Detetion of Antibiotics Resistance Genes in Multidrug Resistant of Salmonella Enterica Serovar Typhi

Muna A. AL-Mazini
Department of Biology, College of Science, University of Basrah

Mohamad A. AL-Hajaj
Department of Biology, College of Science, University of Basrah

Abstract - A total of two hundred and sixty blood samples were collected from patient who visited different hospitals in Basrah city for the period from February to the end of October 2013. Seventy S. Typhi from blood samples of patients were identified by biochemical, serological and API 20E. The antimicrobial susceptibility of isolate showed that 52(86.66%) of the S. Typhi isolates were multidrug resistant (MDR). Molecular study was accomplished first by the extraction of plasmid DNA from isolates and second by using PCR technique to detection of its multidrug resistance genes(tem,cat, cmlA, sul1 and sul2)which encode for resistance to (ampicillin, chloramphenicol and co-trimoxazole).

Keywords: Typhoid fever, Salmonella Typhi, PCR.

I. INTRODUCTION

Typhoid fever is a global infectious disease with prevalence in Africa, South America and greatest risk in the Indian subcontinent [1,2]. The annual incidence of typhoid fever is estimated to be about 17 million cases worldwide [3]. In Africa, it has an estimated crude incidence of 362 cases per 100,000 persons per year [4]. In most endemic areas, approximately 90% of enteric fever is typhoid and caused about 216,500 deaths among children and young adults worldwide [5,6] .

Emerging Multiple Drug Resistance (MDR) is a major problem in control of infectious diseases. In case of typhoid, MDR *Salmonella enterica* serovar Typhi (*Salmonella* Typhi) strains are frequently associated with increased morbidity that leads to toxicity that ultimately results in significantly increased mortality rate [4,7].

The resistance was found to be associated with certain mobile genetic elements. Several types of mobile genetic elements have been described, which play an important role in acquisition, maintenance, and spread of antimicrobial resistance genes. Among these mobile genetic elements, plasmids, transposons, and gene cassettes are the most important. These elements can disseminate horizontally not only between bacteria of the same species, but also among different species or even different genera [8].

II. MATERIALS AND METHODS

Bacterial isolates

Blood sample were collected aseptically from 260 Patients taken from patients suffering from typhoid visited of (Al-Basrah general hospital, Al-Sadr teaching hospital, Al–Fayhaa general hospital, AL-Shafaa General Hospital, AL-Zubair General Hospital, Basrah childrens specialized hospital, Basrah Maternity and Children Hospital and Al-Mawani general hospital in Basrah city) for the period from February to the end of October 2013. Five milliliter of patients blood was inoculated in 50ml of Brain heart infusion (BHI) for culture of *S.* Typhi [9].

Laboratory diagnosis

Subcultures were as follows: from each positive blood bottle, a loopfull was transferred to MacConkey agar and Salmonella-Shigella agar(S.S agar), streaked, incubated for 24 hours at 37 ºC. All presumptive positive suspected *Salmonella* colonies were identified using biochemical tests (Oxidase test, Indol test, Urease test, Methyl Red/ Voges-Proskauer test, Citrate utilization, Kligler test, Urease test and Catalase test), serogrouping using slide agglutination with O, H polyvalent antisera and confirmed by Api20E system.

Antimicrobial susceptibility testing

Antibiotic resistance profiles were determined using the Bauer-Kirby method [10] . Disks used were; ampicillin(10 µg), chloramphenicol(30 µg), co-trimoxazole(1.25/23.75 µg), nalidixic acid(30 µg), tetracycline(30 µg), ciprofloxacin(5 µg), gentamicin(10 µg), cefoxitin(30 µg), ceftriaxone(30 µg), imipenem(10 µg), amikacin(30 µg), rifampicin(5 µg). Results were recorded by measuring the inhibition zone (ml) and interpreted according to Clinical and Laboratory Standards Institute documents [11].

Plasmid isolation of MDR Salmonella Typhi

Plasmid DNA was Extraction from bacteria by *ExiPrep*^{TM}Bacteria Genomic DNA Kit using *ExiPrep*^{TM} 16 Fully Automated Nuclec Acid Extraction System (Bioneer).

Gel was stained with ethedium bromide and photographs were taken under UV trans-illumination according to [12].

Amplification of different drug resistance genes in MDR Salmonella Typhi isolates

Drug resistance patterns were studied by Polymerase Chain Reaction (PCR). The specific primers used for the antimicrobial resistance gene amplification are given in table (1).The conditions for the amplifications are given in table(2). PCR amplification products were determined by agarose gel electrophoresis [12].

Table(1): Oligonucleotide primer sequences used for PCR amplification

Gene	Primer sequence (5-3)	Size/bp	Reference
tem	F:GCACGAGTGGGTTACATCGA R:GGTCCTCCGATCGTTGTCAG	311	13,14
cat	F:CCTGCCACTCATCGCAGT R:CCACCGTTGATATATCCC	623	15
cmlA	F:CGCCACGGTGTTGTTGTTAT R:GCGACCTGCGTAAATGTCAC	394	16
sul1	F:TGGTGACGGTGTTCGGCAT R:GCTAGGCATGATCTAACCCT	841	17
sul2	F:TCAACATAACCTCGGACAGT R:GATGAAGTCAGCTCCACCT	707	17

Table (2): PCR Condition

Cycling Conditions						
Primer	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**No. of Cycles**
tem	94 ^{o}C(5min)	94 ^{o}C(1min)	50^{o}C(1min)	72 ^{o}C(1min)	72^{o}C(7min)	30
cat	94 ^{o}C(2min)	94 ^{o}C(1min)	50 ^{o}C(1min)	72^{o}C(1min)	72^{o}C(5min)	30
cmlA	95 ^{o}C(10min)	95^{o}C(30sec)	55^{o}C(1min)	72^{o}C(1min)	72^{o}C(7min)	30
Sul1	94 ^{o}C(5min)	94 ^{o}C(1min)	50^{o}C(1min)	72 ^{o}C(1min)	72^{o}C(5min)	30
Sul2	94 ^{o}C(5min)	94 ^{o}C(1min)	50^{o}C(1min)	72 ^{o}C(1min)	72^{o}C(5min)	30

III. STATISTIES ANALYSIS

The Chi-square test was used to determine the statistical significance of the data by using SPSS program (Statistical Package for Social Science) version 11, and significance was assumed at $p \leq 0.05$.

IV. RESULTS

Distribution of Salmonella Typhi

A total of the 260 clinically suspected typhoid patients examined in the period between Februray to the end of October 2013. Seventy patients were positive for *S*. Typhi . These (70) isolates which were obtained from blood samples as shown in table (3). In the AL-Zubair, AL-Basrah, AL-Mawani and AL-Fayhaa General Hospital were significant differences at $p \leq 0.05$ from other hospitals.

Table(3): Distribution of *Salmonella* Typhi isolates in different hospitals

No.	Hospital	No. of samples	Isolates	
			No.	%
1	AL-Zubair General Hospital	45	17	37.77*
2	AL-Basrah General Hospital	56	20	35.71*
3	AL-Mawani General Hospital	51	17	33.33*
4	AL-Fayhaa General Hospital	47	11	23.4*
5	AL-Shafaa General Hospital	22	3	13.63
6	AL-Sader teaching Hospital	17	1	5.88
7	Basrah Maternity and Children Hospital	20	1	5
8	Basrah childrens specialty hospital	2	0	0
Total		260	70	26.92

*: Significant differences at $p \leq 0.05$

Isolation and identification of samples

Out of 260 cases, 70 isolates were identified as belong to *S.*Typhi. The isolates were identified as related to the *Salmonella* by their pale colonies (non lactose fermenters) on MacConkey agar. In addition, on SS agar appear as colorless colonies, production of H_2S turn the center of colony to black. All of *Salmonella* (70 isolates) gave alkaline (red) slant and acid (yellow) butt,with production of H2S (blackening of agar) in Kilgler Iron agar, and all of them were positive for methyle red test, this indicated by diffuse red color in medium and indicated positive for fermentation of glucose. They also gave negative reaction for Voges- Proskauer test indicated by absence of development of pink-to red. Also all isolates gave negative reaction for citrate utilization, oxidase, indole and ureas tests.While gave positive for motility and catalase test.

Seventy isolates were serologically diagnosis using *salmonella* antisera to make sure it belonged to the *Salmonella* bacteria.

To confirm and complete the biochemical and serological results, the API 20E system were used for identification of *S.* Typhi. The system which contains 20 different biochemical reactions including 10 enzymatic reactions,10 fermentation oxidation reactions an oxidase test was used. The results were interpreted after 24 h. at 37°C, which revealed that tested isolates (20) belongs to *S.* Typhi . Biochemical reactions on API 20E strip and calculation chart are shown in Figure (1) .

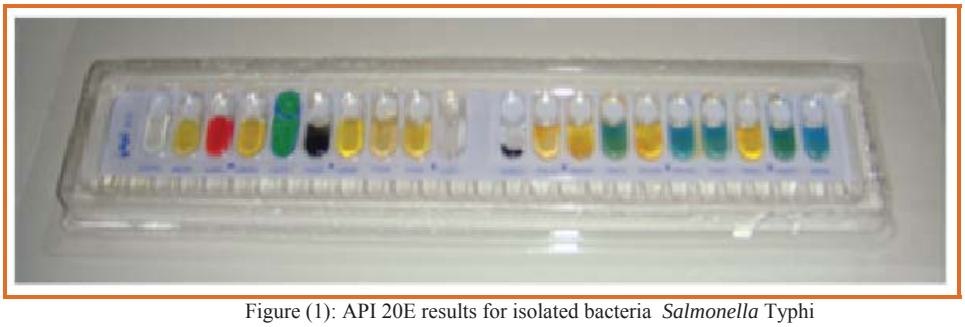


Figure (1): API 20E results for isolated bacteria *Salmonella* Typhi

Antimicrobial susceptibility patterns

Antibiotic sensitivity and resistance rate of 70 isolates of *S.*Typhi to twelve antimicrobial compounds, namely, ampicillin, chloramphenicol, co-trimoxazole, tetracycline, nalidixic acid, gentamicin, ceftriaxone, amikacin, ciprofloxacin, impenem ,cefoxitin and rifampicin are shown in table(4)

Table (4):Frequency of antibiotics susceptibility of *Salmonella* Typhi

Antimicrobial agents	Sensetivity	Intermediate	Resistance
Ampicillin	9(12.85%)	1(1.42%)	60 (85.71%)*
chloramphenicol	14(20%)	0	56 (80%)*
co-trimoxazole	15(21.42%)	1(1.42%)	54 (77.14%)*
Nalidixic acid	32(45.71%)	2(2.85%)	36 (51.42%)
Tetracyclin	44(62.85%)	1(1.42%)	25 (35.71%)
Gentamicin	62(88.57%)	0	8 (11.42%)
Ceftriaxone	66(94.28%)	0	4 (5.71%)
Amikacin	66(94.28%)	0	4 (5.71%)
Ciprofloxacin	58(82.85%)	10(14.28%)	2(2.85%)
Impenem	70(100%)	0	0 (0%)
Cefoxitin	70(100%)	0	0 (0%)
Rifampicin	70(100%)	0	0 (0%)

*: Significant differences at $p \leq 0.05$.

In this study results showed that most isolates were resistant to the antimicrobial agents; (ampicillin, chloramphenicol and co-trimoxazole) table (4) . Out of 70 isolates, 52(86.66%) of them were MDR

Plasmid isolation of MDR Salmonella Typhi

All the 52 MDR *S.*Typhi strains were further processed for plasmid DNA extraction and analysed by agarose gel electrophoresis. There were the same strains which were used for curing. It was found that the separation of large and small plasmids according to their molecular weight as shown in figure(2). All MDR *S*. Typhi isolates (52 isolates) contained large plasmids. There were also small plasmids present within the *S.* Typhi isolates (2) plasmid bands for 9 isolates (17.3%) and 3 band for 3 isolates (5.7%).

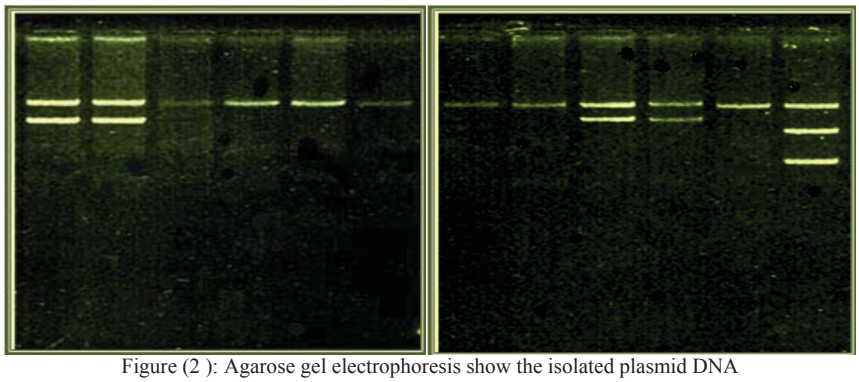


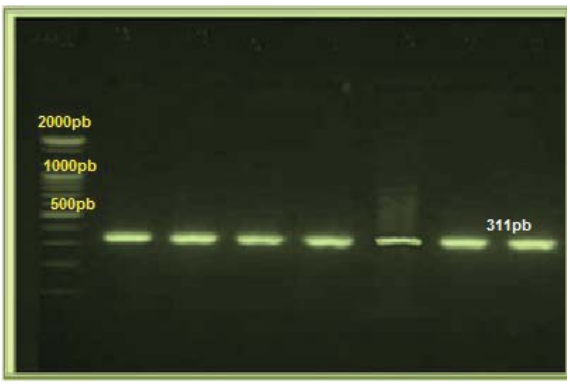
Figure (2): Agarose gel electrophoresis show the isolated plasmid DNA

Detection of antibiotic resistance related genes by PCR

The present of three drug resistance genes was checked by PCR. The result are showed as following:-

Amplification of genes conferring resistance to ampicillin:

The gene related to ampicillin resistance, *tem* gene was targeted by specific primer. The results revealed that all *S*. Typhi isolates 52 isolates (100%) were PCR positive for the *tem* gene with PCR product 311bp (figure 3). DNA bands have been confirmed by comparing its molecular weight with 100bp DNA ladder .



Figure(3):Gel electrophoresis of PCR amplified products from extracted *Salmonella* Typhi DNA amplified with primer *tem.* M= DNA ladder (100-2000 bp)

Amplification of genes conferring resistance to chloramphenic

cat and *cmlA* are genes that confer resistance to chloramphenicol antibiotic. The present study showed high prevalence of *cat* gene for all *S.*Typhi isolates, positive result represented 52(100%) isolates with PCR product 623pb (Figure 4). While amplification with primer of *cmlA* gene did not show any positive result in our study with PCR product 394pb (Figure 5).

Figure (4):Gel electrophoresis of PCR amplified products from extracted *Salmonella* Typhi DNA amplified with primer *cat.* M= DNA ladder (100-2000 bp)

Figure (5):Gel electrophoresis of PCR amplified products from extracted *Salmonella* Typhi DNA amplified with primer *cmlA.* M= DNA ladder (100-2000 bp)

Amplification of genes conferring resistance to co-trimoxazole

*Sul1*and *sul2* are those responsible for conferring resistance to co-trimoxazole drug. PCR amplification was carried out for both these genes by specific primers. Results showed that 2(3.84%) of *S.* Typhi isolates gave positive result for *sul1* gene with PCR product 841bp (Figure 6). On the other hand, the prevalence of *sul2* gene was relatively high, where results showed its presence in 50(96.15%) isolates, and showed an amplification product of 707bp (Figure 7).

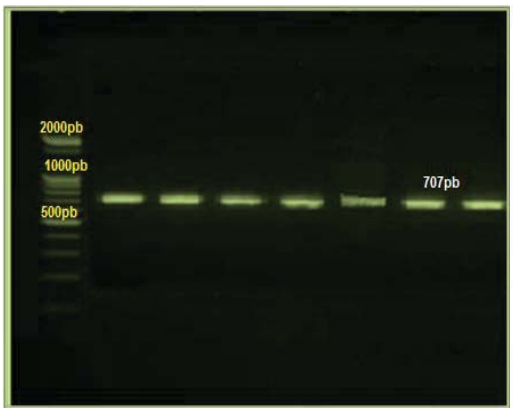


Figure (6):Gel electrophoresis of PCR amplified products from extracted *Salmonella* Typhi DNA amplified with primers *sul1*. M= DNA ladder (100-2000 bp)

Figure (7):Gel electrophoresis of PCR amplified products from extracted *Salmonella* Typhi DNA amplified with primers *sul2* . M= DNA ladder (100-2000 bp)

V. DISCUSSION

In spite of recent advances in public health and sanitation, Typhoid fever continues to be a major cause of morbidity and mortality [18]. . It is clear from table (3) that the majority of patients are AL-Zubair General Hospital, AL-Basrah General Hospital, AL-Mawani General Hospital and AL-Fayhaa General Hospital respectively. During present study the detection of multidrug resistance among isolates was performed soon after the identification of *Salmonella* isolates from blood samples. Data also showed that the prevalent resistance patterns were that against (ampicillin, chloramphenicol and co-trimoxazole) which represent the first line therapy of enteric fever table(4).Out of 70 isolates, 52(86.66%) of them were MDR.

Historically, the drugs of choce were chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (co-trimoxazole). However, antimicrobial resistant *S.*Typhi isolates emerged in the 1970s in Latin America and Asia. A large number of *S.* Typhi strains have been isolated in Korea, but no resistant strain was documented until 1992, when a chloramphenicol, ampicillin and co-trimoxazole resistant strains were isolated from a patient who returned from a southeast Asian country [19].

A study conducted in Iraq (sulaimani)[20] shown that resistance to chloramphenicol, ampicillin and co-trimoxazole were exceeding 97% of *S.* Typhi isolates. (21) finding revealed that most of the isolates were marked by MDR, and that may be attributed to the continuous use of antibiotics for treatment of typhoid fever,in addition the unclear food and water supply. Reports of (22) confirmed the conyinuous use of antibiotics for treatment; this phenomena pause a big challenge to the scientists when the resistance to antibiotics increased and make a global problem.

Nucleic-acid targeted detection systems as the polymerase chain reaction (PCR) offer rapid and sensitive method to detect the presence of resistance genes and is crucial in the elucidation of resistance mechanisms [23].

The results of this study confirmed to this general trend as chloramphenicol that that the cat gene was amplified in 52 (100%)isolates from 52 MDR isolates (were positive for *cat* gene) but non (negative) for *cmlA* gene. In addition, showed that the Temoniera (*tem*) gene was amplified in 52(100%) isolates from 52 MDR isolates. Out of 52 MDR isolates,*sul2* gene was detectable in 50(96.15%) isolates while 2(3.84%) of these isolates showed as *sul1* gene.

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