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Research Article
Molecular Detection of *gyrA* Gene in *Salmonella enterica* serovar Typhi Isolated from Typhoid Patients in Baghdad

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Abstract

Background and Objective: Typhoid fever is endemic in most countries, causing major public health problems with high morbidity and mortality, the resistance of *Salmonella enterica* serovar Typhi (S. Typhi) towards antimicrobials is recently increased. The aim was to detect the harboring *gyrA* gene in Typhi *Salmonella enterica* serovar. **Materials and Methods:** Twenty *Salmonella enterica* serovar Typhi isolates were obtained from the Teaching laboratories of the medical city in Baghdad, the isolates were obtained from blood specimens from typhoid patients. Colonies of *Salmonella enterica* serovar Typhi appeared on CHROM agar and Xylose Lysine Deoxycholate Agar (XLD) as light mauve to mauve-colored and as red with black center colonies, respectively. Polymerase Chain Reaction (PCR) technique was used to detect the presence of the *gyrA* gene within the twenty isolates with specific primer. **Results:** All twenty isolates show the highest resistance rates to Cefazolin 18 (90%), Ciprofloxacin 16 (80%), and Nalidixic acid 15 (75%), while it shows highest sensitivity rates to Cefepime 18 (90%) and Tobramycin 16 (80%) antibiotics. The MIC values show equal to or more than breakpoint of Nalidixic acid and Ciprofloxacin were 15 (75%) and 16 (80%) isolates, respectively. **Conclusions:** PCR results showed the presence of *gyrA* gene (488 bp) in 14 (70%) of isolates (isolate number 1, 2, 3, 4, 7, 8, 9, 10, 12, 13, 14, 15, 18 and 19). On the other hand, 6(30%) isolates (isolate numbers 5, 6, 9, 11, 16, 17, and 20) don't harbor the *gyrA* gene.

Key words: Salmonella enterica serovar, Typhi, nalidixic acid, cefepime, tobramycin, ciprofloxacin, fluoroquinolones, gyrA gene

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In humans, the majority of *Salmonella* sp. causes various types of clinical syndromes and have been broadly categorized into 2 groups. The first group, enteric fever, which is caused by *Typhoidal salmonella* (TS) like *Salmonella typhi* (typhoid fever), *Salmonella Paratyphi* A, and B (Paratyphoid fever) and they are mostly transmitted through the contaminated water and food. The second group of clinical syndromes like diarrhea and gastroenteritis is caused by the non-*typhoidal Salmonella serovars*[1]. *Salmonella enterica* serovar Typhi (*S. typhi*) is the particular *Salmonella serovar* that causes typhoid fever, which is a major public health problem in developing countries[2].

Typhoid fever is the systemic disease, without therapy, the illness may last for 3 to 4 weeks and the death rate ranges between 12 and 30%. *Salmonella typhi* is a motile organism, facultative anaerobe, and is susceptible to various antibiotics. Currently, 107 strains of *Salmonella* have been isolated; containing many varying in metabolic characteristics, levels of the virulence, and the multi-drug resistance genes that complicate treatment in areas that resistance is prevalent[3].

Despite the emergence of the newer antibacterial drugs, the enteric fever has continued to be a major health problem[4]. In the past, ampicillin, trimethoprim/sulfamethoxazole and chloramphenicol were the antibiotics used for the treatment of enteric fever[5]. Due to the emergence of multidrug-resistant among *salmonella* isolates in the late 1980s, the fluoroquinolones namely ciprofloxacin and the extended-spectrum cephalosporins such as cefotaxime and ceftriaxone became the drugs of the choice[6].

More recently the azithromycin drug is effective in the treatment of enteric fever[7,8]. The resistance of *S. typhi* to ceftriaxone and the cefotaxime due to the production of the ESBL and also the reduced susceptibility to the ciprofloxacin resulting in the treatment failure which is being reported in many countries[9]. The emergency of multidrug resistance to the commonly used antibiotics has further complicated the treatment and management of enteric fever and is recognized as one of the greatest challenges in the management of this disease[10].

In *Salmonella*, the primary target of fluoroquinolones is DNA gyrase, which is consists of two subunits, A and B, which are encoded by *gyrA* and *gyrB* genes[11]. Resistance to the fluoroquinolones has been emerged in the region and represents a significant threat to the typhoid fever treatment[12]. A single point mutation in the Quinolone Resistance Determining Region (QRDR) of the *gyr*A can be mediate the non-fluorinated quinolone (NAL) resistance and then reduce the susceptibility to the fluoroquinolones (ciprofloxacin)[13,14].

This study aimed to determine the antibiotic susceptibility pattern, estimation the minimal inhibitory concentration (MIC) to fluoroquinolones and detect the presence of the *gyrA* gene by PCR assay in all *Salmonella enterica* serovar typhi isolates in this study.

MATERIALS AND METHODS

Isolation and Identification of *Salmonella enterica* serovar Typhi isolates: The study was carried out at the Microbiology Department, Quality Control Lab from Jan2018-March2019). In this study, twenty *Salmonella enterica* serovar Typhi isolates were isolated from the blood specimens of typhoid patients at the Teaching Laboratories/Medical city in Baghdad. All clinical isolates of *Salmonella enterica* serovar Typhi were identified in the laboratories of Teaching Laboratories of Medical city by the use of API-20E System (Bio-Merieux, France) and according to the manufacturer's instructions. On the other hand, Xylose Lysine Deoxycholate agar (XLD agar) (Hi media/India) and CHRO Magar for *Salmonella* (BBL/USA) was also used to watch the characteristics of *Salmonella* isolates colonies on these media.

Antimicrobial susceptibility test: Kirby-Bauer method was used as described by WHO[15] to carry out the antimicrobial susceptibility test for different 12 antimicrobial drugs which including; Amoxicillin Clavulanic acid, Ampicillin-Sulbactam, Amikacin, Ceftazidime, Cefotaxime, Cefepime, Cefazolin, Cefoxitin, Ciprofloxacin, Gentamicin, Tobramycin and Nalidixic acid (Mast/USA). Inhibition zones developed around the discs were measured by millimeter (mm) using a metric ruler according to Clinical and Laboratory Standards Institute CLSI (2011)[16].

Estimation of Minimal Inhibitory Concentration (MIC):

- **Antibiotic stock solution:** The antibiotic stock solution was prepared at a final concentration of 10 mg mL^{-1} according to the CLSI (2011)[16], the methods were done as the follows Stock solution of the Ciprofloxacin antibiotic prepared by dissolving 0.5 g of Ciprofloxacin antibiotic in 50 mL of D.W., in the other hand, the stock solution of Nalidixic acid antibiotic prepared by dissolving 0.5 g of Nalidixic acid antibiotic in 25 mL of D.W. +25 mL of NaOH (1 mol L^{-1}) which added drop by drop for the dissolving of Nalidixic acid antibiotic and ultimately get the final

volume (50 mL), followed by sterilized step by filtration using 0.22 µm membrane filter

- **Agar dilution method:** Serial dilution agar method was used for MIC to Ciprofloxacin and Nalidixic acid antibiotic were used as follow[17]
  - Double folded serial dilutions (0.25-1024) µg mL^{-1} of the Nalidixic acid antibiotic were prepared from the stock solution of Nalidixic acid antibiotic which previously prepared. Double folded serial dilutions (0.025-32) µg mL^{-1} of the Ciprofloxacin antibiotic were also prepared from the stock solution of the Ciprofloxacin antibiotic which previously prepared
  - Separately, dilutions were added to Mueller Hinton agar at 50°C into sterile glass tubes each one alone and mixed well then poured into sterile Petri-dishes. Finally cooled to 37°C left to solidify at room temperature (25°C), kept at 4°C to use for 24 h
  - Few colonies (4-5) from the overnight culture were transferred to 5 mL of normal saline to prepare bacterial suspension which adjusted to 0.5 McFarland (1.5×10^8 CFU mL^{-1})
  - Five microliter of each bacterial suspension were drowning by micropipette and left for 10 min
  - The plates were incubated at 37°C for18-24 h
  - The result of MIC was recorded for each antibiotic
  - The result was compared with MIC interpretive standard according to CLSI (2011)[16], as illustrated in Table 1

Genotypic detection of *gyrA* Gene by Polymerase chain reaction (PCR) technique

Extraction of genomic DNA: DNA was obtained by suspending 2-3 colonies of each tested *Salmonella enterica* serovar Typhi isolate (which grow on the Nutrient agar plates) in 500 µL of D. W. and heating at 90°C for 10 min BY using a water bath. Samples then were spun at 10000 rpm for 10 min. The samples after that were used as the bacterial DNA template for the PCR technique[18].

Amplification reaction of PCR: The *gyrA* forward in reverse primers (Alpha DNA, Canada) was provided in a lyophilized form then dissolved in sterile deionized water(D.W) to reach the final concentration of 100 picomole/µL as recommended by the provider and then stored in a deep freezer till using. The forward *gyrA* primer: (5'-TACGCGATGAGCGTGATCGTC-3') and the reverse *gyrA* primer: (5'-GTTGTGCGGCGGGATGTTGGT-3') were chosen according to Han and Andrade[19]. The PCR master mix 2X (Promega, USA), extracted DNA and primers were thawed at 4°C. The PCR mixture was set up in a total volume of 25 µL which included: 12.5 µL of PCR master mix, *gyrA* forward primer (1.5 µL) and reverse primer (1.5 µL), 4 µL of template DNA and the rest volume was completed with sterile D.W. PCR reaction tubes were vortexes and finally placed into a thermocycler PCR instrument. DNA was amplified as indicated in Table 2.

Table 1: MIC Interpretive standard according to CLSI

	MIC Interpretive standard (µg mL^{-1})		
Antibiotic	Susceptible	Intermediate	Resistant
Ciprofloxacin	0.06≤	0.12-0.5	1≥
Nalidixic acid	16≤	_	32≥

Table 2: The program used in the thermocycler PCR

Stage	Temperature (time)
Initial denaturation	95°C (5 min)
30 cycles	
Denaturation	95°C (1 min)
Annealing	57°C (1 min)
Extension	72°C (1 min)
Final extension	72°C (7 min)

Agarose gel electrophoresis: The electrophoresis gel was used to identify the PCR products of the *gyrA* gene which visualized by using Lithium bromide and UV Transilluminator documentation system as depicted by Sambrook *et al.*[20]. The PCR products have been confirmed by comparing its molecular weight with the 100 bp DNA Ladder (Kapa, India). Isolation and Identification of *Salmonella enterica* serovar Typhi isolates

Twenty isolates of *Salmonella enteric* serovar Typhi were isolated from blood specimens of typhoid patients at Teaching Laboratories of Medical City in Baghdad. All *S. typhi* isolates were come to the teaching laboratories for culture and identified in the same laboratories by the use of the API- 20E system.

Genotypic detection of *gyrA* Gene by Polymerase Chain Reaction (PCR): One of the main objectives of this study is to detect the presence of a *gyrA* gene that encoding for DNA gyrase, within twenty isolates of *Salmonella enterica* serovar Typhi by PCR with specific primer. Each extracted DNA sample was subjected to the PCR reaction with a specific primer.

Statistical analysis: All the collected data were subjected to statistical analysis using SPSS.

RESULTS

All isolates under study were cultured on Xylose Lysine Deoxycholate agar (XLD agar) and CHROM agar for *Salmonella*to to observe the characteristics of *Salmonella* isolates colonies on these media Fig. 1. *Salmonella enterica* serovar Typhi colonies on Xylose Lysine Deoxycholate agar (XLD) appeared as red with black centers colonies, while on CHROM agar, the colonies appeared as light mauve to mauve-colored colonies.

Antimicrobial susceptibility test: The results of susceptibility test to all 20 *Salmonella enterica* serovar Typhi isolates toward 12 different antibiotics showed that the highest resistance rates were to Cefazolin 18 (90%), Ciprofloxacin 16 (80%) and Nalidixic acid 15 (75%), while the highest sensitivity rates were to Cefepime 18 (90%) and Tobramycin 16 (80%) antibiotics. Bacterial isolates were revealed different degrees of resistance towards the remaining antibiotics under the study as shown in Table 3.

Estimation of Minimal Inhibitory Concentration (MIC): Minimum Inhibitory Concentration (MIC) for two antibiotics (Ciprofloxacin and Nalidixic acid) were determined by using the standard agar dilution method on Mueller-Hinton agar (Fig. 2) and the results of this test were interpreted after 18-24 h of incubation at 37°C according to the Clinical Laboratories Standards Institute CLSI (2011). Depending on breakpoint of the Ciprofloxacin and the Nalidixic acid

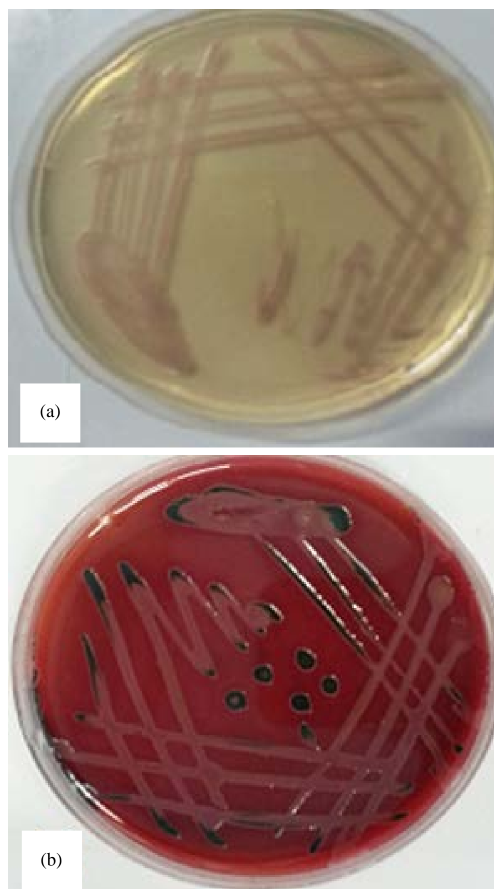

Fig. 1(a-b): *Salmonella enterica* serovar Typhi colonies on (a) CHROM agar and (b) XLD agar after 24 h of incubation at 37°C

Table 3: Antimicrobial resistance patterns of Salmonella enterica serovar Typhi isolates

Isolate No.	CIP	NA	TN	FOX	CAZ	CPM	AK	CTX	AUG	CZ	SAM	GM
1	R	R	S	S	S	S	S	S	R	R	R	S
2	R	R	R	S	S	S	S	R	S	R	R	R
3	R	R	S	R	R	S	R	R	S	R	S	S
4	R	R	S	S	S	S	S	R	S	R	R	R
5	S	S	S	S	R	S	S	R	R	S	S	S
6	S	S	R	S	S	S	S	R	S	R	R	R
6	R	R	S	S	S	S	S	R	R	R	R	S
8	R	R	S	S	S	S	S	R	S	R	S	S
9	R	R	S	S	S	S	R	R	S	S	R	R
10	R	R	R	S	R	R	S	R	S	R	S	R
11	S	S	S	S	S	S	S	S	S	R	S	S
12	R	R	S	S	S	S	S	S	R	R	R	S
13	R	R	R	R	S	S	S	R	R	R	R	R
14	R	S	S	S	R	S	R	S	R	R	R	S
15	R	R	S	S	R	S	S	R	R	R	R	R
16	R	R	S	S	S	S	S	R	S	R	S	S
17	R	R	S	S	R	S	S	R	R	R	R	S
18	R	R	S	S	S	S	S	R	S	R	S	S
19	R	R	S	S	R	S	S	S	S	R	S	S
20	S	S	S	S	R	R	S	S	S	R	S	S

S: Sensitive, R: Resistance, AUG: Amoxicillin-Clavulanic acid, SAM: Ampicillin-Sulbactam, AK: Amikacin, CPM: Cefepime, CAZ: Ceftazidime, CTX: Cefotaxime, CZ: Cefazolin, FOX: Cefoxitin, CIP: Ciprofloxacin, GM: Gentamicin, TN: Tobramycin and NA: Nalidixic acid

antibiotics for each isolate, if the value was equal or more than the breakpoint, that means the bacterium was resistant to the antibiotic under test, the breaking point of Nalidixic acid is $\leq$32 mg mL^{-1} according to CLSI (2011).

The numbers of *Salmonella enterica* serovar Typhi isolates that gave MIC values equal to or more than breakpoint was 15(75%) isolates. On the other hand, the breakpoint of Ciprofloxacin is $\leq$1 mg mL^{-1} according to the CLSI (2011), the numbers of isolates that gave MIC values equal to or more than the breaking point were 16 (80%) of isolates as shown in Table 4.

Table 4: Minimal Inhibitory Concentration (MIC) values of Ciprofloxacin and Nalidixic acid antibiotics for Salmonella enterica serovar Typhi isolates

Isolates No.	MIC Nalidixic acid	MIC Ciprofloxacin
1	512	2
2	64	2
3	512	2
4	512	4
5	4	0.5
6	16	0.25
7	512	4
8	512	2
9	512	16
10	512	4
11	4	0.5
12	128	4
13	1024	4
14	4	4
15	64	2
16	128	1
17	32	4
18	1024	4
19	256	2
20	16	0.5

The results of PCR assay indicate the presence of the *gyrA* gene among *Salmonella enterica* serovar Typhi isolates under study and the PCR products have been confirmed by comparing its molecular weight with 100bp DNA Ladder as shown in Fig. 3. The results of *gyrA* Gene detection by PCR shows the presence of *gyrA* gene (488 bp) in 14 (70%) of *Salmonella enterica* serovar Typhi isolates (isolate number 1, 2, 3, 4, 7, 8, 9, 10, 12, 13, 14, 15, 18 and 19). On the other hand, 6(30%) isolates of *Salmonella enterica* serovar Typhi (isolate number 5, 6, 9, 11, 16,17 and 20) don't harbor *gyrA* gene. All *Salmonella enterica* serovar Typhi isolates that gave positive results for *gyrA* Gene were shown in Fig. 3.

Fig. 2: Minimal Inhibitory Concentration (MIC) test for *Salmonella enterica* serovar Typhi isolates after incubation at 37°C for 24 h

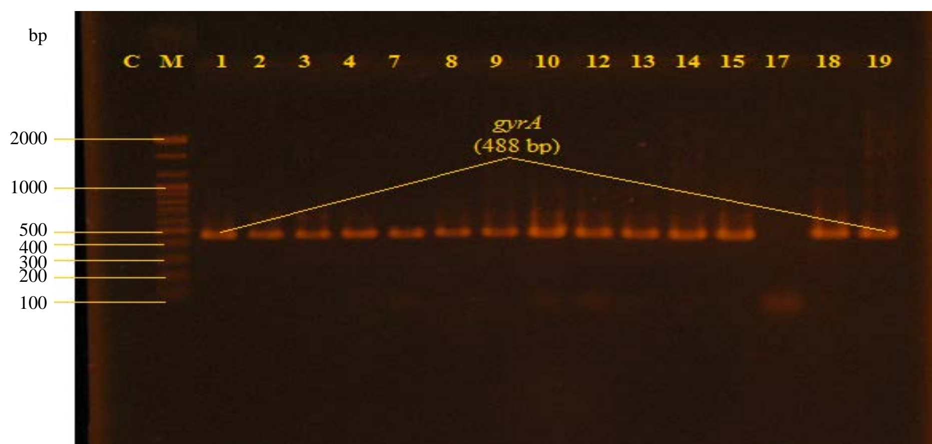

Fig. 3: Gel electrophoresis of PCR product for the *gyrA* gene (488 bp) in *Salmonella enterica* serovar Typhi isolates. Lane M, 100 bp DNA ladder; lanes (1, 2, 3, 4, 7, 8, 9, 10, 12, 13, 14, 15, 18 and 19), *Salmonella enterica* serovar Typhi isolates which harboring *gyrA* gene; Lane 17, *Salmonella enterica* serovar Typhi isolates which don't harbor *gyrA* gene; lane C: Negative control. Detection was done on an agarose gel (1%) at 5 V/cm for 1.5 h, stained with the ethidium bromide, and visualized on the UV transilluminator documentation system

DISCUSSION

In this study, the results of the antibiogram show that most of the studied that isolates of *Salmonella enterica* serovar Typhi were multiple-drug resistant which resist both quinolones antimicrobials ciprofloxacin and nalidixic acid, this was confirmed by its minimum inhibitory concentration test. This finding may indicate that the local isolates of *Salmonella enterica* serovar Typhi have highly resistant to a different group of antimicrobials including fluoroquinolones.

The propagation of *Salmonella enterica* serovar Typhi is particularly a concern with the lack of feasible alternatives and the explicit correlation between increasing MICs to the fluoroquinolones and the treatment failure[21]. The alarming rise in the multi-resistant isolates of *Salmonella enterica* serovar Typhi which was also quinolone-resistant may soon take place. This can cause a problem to the health services in the developing countries where such drugs may not be available[22]. Twenty isolates of *Salmonella enterica* serovar Typhi were subjected to the PCR technique to investigate the existence of the *gyrA* gene that encoding for the DNA gyrase. 14 (70%) isolates were given positive results with 488 bp amplified product of *the gyrA* gene.

The researchers in the current study noticed that most of the isolates were possessed *gyrA* gene, all of the *gyrA*-harboring isolates were resistant to both ciprofloxacin and nalidixic acid. The clinicians are now highly dependent on fluoroquinolones for typhoid therapy. However, a widespread of fluoroquinolone uses has been followed by the emergence of the isolates with elevated MICs[23]. These isolates are characterized by the point of the mutation within the *gyrA* (DNA gyrase) gene and sometimes an additional nucleotide substitution in the *parC* gene[24]. Ling *et al.*[25] have been noticed that the presence of *gyrA*, *parC*, and *parE* mutations in *Salmonella enterica* serotype Typhimurium, is the most common Salmonella serotype resistant to high concentrations of the fluoroquinolones, and the presence of *gyrA* mutations in *Salmonella enterica* serotypes Typhi and *Paratyphi* are a serious concern and call for the continuous monitoring of the fluoroquinolone-resistant salmonellae[26].

CONCLUSION

PCR results indicates the presence of *gyrA* gene (488 bp) in 14 (70%) of isolates (isolate number 1, 2, 3, 4, 7, 8, 9, 10, 12, 13, 14, 15, 18 and 19). gyrA genes encode a protein forming a subunit of a DNA gyrase which indicates the resistance to the antibiotic ciprofloxacin. On the other hand, 6(30%) isolates (isolate numbers 5, 6, 9, 11, 16, 17 and 20) don't harbor the *gyrA* gene.

SIGNIFICANCE STATEMENT

This study discovers the presence of *gyrA* gene (488 bp) in *Salmonella enterica* serovar Typhi isolates that were obtained from the Teaching laboratories of the medical city in Baghdad that can be beneficial to detect the harboring *gyrA* gene in Typhi Salmonella enterica serovar. This study will help the researcher to search for other than *Salmonella enterica* serovar Typhi isolates to harboring the *gyrA* gene. Thus, a new source must be found to ensure the possibility to harbor the *gyrA* gene.

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