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Molecular Study on Multidrug Resistant of *Salmonella Enterica* Isolated from patient with Enteric Fever in Najaf - Province/Iraq

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Abstract

Minimum Inhibitory concentration of four antimicrobials used in treatment of Salmonella typhi invasive infections were determined by two different methods , the agar dilution method and HiComb kit. For Ampicillin MIC was more than 32 µg/ml. Chloramphenicol MIC ranged between (128-256) µg/ml. On other hand MIC of nalidixic acid was varied from (0.5 – 2) µg/ml. Regarding ciprofloxacin MIC range from (0.001-0.01) µg/ml. Molecular study was accomplished first by the extraction of DNA from S. typhi isolates and second by using PCR technique to amplify seven different primers to determine the presence of integrons. Genes were int, intI, for integrase gene where the first sequence gave (30%) positive while second sequence showed (20%). Sul genes were also used, the first sequence of SulI gene showed (15%) while second one gave no positive result , while sul-2 gene recorded (50%) positivity. Qac genes located on 3' end of class-I integron were also investigated , qacE did not show any positive result, while the mutant type qacEΔ recorded high prevalence among S. typhi isolates. Finally four pairs of primers targeted the detection of Extended Spectrum Beta-Lactamases (ESBLs) genes which comprised bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M}. Amplification with the first three primers did not yield DNA products, while the fourth showed 50% positivity.

Keywords: Salmonella typhi, int, intI, sulI, sul-2, qac, qacE, bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}.

Introduction

Enteric fever is a systemic infection caused by numerous *Salmonella enterica* serotypes including *S. typhi* and *S.* paratyphi A, B, or C[1]. Some studies was witnessed emergence of multiple drug resistance among *Salmonella* strains causing enteric fever increasing the potency of risks accompanied to this bacterium, which result in treatment failures and limiting therapeutic options[2,3].

A genetic element, the integron, is possibly a main agent in the propagation of multidrug resistance among gram-negative bacteria[4]. Class 1 integrons have been inspected the most extensively. They consist of a variable region bordered by 5' and 3' conserved regions.

The 5' region is made up of the *int* gene, *attI*, and the promoter which drives transcription of genes within the variable region. The 3' region comprises of an ethidium bromide resistance locus (*qacED1*) and a sulfonamide resistance gene (*sulI)*[5]. Minority or rarity of studies on integrons in our country and unavailability of molecular studies on *S. typhi* in Al- Najaf made it of high necessity to investigate these aspects of this potent disease caused by this pathogen which has been lately increased due to decreasing of typical drinking water purification and its contamination with sanitation[6].

Material and Methods

This method was used to determine the minimum inhibitory concentration of 20 *S. typhi* isolates to antibiotics that showed decreased effectiveness in the antibiotic sensitivity test. Two procedures were used: i. Agar plate dilution method according to Andrews J.[7], for ampicillin and chloramphenicol. ii. Dilution and Diffusion (HiComb test by Himedia, India) for nalidixic acid and ciprofloxacin.

PCR Supplies Assembling and Thermo cycling Conditions: The DNA templates were subjected to PCR using 11sets (F and R) of primers targeting two groups of genes: the first group listed in table-1 to determine the integron coded genes and the second group listed in table-2 to determine ß-lactam antibiotics resistance genes. The reaction mixture moreover contains Go Taq® Green Master Mix, X2 (promega,USA), which is premixed ready-made solution containing bacteriology derived Taq DNA polymerase d NTP, $MgCl_2$ (promega,USA), and reaction buffers at optimal concentrations and is recommended for any amplification reaction 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 0.2ml 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. Polymerase chain reaction assays were carried out in a 25 µI reaction volume, and the PCR amplification conditions performed with a thermal cycler were precise to each single primer set depending on their reference procedure, as shown in tables 3, 4.

Table-1
Integrons Detection Primers

Primer name	Primer sequence		Reference
IntI	F	GGC ATC CAA GCA GCA AGC	(9)
	R	AAG CAG ACT TGA CCT GAT	
Int	F	CCT GCA CGG TTC GAA TG	(19)
	R	TCG TTT GTT CGC CCA GC	
Sul I	F	CGG CGT GGG CTA CCT GAA CG	(20)
	R	GCC GAT CGC GTG AAG TTC CG	
Sul 2	F	CCT GTT TCG TCC GAC ACA GA	(21)
	R	GAA GCG CAG CCG CAA TTC AT	
QacE	F	TAA GCC CTA CAC AAA TTG GGA GAT AT	(19)
	R	TTA GTG GGC ACT TTG CTT TGG AAA G	
QacEΔ	F	TAA GCC CTA CAC AAA TTG GGA GAT AT	(19)
	R	GCC TCC GCA GCG ACT TCC ACG	
SulR	F	TAA GCC CTA CAC AAA TTG GGA GAT AT	(19)
	R	GGG TGC GGA CGT AGT CAG C	

Table-2
Extended spectrum beta lactamasaes genes primers

Primer name	Primer sequence		Reference
bla_{TEM}	F	AAACGCTGGTGAAAGTA	(22)
	R	AGCGATCTGTCTAT	(23)
bla_{SHV}	F	ATGCGTTATATTCGCCTGTG	(22)
	R	TGCTTTGTTATTCGGGCCAA	(23)
bla_{CTX-M}	F	CGCTTTGCGATGTGCAG	(24)
	R	ACCGCGATATCGTTGGT	(23)
bla_{OXA}	F	ATATCTCTACTGTTGCATCTCC	(25)
	R	AAACCCTTCAAACCATCC	

Results and Discussion

The result of minimum inhibitory concentration of ampicillin which was resolute by using the agar dilution method showed that 15, (75%) of isolates grew in the presence of 32 µg/ml of ampicillin reflecting that the MIC was higher than 32 µg/ml. while that of chlorampenicol gave a range was between (128-256) µg/ml where most isolates showed 256 µg/ml MIC value. Regarding nalidixic acid results showed that most isolates 12(60%) were resistant to the highest concentration 240 µg/ml, while others recorded values: 2 µg/ml 4(20%), isolates, 2 (10%) of isolates with 0.5 µg/ml, and 2 isolates (10%) with 0.1 µg/ml**.** Results of ciprofloxacin showed that MIC range was between 0.001- 0.01 µg/ml. This range is still within susceptible range since MIC less than 1 µg/ml.

Integron Detection: The result of PCR amplification with the first sequence detecting *Int* gene were positive in (30%) of isolates**.** Also we found that there were only 4 isolates gave the positive results using the second sequence in (20%) of isolates. Detecting the 3′ region of the integron, results showed that three , (15%) of *S*. Typhi isolates gave positive result for *sul*1 gene by using the primer sequence: (F: 5′-CGG CGT GGG CTA CCT GAA CG -3′, R: 5′-GCC GAT CGC GTG AAG TTC CG -3′). On the other hand, the prevalence of *sul*- 2 gene was relatively high , where results showed its presence in 10 isolates numbered:(ST2, ST3, ST4, ST5, ST6, ST10, ST13, ST 15, ST 19, ST 20) ,(50%) of isolates**.** The present study showed high prevalence of *qacE*Δ gene among *Salmonella* Typhi isolates, positive result represented 60% of total (table -5)

Extended Spectrum Beta Lactams: In order to detect the possession of bacterial isolates of ESBLs genes the present study recorded prevalence of bla_{CTX-M} gene in (50%) of *Salmonella* Typhi isolates as revealed by PCR amplification.

Discussion: The minimum inhibitory concentration of ampicillin result was similar to that one of Mulvey M. et al[8], Rao S.[9], who found MIC of antibiotic was ≥ 32 µg/ml. As compared to other studies[10], mentioned that their MIC for chloramphenicol was equal to 16 µg/ml. while Mulvey M. et al [8] recorded MIC of this drug ≥32 µg/ml. Resistance to chloramphenicol may be intervened enzymatically through chloramphenicol acetyltransferase or non-enzymatically through the *cml*A or *flo* genes, which both encode putative drug efflux pumps. The *flo* gene is similar in primary structure to *cml*A and develops the resistance to both florfenicol and chloramphenicol[11]. Comparing with other studies[12], significant resistance (97.8%) was noted against first generation quinolone, nalidixic acid (MIC > 256 mg/ml). These drugs may cross the outer membrane in two different methods: through the précis porins or by diffusion through the phospholipid bilayer. The degree of diffusion of a quinolone is greatly associated with, and dependent on its level of hydrophobicity[13]. Our study had come in accordance with a study[14], who generalized that in Turkey the resistance to fluoroquinolones is still not high, however the isolates with decreased susceptibility to ciprofloxacin seem to be increasing. Moreover, strains that are already resistant to nalidixic acid may need fewer exposures to fluoroquinolones to develop high level resistance to

ciprofloxacin, than the strains that are fully ciprofloxacin susceptible[15].

To our knowledge our study is the first to detect the integrons of bacteria in Iraq, and from the minority to study *Salmonella* Typhi genetically as well. Our study came in accord with other studies[16,17] in the prevalence of class 1 integrons in case of using the first sequence targeting the *int* gene. Results showed that a large number of isolates did not produce the gene as it was not detected by PCR amplification.

This may be attributed to presence of other sequence of the enzyme that can be detected by other primer sequence. *qacE* and *qacE*Δ1 are genes that confer resistance to QACs and dyes such as ethidium bromide. *qacE*Δ1, a mutant version of qacE, seems to be partially functional as a multidrug transporter[18] and is widely distributed throughout gram-negative bacteria because of its location on the 3´ conserved region of class 1 integrons[19] Correlation when made between int genes and *qac E*Δ gene in present study ,we observe simultaneous occurrence in 5 isolates (25%) of total , table -5, but co-existance of all genes of 5´and 3´ conserved segments was in 4 isolates which may reflect the number of isolates carrying class 1 integron.

Our result showed vast variance as compared to other studies regarding the genes targeting ESBLs mentioned above, except of that of *blaCTX*-M. Emergence of *CTX*-M-15 type extended-spectrum beta-lactamase-producing *Salmonella spp* was also noted in Kuwait and the United Arab Emirates, where PCR and sequencing were used to conclude the genetic determinant(s) responsible for ESBL phenotypes[20].

Conclusion

The most prevalent serotype of Salmonella enteric causing enteric fever during the study duration was serovar typhi. All the isolates of Salmonella were intermediately responsive to ciprofloxacin reflecting increased resistance to the drug which represent drug of choice in enteric fever therapy. The possession of the isolate Salmonella typhi of the gene responsible for the integration process (intergrase) int gene. Availability of sul resistance gene among Salmonella isolates which have correlation with integrons presents. Occurrence of qac EA gene which is highly related with integrons elements in sixty percent of isolates. Finley the presence of integrons in the isolates of Salmonella typhi was in a fifteen percent.

Table-3
Thermo cycling conditions of integron primers

Primer name	Denaturation	Annealing			Extension	Cycles number
IntI	94* 3 min**	94 30 sec	55 1 min	72 1 min	72 7 min	35
Int	95 5 min	95 45 sec	50 45 sec	72 1 min	72 10 min	30
SuII	95 5 min	94 30 sec	55 30 sec	72 30 sec	72 10 min	35
qacE	95 5 min	95 45 sec	67 45 sec	72 45 sec	72 10 min	30
*qacE*Δ	95 5 min	95 45 sec	67 45 sec	72 45 sec	72 10 min	30
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

Table-4
Thermo cycling conditions of ESBLs primers

Primer name	Denaturation	Annealing			Extension	Cycles number
bla_{TEM}	94* 30 sec**	94 30 sec	45 1 min	72 1 min	72 10 min	35
bla_{SHV}	94 30 sec	94 30 sec	60 1 min	72 1 min	72 10 min	35
bla_{CTX-M}	94 30 sec	94 30 sec	60 1 min	72 1 min	72 10 min	35
bla_{OXA}	94 5 min	94 50 sec	55 50 sec	72 30 sec	72 5 min	30

* The number in the first line of the columns :2nd , 3rd ,4th , 5th ,6th of tables 3,4 refers to temperature of the thermo cycling in Celsius centigrade's (°C), **The number in the second line of the columns: 2nd , 3rd ,4th , 5th ,6th of tables 3,4 refers to time of thermo cycling.

Table-5
Results of class-1 integron genes detection

Isolate number	Class 1 Integron detection primers						
	5′ conserved segment primers		3′ conserved segment primers				
	Int	***intI***	***sul 1***	***sul 1R***	***Sul2***	***Qac EΔ***	***qac E***
ST1	-	+	-	-	-	+	-
ST2	+	+	-	-	+	+	-
ST3	+	+	-	-	+	+	-
ST4	+	+	+	-	+	+	-
ST5	+	-	-	-	+	-	-
ST6	+	-	-	-	+	+	-
ST7	-	-	+	-	-	-	-
ST8	-	-	+	-	-	+	-
ST9	-	-	-	-	-	-	-
ST10	+	-	-	-	+	-	-
ST11	-	-	-	-	-	-	-
ST12	-	-	-	-	-	+	-
ST13	-	-	-	-	+	+	-
ST14	-	-	-	-	-	-	-
ST15	-	-	-	-	+	+	-
ST16	-	-	-	-	-	-	-
ST17	-	-	-	-	-	+	-
ST18	-	-	-	-	-	+	-
ST19	-	-	-	-	+	-	-
ST20	-	-	-	-	+	+	-

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