhi isolates from two major hospitals in Tehran belonged to ST19(46). Some research suggests that ST19 is also widely spread in Asia(47,48). Each ST of S.typhi in that study displayed various antibiotic resistance profiles. Another ST found in this study is ST34, which has been noted to be one of the most common STs in S.typhi in Asia (22,27). In this study, both S.typhi isolates as ST34 possessed the qnrS gene, whereas Sohyun Lee found that 9 S. Typhi isolates as ST34 had qnrS gene in South Korea from 2016 to 2019(49). S.typhi ST36 was reported in one isolate, and some S.typhi strains obtained from human clinical samples in Denmark were identified as ST 36 and had qnrB(50).

CONCLUSIONS

Fortunately, our study demonstrated that cephalosporins (ceftriaxone and cefixime), carbapenems (imipenem and meropenem), and azithromycin are fully effective against S.typhi clinical isolates from typhoid fever patients. The findings of this study are the first to report the presence of plasmid-mediated quinolones-resistant genes in eight MDR S.typhi clinical isolates from Iraq. The qnrB gene was more prevalent than the qnrS and qnrA. These genes may contribute to the quinolone resistance of S. Typhi. MLST revealed that ST19 was the predominant lineage in S.typhi clinical isolates that harbor qnr genes. With the appearance of plasmid-mediated quinolones-resistant genes in S.typhi, it's important to limit the use of quinolones and have good infection control to keep resistant strains from spreading.

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