Molecular Study on Distribution of Sul-1 and Sul-2 genes among Salmonella enterica causing Enteric Fever

Abdullah K. Hindi  
Prof. 
Science college/University of Babylon

Ebtehal E. Shubbar  
Assis Teacher 
Science college/university of Kufa

Salman A. Addos  
Assis Prof 
Medicine college/University of Kufa

Abstract
This study comprised 200 blood samples taken from patients visited some of Al-Najaf province. The most prevalent species was Salmonella Typhi, where 20 isolates had been identified. Serological typing was done by using both poly and monovalent antisera, which revealed that all isolated Salmonella was belong to serovar Typhi. DNA was extracted from all Salmonella Typhi isolates. Extracted DNA was used in Polymerase Chain Reaction technique. Three pairs of primers was used to detect the presence of targeted genes (two for sul-1 gene, and one for sul-2). Data showed the occurrence of sul-1 gene the first sequence in 15% of total, while second sequence gave no positive result. Sul-2 gene on the other hand, showed 50% positivity.

Introduction
Enteric fevers have continued to pose a serious threat to public health causing morbidity and mortality especially in economically poor countries where level of hygiene is below standards and sanitary conditions are poor. (Dimitrov et al., 2009). Enteric fever (typhoid or paratyphoid fever) is a systemic infection caused by several Salmonella enterica serotypes including S. Typhi and S. Paratyphi A, B, or C (Udeze et al., 2010). It affected an estimated 16 million people in the 1990s, with 600,000 deaths reported annually worldwide (Lee et al., 2004)

The use of antimicrobial agents in food animals has been a major factor in the emergence of Salmonella with decreased susceptibility to antibiotics. A basic role in the spread of antimicrobial resistance in Salmonella has been attributed to class 1 and class 2 integrons. (Antunes et al., 2005)

Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes sul1 and sul2, encoding forms of dihydropteroate synthase that are not inhibited by the drug. The sul1 gene is normally found linked to other resistance genes in class 1 integrons (Liu et al., 2009)

The correlation has been made between the sul genes and the presence of integrons and hence this study was carried out to achieve the following aims:

1. The detection of Salmonella causing enteric fever by blood culture.
2. The investigation of sul genes among Salmonella Enterica serovar Typhi.

Procedures
1-Samples Collections
Blood samples (200) samples were collected from patients suffering from enteric fever symptoms in Al-Najaf province hospitals. Patients whose Widal test titer was ≥160 were submitted to venipuncture procedure.

Patients comprised 105 males and 95 females, whom aged from 15-45 years.

2- DNA Extraction (salting out method)
According to Pospiech and Neumann (1995), the DNA extraction was done as follow: a loopfull of bacterial growth were inoculated in 5 ml nutrient broth at 37 °C for 24 hours. The bacterial growth was centrifuged at 6000 rpm for 5 minutes. The precipitate was washed in TE buffer by adding 2 ml of TE buffer and vibrated by vortex then centrifuged at

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6000 rpm for 10 minutes, and the washing repeated twice. The pellet resuspended in 2.5 ml SET buffer. 300 µl of freshly made 25% SDS were added to the lysate. The lysate was mixed by inversion, incubated at 55°C for 5 minutes, then mixed again thoroughly by inversion. 1 ml of 5M NaCl solution were added and cooled to 37°C. A mixture of equal volume (3.8 ml) of [phenol: chloroform: isoamyl alcohol (25:24:1) was added to the lysate and mixed by inversion for 30 minutes at room temperature. It was spin by centrifuging at 6000 rpm for 15 minutes. The aqueous phase was transferred to a fresh tube, then 0.6 volume of isopropanol was added to the extract and mixed by inversion for 3 minutes; the DNA spooled on to a pasteur pipette. DNA rinsed in 5 ml of 70% ethanol, air dried for 15 minutes, and dissolved in 1 ml TE buffer, heated to 55°C in water-bath, and the DNA extract was stored in freezer at -20°C until used.

3- Polymerase Chain Reaction Assay

A. Preparing the primers

The Biocorp DNA primers, table (1) were prepared depending on manufacturer instruction by dissolving the lyophilized product with TE buffer molecular grad after spinning down briefly. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

Table(1) primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sul 1</td>
<td>F CGG CGT GGG CTA CCT GAA CG</td>
<td>Sunde ,2005</td>
</tr>
<tr>
<td></td>
<td>R GCC GAT CGC GTG AAG TTC CG</td>
<td></td>
</tr>
<tr>
<td>Sul 2</td>
<td>F CCT GTT TCG TCC GAC ACA GA</td>
<td>Chang et.al,2007</td>
</tr>
<tr>
<td></td>
<td>R GAA GCG CAG CCG CAA TTC AT</td>
<td></td>
</tr>
<tr>
<td>sulR</td>
<td>F TAA GCC CTA CAC AAA TTG GGA GAT AT</td>
<td>Chuanchuen et. al, 2007</td>
</tr>
<tr>
<td></td>
<td>R GGG TGC GGA CGT AGT CAG C</td>
<td></td>
</tr>
</tbody>
</table>

B. PCR supplies assembling and thermocycling conditions

The DNA templates were subjected to PCR using 3 sets (F and R) of primers targeting genes listed in table (1). The reaction mixture moreover contain Go Taq® Green Master Mix, X2 which is premixed ready-to-use solution containing bacteriology derived TaqDNA polymerase dNTP, MgCl₂, and reaction buffers at optimal concentrations and its recommended for any amplification reaction that to visualized by agarose gel electrophoreses and ethidium bromide staining.

Assembling PCR materials were done according to the procedure of Promega corporation (USA), using PCR reaction mixtures prepared in 0.2 ml eppendorf tube with 25 µl reaction volumes, which contain: 12.5 µl Go Taq® Green Master Mix X2, 2.5 µl upstream primer, 2.5 µl downstream primer, 5 µl DNA template, 2.5 µl nuclease-free water. All the appending was done in laminar flow on ice.

C. The PCR Cycling Profiles

Polymerase chain reaction assays were carried out in a 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows:

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Table (2) PCR cycling Profiles

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycles number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SulI</td>
<td>95</td>
<td>94</td>
<td>55</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>30 sec</td>
<td>30 sec</td>
<td>10 min</td>
</tr>
<tr>
<td>SulR</td>
<td>95</td>
<td>95</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>45 sec</td>
<td>1 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Sulf 2</td>
<td>95</td>
<td>94</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>30 sec</td>
<td>30 sec</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Results and Discussion

1. Isolation and Identification

Identification was done by subculturing of inoculated bottles on Blood agar, MacConkeys agar, Xylose Lysine Deoxycholate (XLD) agar, Salmonella Shigella (S.S.) agar and Triple Sugar Iron (TSI) agar. *Salmonella* colonies appeared pale on MacConkey's agar (non lactose fermenter), red with black center on (XLD) agar, also formed black colonies on (S.S.) agar. Further identification was confirmed by using api-20 E system.

Our study came in some similarity with that of Fairuze-Ali, (2006), where she recorded (22.6%) positive culture from bacteremias and *Salmonella* still the first in rate of isolation. Although the two studies that made in Najaf, our study showed a difference in *Salmonella* prevalence, this may be attributed to that: patients comprised in present study only those who had symptoms of enteric fever and high Widal test titres≥ 160, but it still higher than finding of Antunes et al., (2006) who obtained (50%) and from that of Enabulele et al.,(2006) which was (39%) out of the 1431 gram negative bacilli.

On the other hand, Obara et al., (2011) yielded Three agents from bacteremia cases; *Staphylococcus aureus* (20.9%), *Salmonella typhi* (20.9%) and *Acinetobacter* (12.3%) counted for over half of the positive cultures, while *Streptococcus pneumoniae* and non-typhi Salmonellae each accounted for (7.6%).

2. The detection of sul genes:

*Sul* genes are those genes responsible for conferring resistance to sulfonamide drugs.

In this study we used three different sequence of oligos primers, two of them to detect the *sul* 1 gene and the other is to detect the presence of *sul* 2 gene.

Results showed that three, (15%) of *S. Typhi* isolates gave positive result for *sul* 1 gene by using the primer sequence: (F:CGG CGT GGG CTA CCT GAA CG, R:GCC GAT CGC GTG AAG TTC CG), which numbered (4,7,8) while the sequence (F:TAA GCC CTA CAC AAA TTG GGA GAT AT, R: GGG TGC GGA CGT AGT CAG C) Figure (4-12) gave no DNA product after amplification by PCR.

On the other hand the prevalence of *sul*-2 gene was relatively high, where results showed its presence in 10 isolates numbered:(ST2, ST 3, ST 4, ST 5, ST 6, ST 10, ST 13, ST 15, ST 19, ST 20) , (50%) of isolates. Figure(1). On the basis of relationship between the two above results, we found that only one isolate (5%) had both genes. This result is converging with a study in Denmark made to detect the *sul* 1 and *Sul* 2 genes where they
had 54 of 292 isolates with sul 2 gene, and 30 had sul 1 gene while those with both were 20 isolates, and three lacking any of these genes (Kerren et al., 2002).

Another study performed in Central African Republic showed that sul1 gene was found in 67 isolates, the sul2 gene in 72 isolates and both genes in 62 isolate and this resembles present study in this result. (Frank et al., 2007).

Table (3) Prevalence of Sul gene among S. Typhi isolates

<table>
<thead>
<tr>
<th>Result</th>
<th>Number</th>
<th>Percentage</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive for Sul-1</td>
<td>3</td>
<td>15%</td>
<td>ST 4, ST 7, ST 8</td>
</tr>
<tr>
<td>Negative for Sul-1</td>
<td>17</td>
<td>85%</td>
<td>ST 1, ST 2, ST 3, ST 5, ST 6, ST 9, ST 10, ST 11, ST 12, ST 13, ST 14, ST 15, ST 16, ST 17, ST 18, ST 19, ST 20</td>
</tr>
</tbody>
</table>

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By using PCR dependent method for screening the presence of sul genes in Salmonella from various sources in United kingdom Bailey et al (2009) revealed that of the 531 bacterial isolates the most common genotype was sul 1 gene followed by sul 2 then by sul 3. The occurrence of the three genes was only in (2.3%). Our study had disagreed with Antunes et al ., (2005) study which made to detect the sul genes among the Portuguese Salmonella isolates were sul-1 then preceding by sul-2 and sul-3 genes where out of 200 isolates results were :152, 74, and 14 respectively.

Sulfonamides have been used as antimicrobial agents since 1930s and these compounds started the modern era of chemotherapy (Radstrom and Swedberg 1988). The resistance of these drugs is plasmid mediated that is very frequently found in Gram negative clinical isolates and often in combination with other antibiotic resistance traits (Lopez et al ., 1987). These drugs act as structural analogue of P-aminobenzoic acid and bind dihydropteroate synthase, a catalytic enzyme in the folic acid biosynthesis pathway result in inhibition of dihydrfolic acid formation. (Bailey et al .,2009). The resistance was conferred by mutation in dihydropteroate synthase (DHPS) gene or from acquisition of an alternative gene (sul). Enzymatic and genetic characteristics divide the plasmid-borne, sulfonamide resistance dihydropteroate synthases (DHPS) into two groups, type I and type II. In a cell extract, type I DHPS loses its activity rapidly, whereas the type II enzyme is stable. (Perreten, and Boerlin 2003). The sulI gene is often located on transposons related to Tn2l and on large self-transmissible resistance plasmids that show similarity to Tn2l in the resistance region .The gene for the type II DHPS is often found on small nonconjugative R plasmids (Perreten and Boerlin 2003).

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted S.Typhi DNA amplified with primers Sul-2F and Sul-2 R. Lane (M), DNA molecular size marker(100-bp ladder).
Lane (ST2) S.Typhi isolate number 2 shows positive results with Sul-2 gene.
Lane (ST3) S.Typhi isolate number 3 shows positive results with Sul-2 gene.
Lane (ST4) S.Typhi isolate number 4 shows positive results with Sul-2gene.
Lane (ST5) S.Typhi isolate number 5 shows positive results with Sul-2gene.
Lane (ST6) S.Typhi isolate number 6 shows positive results with Sul-2 gene.

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Lane (ST10) S. Typhi isolate number 10 shows positive results with Sul-2 gene.
Lane (ST13) S. Typhi isolate number 13 shows positive results with Sul-2 gene.
Lane (ST15) S. Typhi isolate number 15 shows positive results with Sul-2 gene.
Lane (ST19) S. Typhi isolate number 19 shows positive results with Sul-2 gene.
Lane (ST20) S. Typhi isolate number 20 shows positive results with Sul-2 gene.

Lanes (ST1, ST7, ST8, ST9, ST11, ST12, ST13, ST14, ST16, ST17, ST18) S. Typhi isolates show negative result to Sul-2 gene.

From the study of class I integron the region of sul I gene is located on the 3 conserved region and is frequently identified with these potentially mobile elements in slurry and soil environment. (Bailey et al., 2009). Till now the known genes encoding plasmid borne sulfonamides resistance do not exceed the sul1, sul2, and sul3. Although many studies had been made for the prevalence of sul genes, they showed vast variation depending on environment, and bacterial species sampled. Our study came in accordance with that of Trobos et al., (2009) were among the three studied genes the sul 2 was the most prevalent one in E.coli from pig and pork poultry. While Antunes et al., (2005) found that sul 1 was the most prevalent one and hence the most frequent mechanism of resistance to sulfonamides in Portugal. In contrast recently the spread of sul 2 seem to have increased in other European countries (Kerrn et al., 2002).

Presence of sul2 gene is related with class 1 integron, since class1 integrons were always associated with sul genes, including sul2 and sul3.(Antunes et al., 2005). The same study confirmed that sul1 gene was consistent marker for presence of this class of integrons because it was found in all isolates having them and resistant to sulfonamides. But in our study number two isolates (7, 8) were positive for the sul1 gene but negative for class 1 integron, this does not mean that they do not have class 1 integron according to the study of Frank et al., (2007) where they found that three of isolates of E.coli had the same result and they attributed it to that one plasmid has been described carrying class 1 integron and truncated int gene which could have given similar result.

Table (4): Prevalence of Sul-2 gene among S. Typhi isolates

<table>
<thead>
<tr>
<th>Result</th>
<th>Number</th>
<th>Percentage</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive for Sul-2</td>
<td>10</td>
<td>50%</td>
<td>ST 2, ST 3, ST 4, ST 5, ST 6, ST 10, ST 13, ST 15, ST 19, ST 20</td>
</tr>
<tr>
<td>Negative for Sul-2</td>
<td>10</td>
<td>50%</td>
<td>ST 1, ST 7, ST 8, ST 9, ST 11, ST 12, ST 14, ST 16, ST 17, ST 18</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>100%</td>
<td>20</td>
</tr>
</tbody>
</table>

References

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