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

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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. The annual incidence of typhoid fever is estimated to be about 17 million cases worldwide. In Africa, it has an estimated crude incidence of 362 cases per 100,000 persons per year. In most endemic areas, approximately 90% of enteric fever is typhoid and caused about 216,500 deaths among children and young adults worldwide.

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.

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.

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.

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 °C. 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. 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 (mm) and interpreted according to Clinical and Laboratory Standards Institute documents.

**Plasmid isolation of MDR Salmonella Typhi**

Plasmid DNA was extracted from bacteria by ExiPrep™Bacteria Genomic DNA Kit using ExiPrep™ 16 Fully Automated Nucleic Acid Extraction System (Bioneer).

Gel was stained with ethidium 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>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5-3)</th>
<th>Size/bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tem</td>
<td>F:GCACGAGTGGTACATCGA R:GGTCTCCCGCATGTTCAG</td>
<td>311</td>
<td>13, 14</td>
</tr>
<tr>
<td>cat</td>
<td>F:CTGCCCTCTATCGAGT R:CCACCCTGATATATCC</td>
<td>623</td>
<td>15</td>
</tr>
<tr>
<td>cmlA</td>
<td>F:CGCCCGTGGTTGAGTCATG R:GCGACCTGCCGTAATTGTCAC</td>
<td>394</td>
<td>16</td>
</tr>
<tr>
<td>sul1</td>
<td>F:TGTGACGGTGATCCGACAT R:GCTAGGCATGATCTAACCCT</td>
<td>841</td>
<td>17</td>
</tr>
<tr>
<td>sul2</td>
<td>F:TCACTACATCGGACAGT R:GATGAAGTGCATGCACCT</td>
<td>707</td>
<td>17</td>
</tr>
</tbody>
</table>

**III. STATISTICS 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 \( p < 0.05 \).

**IV. RESULTS**

**Distribution of Salmonella Typhi**

A total of the 260 clinically suspected typhoid patients examined in the period between February 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 \( p < 0.05 \) from other hospitals.
Table (3): Distribution of *Salmonella* Typhi isolates in different hospitals

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

*: Significant differences at *p* ≤ 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 H₂S 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](image)

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).

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

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

*: Significant differences at p≤ 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](image)

**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.
Amplification of genes conferring resistance to chloramphenicol

*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).

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 841pb (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).
In spite of recent advances in public health and sanitation, Typhoid fever continues to be a major cause of morbidity and mortality. 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 \textit{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 choice 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.

A study conducted in Iraq (sulaimani) 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 continuous 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. The results of this study confirmed to this general trend as chloramphenicol 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.

REFERENCES


