

Detecting Phenotypic and Genotypic of the Antibiotic Resistant *Salmonella enterica* Serotype Paratyphi Isolated from Blood Samples in Najaf Province /Iraq

Al-Muhannak, Fadhil H. N.⁽¹⁾; Al-Sherees, Hashim Ali Abdulameer⁽¹⁾; Abdul Sada, Ibrahim Abed Ali⁽²⁾; Mahmood, Thikra Abdullah⁽¹⁾; Obaid, Rasha Fadhel⁽³⁾

⁽¹⁾ Department of Medical Microbiology, faculty of Medicine, University of Kufa /Iraq, ⁽²⁾ Najaf Health Directorate/ Najaf, Iraq, ⁽³⁾ Al-Mustaqbal University college Babylon/Iraq

Corresponding author: Hashim Ali Abdulameer Al-Sherees, hashimaa49@yahoo.com

Abstract

Background & Objectives: *Salmonella* Paratyphi is a leading cause of human paratyphoid fever in developing countries, causing deaths in humans worldwide. There are several paths for catching paratyphoid fever, but the close contact between patients and healthy humans is so far the most frequent cause of human infections. No study is found about the antibiotic resistance (phenotypic and genetic) of *S. Paratyphi* isolated from patients in Al-Najaf Province/Iraq, to the researcher's best knowledge. Hence, this study aimed to determine the prevalence of *S. Paratyphi* isolates from blood specimens and the antibiotic resistance determinants of them, as well as the genetic relationship among isolates.

Methods and Results: Blood specimens from 1743 patients with suspected enteric fever were cultured for the identification of *Salmonella enterica* during the period from first April to the end of October 2017. 107 (6.14%) *S. enterica* isolates were recovered and only two (1.87%) of isolates were *S. enterica* serovar Paratyphi B. These two isolates (ST39 and ST89) were tested against 23 antibiotics using the disc diffusion method on Muller-Hinton agar and the genotypic antibiotic resistance determinants by PCR. ST39 isolate was sensitive to all antibiotics while ST89 isolate was resistant to only cefepime, piperacillin and tobramycin. ST39 isolate did not carry integrons (1 or 2) and any resistance determinants, while ST89 isolate carried integron class1 as well as blaTEM, blaCTX-M and aac(6')-Ib genes.

Conclusions: The findings of *S. Paratyphi* isolates with integron 1 and resistant antibiotic genes indicating public health risks.

Key words: aac(6')-Ib genes, blaCTX-M, blaOXA, blaSHV, blaTEM, ESBLs, and *S. Paratyphi*,

Introduction

Paratyphoid fever which is a specific disease for humans, is a type of enteric fever caused by *Salmonella enterica*, subspecies *enterica* serovar *Paratyphi* A, B and C. *S. Paratyphi* A, B and C are differentiated by their various surface antigenic structures ⁽¹⁾. The symptoms of paratyphoid fever are similar to those of typhoid fever, making these two conditions difficult to differentiate clinically ^(2,3). *S. Paratyphi* B infections can cause enteric fever (paratyphoid fever) or gastroenteritis. In some cases, serious complications can occur (septicaemia, meningitis). The most affected age group is infants, young children and immunocompromised patients ⁽⁴⁾. Clinical reports show that *S. Paratyphi* can persist in the gallbladder of asymptomatic carriers ⁽⁵⁾.

The main factor in the development of antibiotic-resistant strains is the ability of the bacterium to acquire and spread the extra-chromosomal DNA through mobile genetic elements, such as plasmids, transposons and integrons ^(6,7,8). The increased prevalence of antibiotic-resistant bacteria has led to a great interest of researchers in the genetic mechanisms of resistance offered by these bacteria. Environmental factors influence the development of antimicrobial resistance in bacteria. Studies have shown that bacteria isolated from faecal-contaminated areas have more resistance to antibiotics due to their acquisition of resistance genes from faecal-associated bacteria. Therefore, contaminated foods by faeces containing *S. Paratyphi* will be more serious ⁽⁹⁾. Hence, The Enterobacterial Repetitive Intergenic Consensus polymerase chain reaction (ERIC-PCR) technique is used to identify genetic diversity in bacteria by separating specific chromosomal segments. These chromosomal segments are various in different species, genus and

strains. The ERIC sequences depended on the amplification of the chromosomal segments, which is appear by gel electrophoresis method as genomic bands (fingerprints) in different molecular weights. This method is used to reveal the epidemiological relationship between bacterial isolates and their degree of genetic diversity ^(10,11,12). The aim of this study is to determine the prevalence of *S. Paratyphi* isolates, the phenotype and genotype of their antibiotic resistance, and to identify the integrons and the genetic relationship among isolates.

Methods:

Sample Collection and Isolation

A total of 1743 blood samples were taken from patients with suspected enteric fever, in Najaf hospitals, Iraq; they all were aseptically collected during the seven months period of the study from April to October 2017. They were subjected to isolation by being cultured in brain heart infusion broth and incubated at 37°C for 5-7 days. Each BHI medium was subcultured on blood agar and incubated at 37°C for 24 hr. to give bacteria more chance to grow ⁽¹³⁾. From blood agar colonies, the pure colony was subcultured on the selective media, XLD agar, and incubated at 37°C for 24 hrs.

Identification of S. Paratyphi B

A typical colony from each XLD agar was identified by using standard biochemical tests and confirmed by using the Vitek 2 compact system ^(14,15). All the identified bacterial isolates were preserved in 15% glycerol nutrient broth and stored in the deep freeze at -20°C. Positive isolates were sent to the Central Health Laboratory in Baghdad to confirm the final identification

Susceptibility Testing

Depending on the recommendations of the Clinical and Laboratory Standards (CLSI)

⁽¹⁶⁾, twenty-six antibiotic discs of various classes were chosen for the Kirby-Bauer disc diffusion method ⁽¹⁷⁾. The isolates of *S. Paratyphi* and *E. coli* ATCC 25922 (as a control strain) were tested to determine the antibiotic sensitivity patterns.

Each isolate grown on XLD agar was inoculated in 2-3 ml nutrient broth and incubated at 37°C for 18 hours. According to the turbidity of McFarland tube, a sterile cotton swab was used to spread the suspended culture onto a Müller-Hinton agar, then antibiotic discs were dispensed on plates among which the distance is 15 mm at least, and incubated at 37°C for 18-20 hrs. The diameter of the zone around each disc was measured and compared

according to the breakpoints of each type of the antibiotic in CLSI in order to determine their being sensitive (S), intermediate (I), and their resistance (R).

Primers Oligonucleotide Design

The primer sequences of *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes were obtained from Bali et al. (2010) ⁽¹⁸⁾ while *bla*_{OXA} gene was obtained from Guerra et al. (2001) ⁽¹⁹⁾, *aac*(6')-Ib gene was obtained from Akers et al. (2010) (20), *intl*-1 and *intl*-2 genes were obtained from Dillon et al. (2005) ⁽²¹⁾, and ERIC gene was obtained from Smith et al. (2007) ⁽²²⁾. As in Table 1.

Table 1: PCR primers of *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, *bla*_{CTX-M}, *aac*(6')-Ib, *intl*-1 and *intl*-2 and ERIC genes with their references.

Target	Primer name	Oligo sequence (5'-3')	Product size (bp)	Reference
<i>bla</i> _{TEM}	TEM-F	TTTCGTGTCGCCCTTATTCC	403	Bali et al. (2010)
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG		
<i>bla</i> _{SHV}	SHV-F	CGCCTGTGTATTATCTCCCT	293	Bali et al. (2010)
	SHV-R	CGAGTAGTCCACCAGATCCT		
<i>bla</i> _{OXA}	OXA-F	ACCAGATTCAACTTTCAA	598	Guerra et al. (2001)
	OXA-R	TCTTGGCTTTTATGCTTG		
<i>bla</i> _{CTX-M}	CTX-M-F	CGCTGTTGTTAGGAAGTGTG	754	Bali et al. (2010)
	CTX-M-R	GGCTGGGTGAAGTAAGTGAC		
<i>aac</i> (6')-Ib	F	TATGAGTGGCTAAATCGAT	395	Akers et al. (2010)
	R	CCCGCTTTCTCGTAGCA		
<i>intl</i> -1	intl1- F	CAGTGGACATAAGCCTGTTC	160	Dillon et al. (2005)
	intl1- R	CCCGAGGCATAGACTGTA		
<i>intl</i> -2	intl2 -F	CACGGATATGCGACAAAAAGGT	789	Dillon et al. (2005)
	intl2 -R	GTAGCAAACGAGTGACGAAATG		
ERIC	ERIC-1	ATGTAAGCTCCTGGGGATTAC	variable	Smith et al. (2007)
	ERIC-2	AAGTAAGTGACTGGGGTGAGCG		

Detecting Encoding Genes by PCR Technique

According to the results of the antibiotic resistance, five types of primers were chosen to reveal ESBLs genes (blaTEM, blaSHV, blaOXA and blaCTX-M) and aac(6')-Ib gene as well as intl-1, intl-2 and ERIC genes (Table 1). The DNA was extracted by using a Promega Wizard Genomic DNA Purification Kit which is used as the template in the PCR technique. These templates were stored at -20°C in a sterile Eppendorf tube.

A PCR assay was used for detecting the antibiotic-resistant genes in the total volumes of 25µl containing 1 µl of each primer,

2 µl of template DNA, 12.5 µl of Taq Master Mix (Bioneer AccuPower Gold PreMix, Korea), and 8.5 µl PCR grade water. Thermocycler Biosystem was adjusted as in Tab. 2.

On 1.5% agarose gels, the PCR products and DNA-size ladder (100-1500 bp; GeneDireX INC, USA) were analyzed with electrophoresis which was performed at 60-70 V for 90 minutes with antibiotic resistance encoding genes and 150 minutes with ERIC genes. To evaluate the target product size according to the ladder, the bands of PCR products and the ladder were visualized by ultraviolet light.

Table (2): Programs of PCR thermocycling conditions for all primers used in the present study.

Gene name	Temperature (°C) / Time				
	Initial denaturation	Cycling condition			Final extension
		denaturation	annealing	extension	
TEM	94/5 min	94/30 sec	60/30 sec	72/50 sec	72/5 min
		No. of cycles = 35			
SHV	94/5 min	94/30 sec	60/30 sec	72/50 sec	72/5 min
		No. of cycles = 35			
OXA	94/5 min	94/30 sec	55/30 sec	72/50 sec	72/7 min
		No. of cycles = 35			
CTX-M	94/5 min	94/30 sec	60/30 sec	72/50 sec	72/5 min
		No. of cycles = 35			
aac(6')-Ib	94/15 min	94/45 sec	55/45 sec	72/45 sec	72/10 min
		No. of cycles = 34			
intl-1	94/5 min	94/30 sec	55/30 sec	72/1 min	72/7 min
		No. of cycles = 35			
intl-2	94/5 min	94/30 sec	62/30 sec	72/1 min	72/10 min
		No. of cycles = 30			
ERIC	95/3 min	95/30 sec	51/1 min	72/2 min	72/5 min
		No. of cycles = 35			

Results

The Isolation and Identification of Salmonella Paratyphi B

A sum of 1743 blood samples were obtained from patients with suspected enteric fever from the main hospitals in Najaf province during the period from April to October 2017. The conventional biochemical tests of the culture verified by Vitek 2 compact system has shown that 107 (6.14%) isolates were recovered as *Salmonella enterica* and only two (1.87%) of the isolates were *S. enterica* serovar Paratyphi B.

Antimicrobial Susceptibility of S. Paratyphi B Isolates

The two *S. Paratyphi* isolates were evaluated for susceptibility to 26 different antibiotic discs out of 11 antibiotic classes (according to CLSI / 2020 guidelines as resistant, moderate resistant, and susceptible).

It appears that the 1st isolate (ST89) exhibited resistance only to tobramycin

and piperacillin and intermediate resistant to piperacillin/tazobactam and cefepime, but sensitive to the others while the other isolate (ST39) was sensitive to all antibiotics but intermediate resistant to tobramycin and cefepime.

Detecting the Antibiotic Resistance Encoding Genes by PCR

1. ESBLs Genes (*bla*TEM, *bla*SHV, *bla*OXA, *bla*CTX-M):

ST89 *S. Paratyphi* isolate exhibited resistance to piperacillin and intermediate resistant to piperacillin/tazobactam and cefepime; therefore, it was chosen to reveal the presence of ESBLs genes by using *bla*TEM, *bla*SHV, *bla*OXA and *bla*CTX-M genes. So, it yielded amplification products with *bla*TEM gene (Fig. 1a) and yielded amplification products with *bla*CTX-M gene (Fig. 1b) while *bla*SHV and *bla*OXA genes were absent.

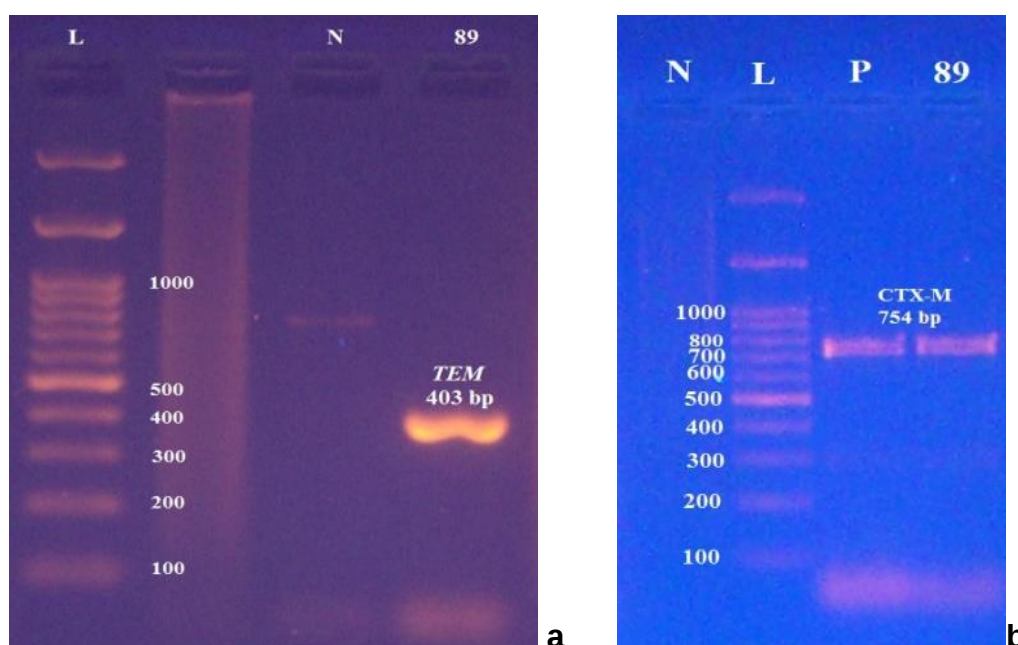


Figure 1: Gel electrophoresis of PCR product showing ESBLs genes (*bla*TEM and *bla*CTX-M genes).

2. Aminoglycoside Resistance Genes

ST89 *S. Paratyphi* B isolate showed positive results with primer *aac(6')-Ib* (Fig. 2).

Class I and II integrons and their distribution among S. Paratyphi B

Both *S. Paratyphi* isolates were tested for the carrying class 1 and class 2 integrons, by PCR technique with specific primers for the *intl-1* and *intl-2* integrase genes. Only

ST89 isolate were found to be carrying class 1 integron, while class II integron was not found in all isolates.

The isolate of integron-positive *S. Paratyphi* B was found carrying *bla*TEM, *bla*CTX-M and *aac(6')-Ib* genes while the isolate of integron-negative *S. Paratyphi* B had no antibiotic resistant genes.

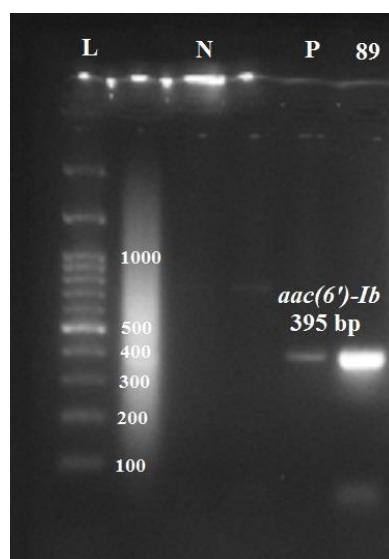


Figure 2: Gel electrophoresis of PCR product showing *aac(6')-Ib* gene.

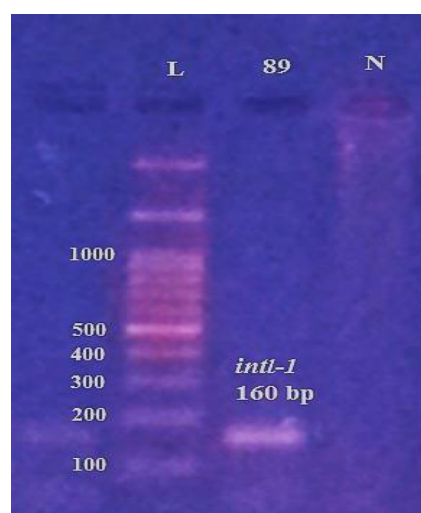


Figure 3: Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *S. Paratyphi* B isolates and amplified with *Intl-1* genes primers. The electrophoresis was performed at 70 volts for 1:30 hr. Lane (L), DNA molecular size marker (100 bp ladder), Lanes (89) show positive results with *Intl-1* gene. Lanes (39) show negative isolate with *Intl-1* gene.

Detecting the epidemiological relationships between isolates by ERIC-PCR

The results of ERIC-PCR fingerprinting of the two *S. Paratyphi* B isolates has shown two PCR amplicons. The numbers of amplified bands range between 2-4 bands with molecular size range between 100 bp and 700 bp (Fig. 4).

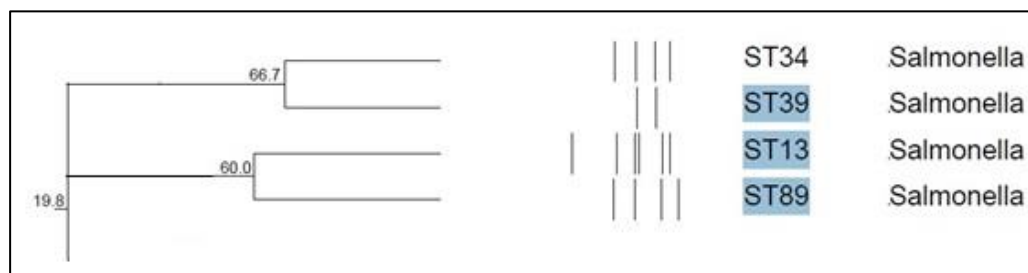


Figure 4: Dendrogram showing two different ERIC-PCR fingerprints of *S. Paratyphi* B isolates at 80% similarity.

Discussion:

During the study period, the examined blood samples revealed that the prevalence rate of *S. Paratyphi* B was 1.87%, and this result was in agreement with the studies of Issa et al. (2006)⁽²³⁾ in Najaf which was one isolate (1%) from 91 positive isolates; Thewaini and Mohameed (2006), in Al-Hilla (5/ 68, 7.35%)⁽²⁴⁾ and Singh and Cariappa (2016) in India shows only one (0.01%) isolate⁽²⁵⁾, as well as Malaysia studies during the years from 1990 to 2000 which were between 3.3-5.5% of the total numbers of *S. enterica*⁽²⁶⁾ while in AlQadesiah (2007), 20 from 65 (30.77%) of *Salmonella* were positive isolates⁽²⁷⁾.

S. Paratyphi B is the human pathogens mostly isolated from poultry⁽²⁸⁾. The results of antibiotic susceptibility test revealed that one of the two *S. Paratyphi* isolates (ST89) has shown resistance to two antibiotics (tobramycin and piperacillin) and intermediate resistance to other two antibiotics (piperacillin/tazobactam and cefepime). However, the other isolate (ST39) have shown intermediate resistance to other two antibiotics (tobramycin

As in Fig. 4, the dendrogram construction based on ERIC-PCR banding including two *S. Paratyphi* isolates has produced two patterns with different clusters at 19.8% similarity.

and cefepime). The resistance to these antibiotics could be either because of the inhibition of antibiotics by the acquired genes or gene mutations; it indicates the use of these antibiotics in the region.

Detecting the Antibiotic Resistance Encoding Genes by PCR

Molecular characterization revealed that ST89 isolate harbored the blaTEM, blaCTX-M and aac(6')-Ib genes. Several studies have shown the emergence of *Salmonella* strains containing ESBLs that carry out ESBL genes worldwide⁽²⁹⁾. However, to the researcher's best knowledge, no other study has investigated the molecular characterization of *S. Paratyphi* B strain harboring the antimicrobial resistant genes in Iraq.

Spread of Class 1 and Class II Integrins Gene Among *S. Paratyphi*:

Due to differences among countries in the consumption of types of antibiotics, different resistance against these antibiotics appeared; accordingly, integrons appeared^(1,30). In this study, class 1 integrons found in only ST89 isolate while class 2 integrons was absent.

ERIC-PCR Fingerprinting

The ERIC genotypes of isolates were used to show the epidemiological relationship of the recovered strains. ERIC analysis revealed different patterns among the two *S. Paratyphi B* isolates. The similarity ratio between ST89 and ST39 isolates was 19.8% which indicates that these isolates were from different sources.

In Najaf, variety of infection sources may happen because it has a feature that especially makes it a center of attraction for many, including the year-round attraction of many pilgrims or visitors, some of them come to tourism, including religious tourism, cultural tourism and business tourism. It has also become a hub for displaced people from hot cities in Iraq, where many displaced families have planned to live, because of stability. In addition to the presence of many universities, colleges and religious and academic institutions, many students from all over the world are drawn to study and research, making the area a continuous movement of passengers arriving and departing from different countries around the world.

Conclusions

This study has shown that the *S. serovar Paratyphi B*, recently acquired resistance determinants like *blaTEM*, *blaCTX-M* and *aac(6')Ib* genes as well as *int1-1*, constitutes major anxiety for public health. Further, surveillance and researches are very necessary to understand their epidemiology and to limit the prevalence of antibiotic resistant *Salmonella* spp.

Recommendations:

According to this research, the recommendations are:

1. The continuous monitoring of paratyphoid dissemination and antibiotic resistance of isolates.

2. Educating food staff and consumers about paratyphoid fever transmission methods.

3. Tracking paratyphoid fever cases among travellers to developing countries.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Data Availability

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

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