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# *Antibiotic resistant and plasmid conjugative study of Salmonella typhi*

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## HIGHLIGHTS

All antibiotic markers were transferred from *S. typhi* to *E. coli* HB 101 with a conjugation frequency of ($0.38\times10^{-5}$).

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## A B S T R A C T

The emergence of multi-drug resistant (MDR) bacteria has endangered the efficacy of antibiotics treatment of pathogenic bacteria worldwide. The aim of this research was to investigate the incidence of *Salmonella enterica* serovar Typhi in Duhok city, Iraq. Specimens of blood and stool were recruited from 267 patients. *S.* Typhi isolates were diagnosed depending on morphology, biochemical and serological tests. *S.* Typhi isolates were tested for their antibiotic resistance. Multi-drug resistant *S.* Typhi isolates were conjugated with *E. coli* HB101. The plasmid profile of transconjugants was investigated. 15/267 (5.6%) *S.* Typhi isolates were identified. Based on their biochemical tests, *S.* Typhi isolates were categorized into two biotypes (I, 26.66% and II, 73.33%). Four resistance patterns were observed. The resistant pattern to ampicillin and tetracycline was the higher (46.6%). Conjugation experiment showed that all antibiotic markers were transferred from *S.* Typhi to *E. coli* HB101 with a conjugation frequency of ($0.38\times10^{-5}$). 13.3% of the *S.* Typhi isolates were multi-drug-resistant resistant and had two small plasmids. Transconjugants *E. coli* acquired the resistance from the multi-drug resistant *S.* Typhi. Antibiotics treatment of the pathogens could be hindered by the constant rise of multi-drug-resistant. Further studies are needed to study the mobile genetic elements and their contribution in antibiotics resistance.



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## 1. INTRODUCTION

*Salmonella enterica* serovar Typhi causes nearly 27 million typhoid cases and about 600 thousands deaths yearly, commonly in developing countries.[1] Antibiotics treatment of the pathogens could be hindered by the constant rise of multi-drug-resistant (MDR) pathogens.[2,3,4] Several mechanisms are exist to confer Salmonella strains to multi-drug resistance (MDR) such as increased mutation rate as a stress response, transfer copies of DNA that codes for a mechanism of resistance to other bacteria by plasmids, decreased cell wall permeability to antibiotics, enzymatic deactivation of antibiotics, efflux mechanisms to remove antibiotics and altered target sites of antibiotic.[5] The multi-drug-resistant *S.* Typhi isolates harbored a transferable plasmid conferring resistance to specific antibiotics. Horizontal exchange of DNA material may occur through plasmids, bacteriophages, transposons and insertion sequences.[6] One of the essential reasons to increase the resistance to multiple drug is the ability of mobile genetic elements to transfer the exogenous genes among bacteria conferring resistance to one or more antibiotics.[7] The aim of this research was to investigate the incidence of *S.* Typhi in patients attending Akre general hospital, Duhok, Iraq. Additionally, to detect the antibiotic susceptibility profile of these isolates. Moreover, to investigate the plasmid profiles of different isolates which show different patterns of drug resistance and to study the role of transmissible plasmids in multidrug resistance.

## 2. MATERIAL AND METHODS

### 2.1 Study design and sample collection

A total of 267 patients who fulfilled the inclusion criteria were studied. The inclusion criteria for the participants were: (a) patients above the age of 1 year; (b) Having both fever and diarrhea; (c) Not using antibiotics for two weeks before enrollment; (d) Willing to participate in the study.

A total of 267 specimens, either blood or stool, from patients who met the criteria, (214 blood and 53 stool) were collected from patients attending Akre general hospital, Duhok, Kurdistan region, Iraq from November 2011 to April 2012. Samples were directly sent to the laboratory, where the analysis was performed. Blood samples were cultivated on BHI broth (Oxoid, UK)[8] while stool samples were cultured on selenite F broth (Oxoid, UK) then incubated for 18 h at 37°C. Then, the broth cultures were examined for significant growth. Next, a loopful from the broth culture was streaked on both MacConkey and Salmonella Shigella agar (Oxoid UK). If bacterial growth was observed, subcultures were prepared into nutrient agar plates and incubated for another 24 h. Isolates were characterized morphologically, gram stain and biochemical reactions.[9,10]. 0.5 ml of bacterial suspension was added to sterile tubes containing 0.5 ml glycerol mixed well, and stored at -20ºC until use.

### 2.2 Antibiotic susceptibility test

*S.* Typhi isolates were tested against three different antibiotics (Bioanalyse, Turkey). Disk diffusion assay was utilized to test the antibiotic susceptibility according to Bauer *et al.* (1966).[11] Bacterial suspensions were prepared in 1.0 ml of sterile normal saline. Then, 0.5 ml of the suspension (OD 0.3 at 600nm) was prepared and spread on Muller-Hinton agar (Difco, USA). Antibiotic discs were placed on the inoculated plates and plates were incubated at 36˚C for 18h. The diameter of inhibition zones were measured according to the recommendations of Clinical and Laboratory Standards Institute (CLSI) guidelines.[12]

2.3 Bacterial conjugation

MDR *S.* Typhi isolates resistant to AMP, TET and CHL antibiotics was used as a donor and *Escherichia coli* HB101 (Rifampicin resistant) was used as a recipient. Bacterial conjugation was performed according to Buu-Hoi and Horodniceanu (1980).[13] Donor and recipient strains were grown in 10 ml BHI broth separately and incubated at 37ºC for 18h. Matings were done by mixing 1 ml of each of donor and recipient cells. Then, 0.2 ml of the mixture was placed directly on a sterile filter paper (0.45µm) and transferred on BHI agar and incubated at 28ºC for 18h. The filter paper was washed with 0.5ml of BHI broth, then the bacterial suspension was diluted to $10^{-5}$, and 0.1 ml of each dilution was spread on BHI agar containing 30µg/ml Rifampicin to select transconjugants. Dilutions ($10^{-5}$, $10^{-6}$, and $10^{-7}$) of the donor isolate culture were prepared and 0.1 ml was spread on BHI agar and incubated for 24h at 37ºC. Then, the frequency of transfer was calculated (The frequency of transfer=No. of transconjugants/No. of donor cells).[13]

2.4 Genomic DNA extraction

Genomic and plasmid DNA extraction was performed according to Roeder and Broda (1987).[14] Fifty (50) ml of bacterial culture in BHI broth was harvested for 10 min at 4000 rpm. The pellet was suspended in 3.5 ml of TE25S buffer (10.26 gm sucrose, 2.5ml of 1M Tris-HCL and 5ml of 0.5M EDTA were mixed and distilled was water used to completed the volume to 100 ml) and 100µl (10 mg/ml) of lysozyme was added and incubated for 1h at 37ºC. Then, 50µl proteinase K and 200 µl SDS (10%) was added and incubated at 55ºC for 1h. 650 µl of NaCl and 650 µl of CTAB/NaCl (10 gm CTAB was added to 14 ml of heated 5M NaCl and the volume was completed to 100 ml with distilled water) were mixed to each tube and incubated for 10 minutes at 55ºC and left at room temperature for 5 minutes. Equivalent size of Chloroform/Isoamyl alcohol (24:1) was added and shake gently for 30 minutes then centrifuged at 4000 rpm for 10 minutes (This step was repeated twice). The supernatant was moved to clean tubes and identical volume of chilly isopropanol was mixed. The tubes were covered with parafilm and mixed gently then kept at -20 ºC for 30 minutes. Then the tubes centrifuged at 4000 rpm for 20 minutes. Next, 1 ml of 70% ethanol was used to wash the pellet and centrifuged at 8000 rpm for 5 minutes at 4°C. The pellet was drained on a filter paper for 30 minutes. The DNA pellet was dissolved in 300 µl TE buffer and stored at -20°C. The DNA was analyzed by 0.8% agarose gel electrophoresis.

## 3. RESULTS AND DISCUSSION

3.1 *Salmonella* Typhi isolation and identification

*S.* Typhi bacteria were diagnosed depending on morphological, serological and biochemical tests. Suspected *S.* Typhi colonies were further identified using API 20E system. Out of 267 (214 blood and 53 stool) tested specimens, only 15 (5.6%) isolates (4 from blood and 11 from stool) were characterized as *S.* Typhi. All these isolates were confirmed by API 20E system. These isolates were differentiated into two different biotypes according to their biochemical reactions. 4/15 (26.66%) isolates were belong to biotype group I, and 11/15 (73.33%) isolates were belong to biotype group II.

3.2 Antibiotic susceptibility test

All *S.* Typhi isolates were tested toward three different antibiotics (ampicillin, chloramphenicol and tetracycline). Results indicated that all isolates

were resistant to ampicillin. Additionally, 9/15 (60%) were resistant to tetracycline and 3/15 (20%) of the isolates were resistant to chloramphenicol.

3.3 Multidrug resistant *S.* Typhi isolates

Bacterial strains that were resistance to one antibiotic in three or more antimicrobial categories are assigned as multi-drug resistant.[5] Results showed four patterns of antibiotic resistance *S.* Typhi isolates (Table 1). 2/15 (13.3%) *S.* Typhi isolates were identified as multi-drug resistant.

Table 1**.** Different patterns of antibiotic resistance *S.* Typhi.

| Resistance pattern | No. of isolates (%) |
|---|---|
| AMP | 5 (33.3%) |
| AMP, TET | 7 (46.6%) |
| AMP, CHL | 1 (6.6%) |
| AMP, TET, CHL | 2 (13.3%) |

3.4 Plasmid profile

To investigate the plasmid profiles of the MDR *S.* Typhi, DNA was extracted from the MDR isolates. Results showed that all these isolates harbor two small plasmid bands which appear almost at the same position on the gel (Figure 1).

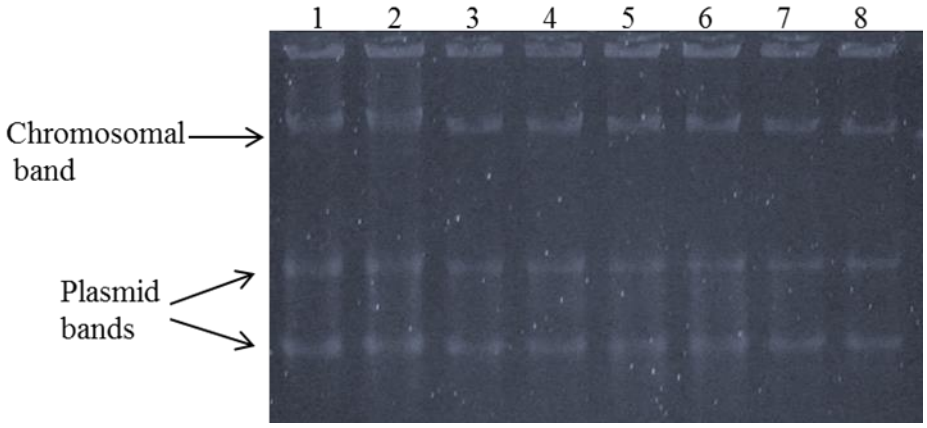


Figure 1. 0.8% gel electrophoresis at 70 Volt for 90 minutes shows the plasmid profile of eight different antibiotic resistances *S.* Typhi isolates

3.5 Conjugation

In order to investigate whether the MDR genes are harboured on transmissible plasmids, the MDR *S.* Typhi isolate resistant to AMP, TET and CHL antibiotics was utilized as a donor strain and a plasmidless refampicin resistant *E. coli* HB101 was used as a recipient. Results showed that the antibiotic markers AMP and TET were transferred to the recipient strain refampicin resistant *E. coli* HB101 (Figure 2). The frequency of transfer was calculated and it was $0.38\times10^{-5}$.

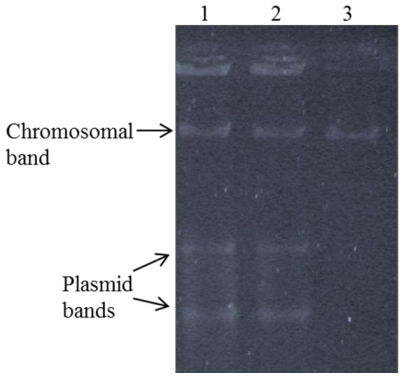


Figure 2. 0.8% agarose gel electrophoresis at 70 Volt for 90 minutes. Lane 1: Genomic DNA of donor strain of MDR *S.* Typhi resistant to (AMX, TET and CHL). Lane 2: Genomic DNA of transconjugant strain *E. coli* HB101 resistant to (AMX and TET). Lane 3: Genomic DNA of Plasmidless refampicin resistant *E. coli* HB101. Conjugation frequency $0.39 \times 10^{-5}$

There are considerable increases of the incidence of human Salmonellosis and its severity.[15] Results revealed that the incidence of *S.* Typhi amongst the 267 recruited patients was 5.6% (15/267). High incidences of typhoid fever were reported in different countries in Asia with difference in occurrence in many places in the same areas.[16,17] According to the biochemical tests, the 15 *S.* Typhi isolates were classified into two different biotypes group I (4, 26.66%) and group II (11, 73.33%). These results were in agreement with other studies worldwide.[18, 19]

The resistance of Salmonella to a variety of antibiotics become a serious public health problem.[20] All *S.* Typhi isolates were resistant to ampicillin. This was in agreement with results of many studies in which the resistant was 100% to ampicillin.[21] High resistance to β-lactam group of antibiotics is due to the ability of *S.* Typhi to produce β-lactamase enzymes.[22] *S.* Typhi isolates were varied in their resistance against tetracycline and chloramphenicol. The alarming ability of the microbe to acquire persistent, high-level resistance to the clinically most relevant antibiotics is a major problem.[23,24,25,26] The *S. typhi* showed different pattern of antibiotic resistance. These results were in agreement with other studies which show different patterns of MDR *Salmonella* Typhi toward different antibiotics.[27,28]

The rise of multidrug-resistance *Salmonella* Typhi is because of the random use of antibiotics in the treatment of enteric fever.[29] AMP, CHL and TET resistance in *Salmonella* Typhi is often plasmid mediated.[30] *Salmonella enterica* plasmids are varying in size (2–200 kb). The antibiotic resistance is due to plasmids group of high molecular weight.[31]

The plasmid profiles of the MDR isolates showed that all isolates harbored two small plasmid bands. In *S.* Typhi, R-plasmid confer resistance to ampicillin, chloramphenicol, cotrimoxazole, and tetracyclin was reported to be transferable.[32] Conjugation is a specific process usually mediated by conjugative plasmids that encode resistance in *Salmonella* Typhi and thus it plays role to the distribution of typhoid fever globally.[33]

The phenotypic characterization of the conjugation experiments showed that the transconjugant *E. coli* HB101 acquired the resistance to AMP and TET antibiotics. Results from various studies revealed the transfer of MDR markers through conjugation experiment from *S.* Typhi to *E. coli*.[34] Mandal and his coworker showed the transfer of MDR markers to a plasmidless *E. coli* (C600) with a frequency of ($0.39 \times 10^{-5}$).[30]

β-lactamase enzymes genes, found on the chromosome or on resistance

plasmids, are transferable among plasmids and chromosomal DNA of different bacteria.[35] Studies showed different plasmids that mediate resistance to antibiotics. In a study, different plasmids, IncHI2 , IncI1 and IncHI2 , were found to mediate resistance to ampicillin in Salmonella.[36] Another study supported the view that acquisition of IncFI plasmids conferred resistance to ampicillin, chloramphenicol and tetracycline.[37] Furthermore, plasmid pFPTB1 mediates resistance to tetracyclines and ampicillin in Salmonella.[38] Multidrug-resistant *S.* Typhi strains may harbor integrons that carry genes of about six antibiotic resistance.[39,40]

## 4. CONCLUSION

In summary, only 2 out 15 (13.3%) of *S.* Typhi isolates were Multi-drug resistant and harboured two small plasmids. The drug resistance markers for ampicillin and tetracycline were capable to transfer to *E. coli*. The treatment of infections could be hindered because of the distribution of Salmonella strains that are resistant to various antibiotics. Further studies are needed to identify the genes conferring to MDR carried by these plasmids and to investigate the pathogenicity islands and their contribution to virulence.

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