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MOLECULAR CHARACTERIZATION OF CHROMOSOMAL AND EXTRA CHROMOSOMAL ELEMENTS RELATED TO ANTIBIOTICS RESISTANCE GENES IN *SALMONELLA TYPHI*

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Abstract

Salmonella typhi the causative agent of typhoid, the consider largest killer disease is becoming a major threat due to emerging antimicrobial resistance. Mechanisms underlying bacterial drug resistance involve developing and/or acquiring of genetic elements in the form of variety of genes, gene complexes or mutations.These genetic elements are either chromosomal and/or extrachromosomal in the form of plasmids. The present study was conducted to investigation of the presence antibiotic-resistance genes (*cat, dfrA7, gyrA, gyrB, strA-strB, sulI, sulII, tetB*) were done by using PCR technique and electrophoresis systems. One hundred percent of *S.typhi* isolates were positive with *gyrA*, while 25 (83%) of isolates carry *gyrB* gene, 19 (63.3%) of isolates carry gene *cat*, *dfrA7*, 12(40%) and 6 (20%) isolates were carry *sulII* and *sulI*, respectively. Thirty (100%) of the isolates gave a negative genes results of (*strA-strB, tetB*).

Key words : *S. typhi*, antibiotics resistance genes.

Introduction

Typhoid fever is an acute, systemic illness caused by infection with *S. typhi* (*Salmonella typhi*), pathogens only specific to humans. Bacterial resistance to antimicrobial agents is a serious problem worldwide with regard to treatment of infectious diseases. The spread of antibiotic resistance is usually associated with either the clonal spread of an epidemic strain or through independent acquisition of the resistance genes on plasmids, transposons or integrons (Martínez *et al*., 2007). Plasmid-associated resistance genes have been discovered for a majority of known antimicrobials. It is not uncommon for a single plasmid to simultaneously mediate resistance to five or more antimicrobials (Hawkey, 2003). This ability to sequester multiple resistance genes is of particular concern to modern medicine because they are responsible for emergence of drug resistance. (Thong *et al.*, 2000). Plasmid associated genes have been implicated in resistance to aminoglycosides, chloramphenicol, penicillin, cephalosporin, erythromycin, tetracycline, sulphonamides and others (Willey *et al.*, 2013). The chromosomal-mediated drug resistance phenomenon against fluoroquinolones has been reported recently as a result of selective pressure on the bacterial population. This has been attributed to a single point mutation in the quinolone resistance determining region (QRDR) of the topoisomerase gene gyrA, which encodes DNA gyrase (Zaki and Karande, 2011). Resistance to trimethoprim is due primarily to mutations in the chromosomal gene that encodes dihydrofolate reductase, the enzyme that reduces dihydrofolate to tetrahydrofolate (Denyer *et al*., 2011). There are many different mechanisms by which Gram-negative bacteria might exhibit resistance to drugs, which include changes in the permeability of the bacterial cell membrane, efflux of the antimicrobials out the bacterial cell, mutation in the target site to prevent antimicrobial agent to reach the attachment sit on cell wall, as well as produce many enzymes that inhibit the effective of

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antimicrobial, which considered as major mechanism of resistance in Gram-negative bacteria (Piddock, 2006).

Materials and Methods

The study was done at Laboratories of Bacteriology and Molecular in Biology Department, Faculty of Sciences, University of Kufa, Iraq.

Specimens collection and bacterial identification

A blood, stool using as a source of 30 samples using in this study with clinical suspicion of typhoid fever who attended different hospitals in Al-Najaf provenance according to ethical approval of ministry of Iraqi health, continuous high fever with temperature 38°C. The median duration of illness at consultation was 6 days (range 6 – 18 days). Each specimen was inoculated on culture of selective media namely XLD and SS agar, then inoculated at 37°C for 18-24 hours (Cheesbrough, 2010).

DNA extraction

Genomic DNA was extracted by using a commercial extraction system (Genomic DNA promega Kit).

Extraction of plasmid DNA

Plasmid DNA was extracted from isolates by using a commercial purification system (Pure Yield™ plasmid Miniprep Kit).

Molecular identification

The PCR assay was performed to detect the antibiotics resistance genes for *S. typhi* shown in table 2. These primers were produced by Alpha DNA Company, Canada as in table 1. The amplified PCR products were detected by agarose gel electrophoresis was visualized by staining with ethidium bromide. The electrophoresis result was detected by using gel documentation system. The positive results were distinguished when the DNA band base pairs of sample equal to the target product size (Bartlett and Stirling, 1998). Finally, the gel was photographed using Biometra gel documentation system.

Results and Discussion

Molecular identification of antimicrobial drug resistance

Chloromphenicol gene (*cat gene*)

The result showed that the chloramphenicol resistance gene (*cat)* (chloramphenicol acetyl transferase) was detected in 19 / 30 *S.typhi* isolates as in fig. 1. In the early stages of antibiotic, chloramphenicol was the universal treatment of choice for typhoid fever and considered the "gold standard" agent for the treatment of this infection. It helped dropping mortality rate to 2%. However, simultaneous resistance against this drug emerged in the early 70's with sudden outbreaks in Mexico, India, Vietnam, Thailand, Korea and Peru. This alarming rise in this resistance is attributed to its unrestricted, over-the-counter availability in many parts of the world, especially in developing countries (Mirza *et al*., 1996). As the resistance against chloramphenicol is continuously being increased, various genes have been linked with this phenomenon. These include efflux pump

Table 1 : Primers used in this study.

Primer type	Primer target	Primer sequence (5'-3')	Amplicon size (bp)	Reference
cat	cat	**F**-CCTGCCACTCATCGCAGT **R**-CACCGTTGATATATCCC	623	Guerra *et al*. (2001)
dfrA7	DHFR1	**F**-GTGTCGAGGAAAGGAATTTCAAGCTC **R**-TCACCTTCAACCTCAACGTGAACAG	191	Wain *et al*. (2003)
gyrA	GYRA	**F**-TGTCCGAGATGGCCTGAAGC **R**-TACCGTCATASGTTATCCACG	347	Griggs *et al*. (1996)
gyrB	GYRB	**F**-CAAACTGGCGGACTGTCAGG **R**-TTCCGGCATCTGACGATAGA	345	Ling *et al*. (2003)
strA-strB	strA StrB	**F**-ATGGTGGACCCTAAAACTCT **R**-CGT-CTAGGATCGAGACAAAG	891	Tamang *et al*. (2007)
sul1	sulI	**F**-GGATGGGATTTTTCTTGAGCCCCGC **R**-ATCTAACCCTCGGTCTCTGGCGTCG	308	Wain *et al*. (2003)
sul2	sulII	**F**-TCAACATAACCTCGGACAGT **R**-GATGAAGTCAGCTCCACCT	707	Chiu *et al*. (2004)
tetB	TB	**F**-CTCAGTATTCCAAGCCTTTG **R**-CTAAGCACTTGTCTCCTGTT	416	Guardabassi *et al*. (2000)

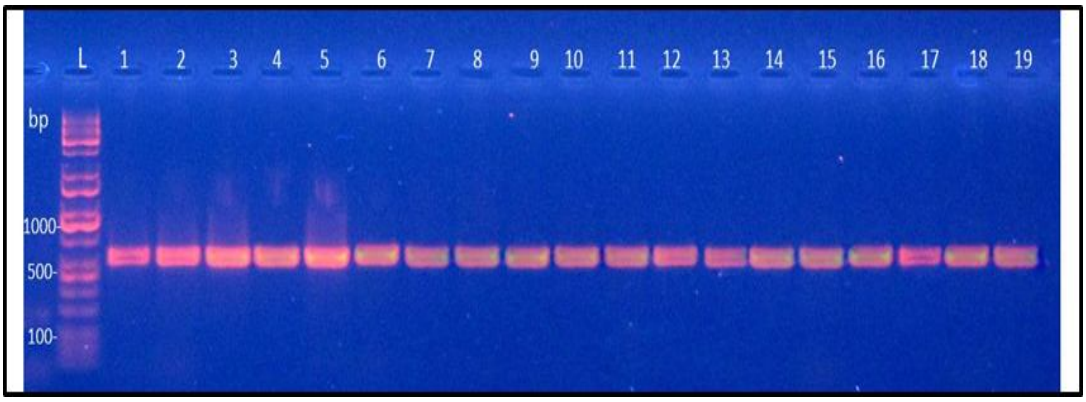

Fig. 1 : PCR amplification products of *S.typhi* isolates that amplified with *cat* gene primers with product 623 bp.Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1 to19) show positive results with *cat* gene.

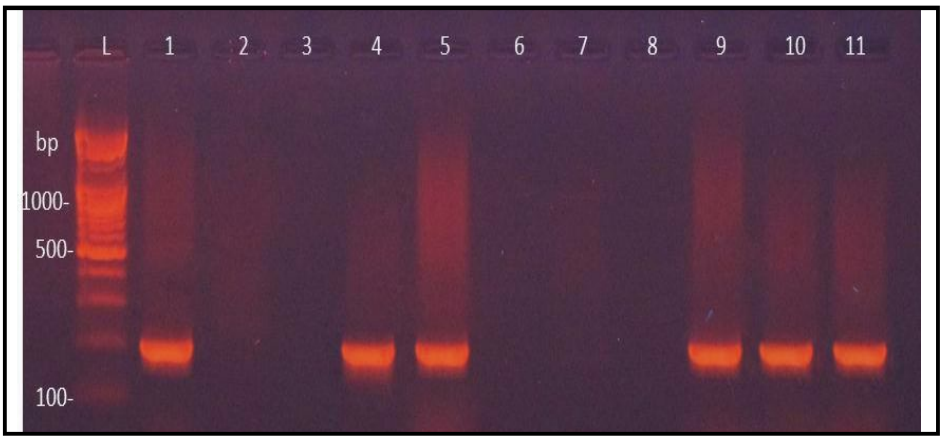

Fig. 2 : PCR amplification products of *S.typhi* isolates that amplified with *dfrA7* gene primers with product 191 bp.Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 4, 5, 9,10 and 11) show positive results with gene *dfrA7*. Lanes (2, 3, 6, 7 and 8) show negative results with *dfrA7*.

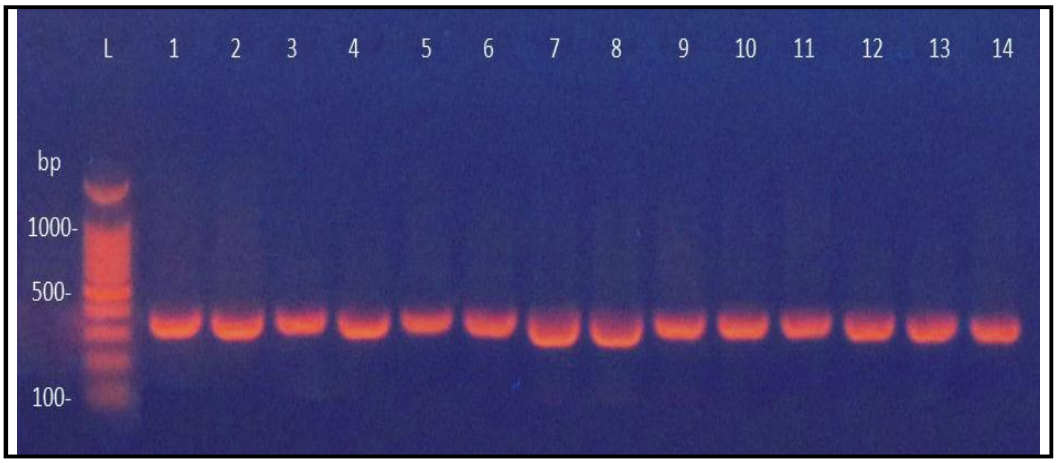

Fig. 3 : PCR amplification products of *S.typhi* isolates that amplified with *gyrA* gene primers with product 347 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1 to 14) show positive results with *gyrA* gene.

related gene *catB* (Zolezzi *et al.*, 2004). This drug inhibits protein synthesis in bacteria by readily penetrating the cells, probably by facilitated diffusion. Chloramphenicol acts primarily by binding reversibly to the 50S ribosomal subunit. Although binding of tRNA at the codon recognition site on the 30 S ribosomal subunit is thus undisturbed, the drug appears to prevent binding of the amino acid containing end of the aminoacyl tRNA to the acceptor site on the 50S ribosomal subunit (Guerra *et al.*, 2003).

The interaction between peptidyltransferase and its amino acid substrate cannot occur, and peptide bond formation is inhibited. Resistance of gram negative

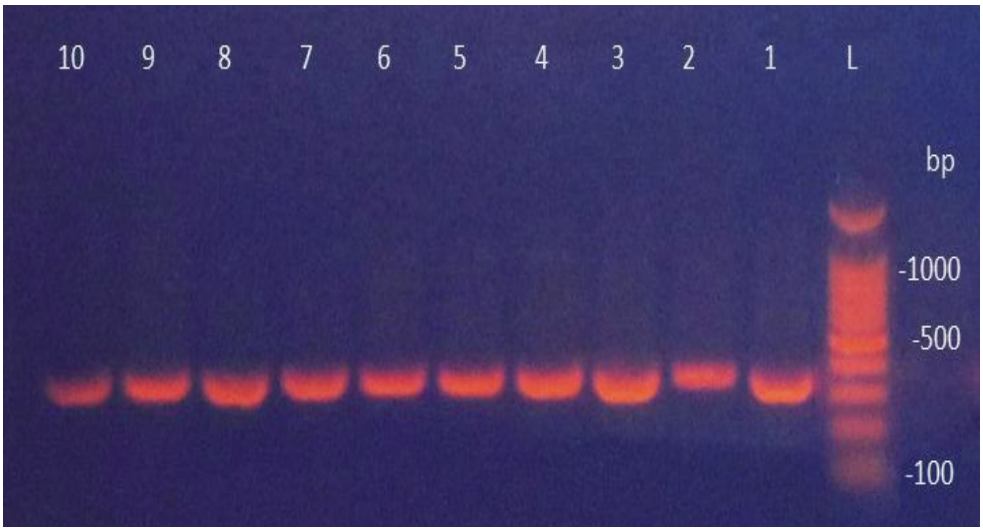

Fig. 4 : PCR amplification products of *S.typhi* isolates that amplified with *gyrB* gene primers with product 345 bp.Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1 to 10) show positive results with *gyrB* gene.

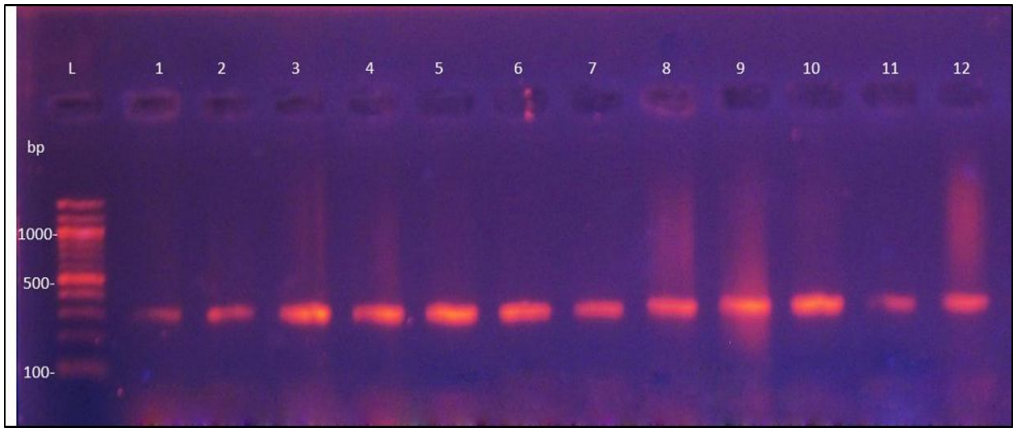

Fig. 5 : PCR amplification products of *S.typhi* isolates that amplified with *sulI* gene primers with product 308 bp.Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1 to 12) show positive results with *SulI* gene.

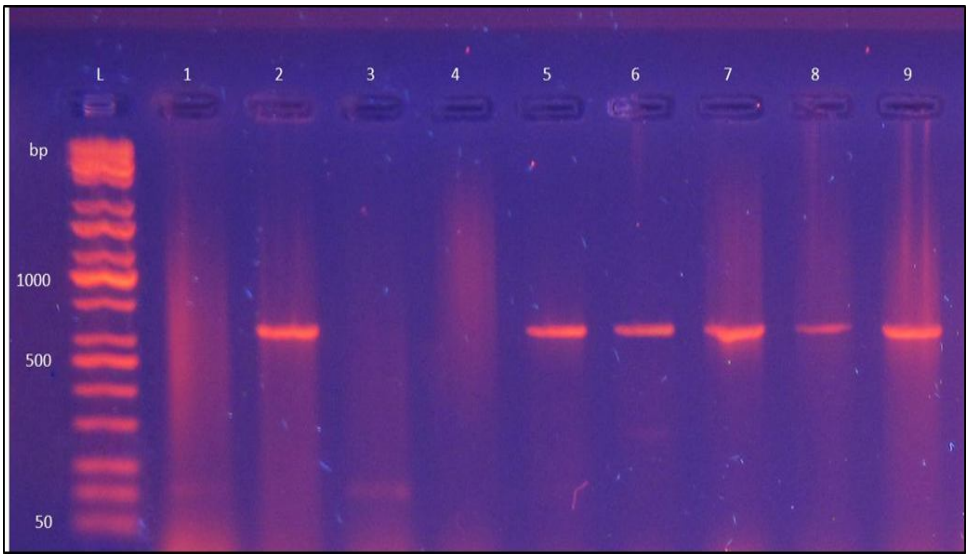

Fig. 6 : PCR amplification products of *S.typhi* isolates that amplified with *sulII* gene primers with product 707 bp.Lane (L), DNA molecular size marker (50-bp ladder), Lanes (2,5,6,7,8 and 9) show positive results with *SulII* gene. Lanes (1, 3, and 4) show negative results with *SulII* gene.

Table 2: PCR programs of primers that apply in the thermocycler.

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
tetB	94ºC for 5 min.	32	94ºC for 1 min	51ºC for 1 min	72 Cº for1min.	72ºC for7min.
cat and *sul2*	94ºC for 5 min.	32	94ºC for 1 min.	50ºC for 1 min.	72ºC for 1 min.	72ºC for7 min.
sul1 and *dfrA7*	95ºC for 5 min.	30	95ºC for 30sec	57.5ºC for 1 min.	72ºC for 1 min	72ºC for 2 min.
strA-strB	94ºC 10 min	32	94ºC 1 min	54ºC for 1 min	72ºC for 1 min	72ºC for 10 min
gyrA and *gyrB*	92ºC for 2 min	32	92ºC for 1 min	62ºC for 1 min	74ºC for 2 min	74ºC for 1 min

bacteria to chloramphenicol usually is caused by a plasmid acquired by conjugation and is due to presence of a specific acetyltransferase that inactivates the drug. At least three types of enzymes have been characterized. Although, resistance to this drug is usually due to its acetylation, both decreased permeability of the microorganism and mutation to ribosomal insensitivity may also be involved. In this respect different genes have been linked with chloramphenicol resistance which include efflux pump related gene *cmlA*, *catP* (Guerra *et al*., 2003 and Zolezzi *et al*., 2004) in additional to *catA* genes (Aarestrup *et al*., 2003) .

Trimethoprim gene (*dfrA7*)

The result showed that the trimethoprim gene was amplified in 18/30 among *S.typhi* isolates as in fig. 2, while 12/30 isolates gave negative result. The gene trimethoprim resistance is mainly caused by *dfr* genes .The resistance to trimethoprim due to interfering with folate synthesis in both Gram-negative and Gram-positive bacteria. It behaves bacteriostatically after competitive and strong binding to dihydrofolate reductase (DHFR) ,which catalyses the formation of tetrahydrofolate from dihydrofolat. Although, DHFRs from eukaryotic cells can also bind trimethoprim, the affinity of the drug toward bacterial enzymes is higher (Chiu *et al.*, 2004).

Gyrase gene (*gyrA* and *gyrB gene*)

The result showed that the gyraseA resistance gene (*gyrA*) was detected in 30(100%) as in fig. 3 and 25/30 isolates gave positive result of *gyraseB* resistance gene(*gyrB*) as in fig. 4. Ciprofloxacin resistance is mainly attributed towards *gyrA* and *gyrB* gene. Quinolones are broad-spectrum antimicrobial agents that have been used widely in clinical medicine. During 1990's quinolones especially ciprofloxacin became the drug of choice in the treatment of MDR typhoid but resistance to quinolones has increased markedly, however, in certain areas of the world during recent years (Threlfall and Ward, 2001). Mechanisms of bacterial resistance to fluoroquinolones were previously considered to fall into two principal categories, alterations in target enzymes (DNA gyrase and/or topoisomerase IV) and alterations in drug accumulation, both resulting from chromosomal mutations mainly in the *gyrA* and *parC* genes due to amino acid substitution or point mutations in the bacterial target genes *gyrA*, *gyrB* encoding DNA gyrase and/or *parC*, *parE* encoding DNA topoisomerase IV. Amino acid substitutions involve the replacement of a hydroxyl group with a bulky hydrophobic residue (Byarugaba, 2009).

Plasmid-mediated quinolone resistance (PMQR) is associated with low level resistance to fluoroquinolones and represent the production of Qnr proteins protecting the targets against the effects of quinolones. Although, quinolone resistance usually results from chromosomal mutations, studies indicate that quinolone resistance can also be plasmid mediated (Wang *et al.*, 2003).

Sulfonamid (*sulI* and *sulII*) gene

The result showed that the Sulfonamide resistance gene (*sulI)* was detected in 12 / 30 isolates *S.typhi* as in fig. 5 and 6 / 30 isolates *S. typhi* gave positive result for *sulII* as in fig. 6) ,this result similar with result of Frank *et al*. (2007). Due resistance sulfonamides to inhibit the first step of bacterial folate synthesis pathway and work bacteriostatically (Ola, 2000). In folate synthesis pathway, sulfonamides act as analogue to *p*-aminobenzoic acid (PABA) and when chosen by enzyme dihydropteroate synthase (DHPS) block folate synthesis. Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes *sulI* and *sulII*, encoding forms of dihydropteroate synthase that are not inhibited by the drug. The *sulI* gene is normally found linked to other resistance genes in class 1 integrons, while *sulII* gene is usually located on small nonconjugative plasmids or large transmissible multi-resistance plasmids (Enne *et al*., 2001).

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