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Molecular Study on Distrbution 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 eneric 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 precipite 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 NaCI solution were added and icooled to 37°C. A mixture of equal volume (3.8 ml) of [phenol: chloroform: isoamylacohol (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 pasture 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

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

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, $MgCl_2$, 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 µI Go Taq® Green Master Mix X2, 2.5 µI upstream primer, 2.5 µI downstream primer, 5 µI DNA template, 2.5 µI 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 µI 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

Primer name	Denaturation	Annealing			Extension	Cycles number
SuII	**95 5 min**	**94 30 sec**	**55 30 sec**	**72 30 sec**	**72 10 min**	**35**
sulR	**95 5 min**	**95 45 sec**	**50 45 sec**	**72 1 min**	**72 10 min**	**30**
Sul 2	**95 5 min**	**94 30 sec**	**60 30 sec**	**72 30 sec**	**72 10 min**	**30**

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

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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).

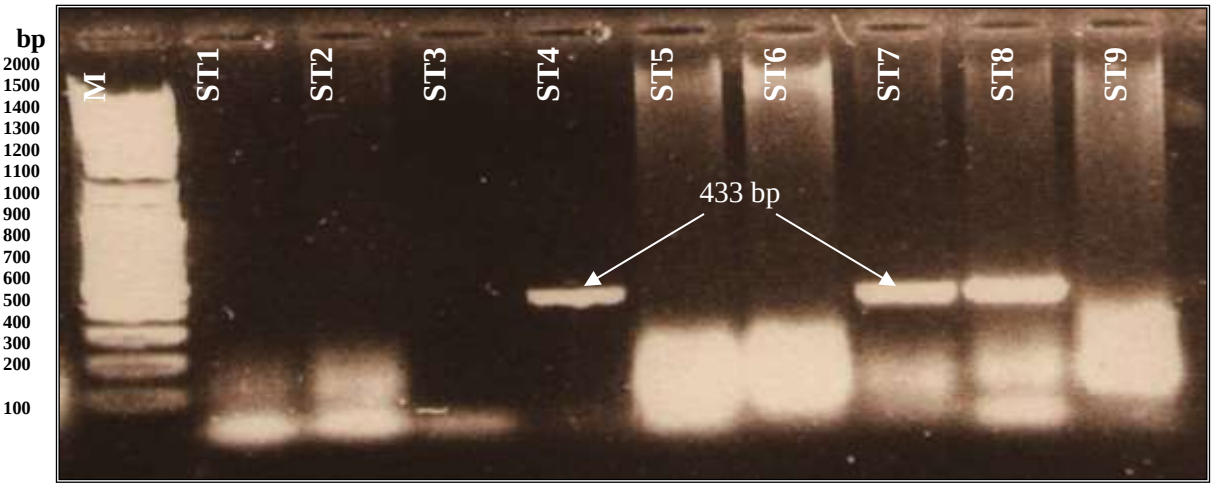

Figure (1): Ethidium bromide-stained agarose gel of PCR amplified products from extracted *S.*Typhi DNA amplified with primers *SulI* F and *SulI* R. Lane (M), DNA molecular size marker(100-bp ladder).
Lane (ST4) *S.*Typhi isolate number 4 shows positive results with *SulI* gene.
Lane (ST7) *S.*Typhi isolate number 7 shows positive results with *SulI* gene.
Lane (ST8) *S.*Typhi isolate number 8 shows positive results with *SulI* gene.
Lanes (ST1,ST2, ST3, ST5,ST9) *S.*Typhi isolates show negative result to *SulI* gene

Another study performed in Central African Republic showed that *sul*1 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

Result	Number	Percentage	Isolates
Positive for *Sul-1*	**3**	**15 %**	**ST** 4, **ST** 7, **ST** 8
Negative for *Sul-1*	**17**	**85 %**	**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

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Total	**20**	**100%**	**20**

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 *sul*I 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).

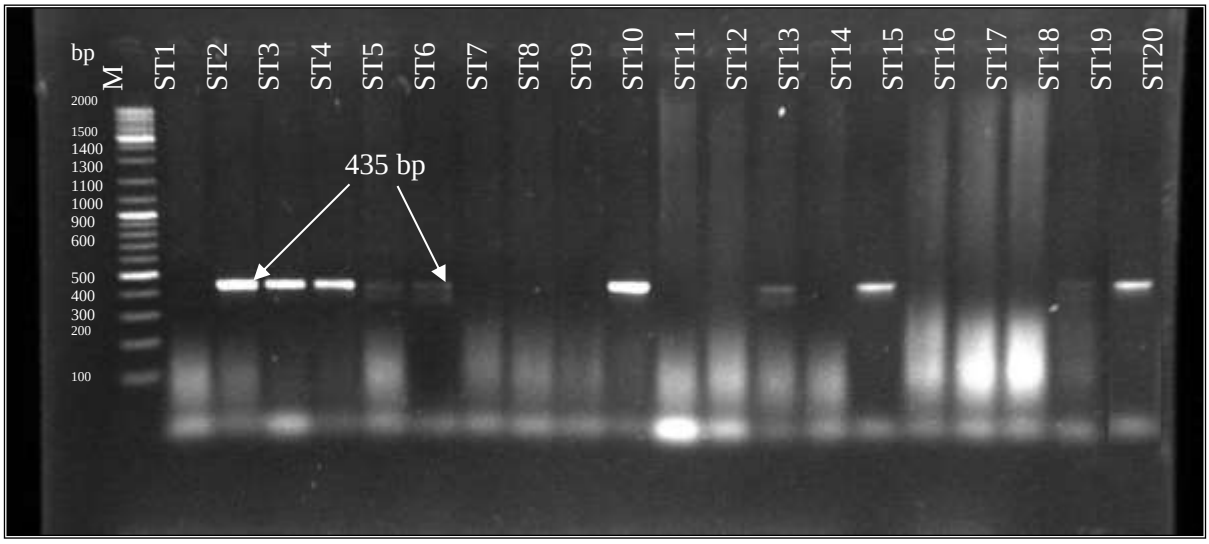

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted *S.*Typhi DNA amplified with primers *Sul-2*F and *Sul-2* R. Lane (M), DNA molecular size marker(100-bp ladder).
Lane (ST2) *S.*Typhi isolate number 2 shows positive results with *Sul-2* gene.
Lane (ST3) *S.*Typhi isolate number 3 shows positive results with *Sul-2* gene.
Lane (ST4) *S.*Typhi isolate number 4 shows positive results with *Sul-2*gene.
Lane (ST5) *S.*Typhi isolate number 5 shows positive results with *Sul-2*gene.
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 *sul*1 , *sul* 2, and *sul*3. 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 Potugal . In contrast recently the spread of *sul* 2 seem to have increased in other European countries (Kerrn *et al* .,2002).

Presence of *sul*2 gene is related with class 1 integron, since class1 integrons were always associated with *sul* genes, including *sul*2 and *sul*3.(Antunes *et al* ., 2005). The same study confirmed that *sul*1 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 *sul*1 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

Result	Number	Percentage	Isolates
Positive for *Sul-2*	**10**	**50 %**	**ST** 2, **ST** 3, **ST** 4, **ST** 5, **ST** 6, **ST** 10, **ST** 13, **ST** 15,**ST** 19, **ST** 20
Negative for *Sul-2*	**10**	**50%**	**ST** 1, **ST** 7, **ST** 8, **ST** 9, **ST** 11, **ST** 12, **ST** 14, **ST** 16, **ST** 17, **ST** 18
Total	**20**	**100%**	**20**

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