International Journal of Multidisciplinary and Current Research ISSN: 2321-3124
Research Article Available at: http://ijmcr.com

Detection of tetracycline resistance genes A and B among clinical isolates of *Salmonella* Typhi from blood samples in Al-Najaf hospitals

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Received 02 July 2020, Accepted 01 Sept 2020, Available online 02 Sept 2020, **Vol.8 (Sept/Oct 2020 issue)**

Abstract

The presence of Salmonella spp. antibiotic resistance genes has a significant impact on human health, especially Salmonella Typhi which causes a serious illness called typhoid fever. Therefore, the aim of this research was to detect the dissemination of tetracycline resistance genes among tetracycline-resistant S. Typhi from Al-Najaf hospitals. A total of 1743 blood samples, 2ml from infants, 5 ml from children and 10 ml from adults (according to WHO recommendations), were obtained from outpatient of the hospitals with suspected typhoid fever. 105 (6.02%) isolates were obtained as S. Typhi. Antimicrobial susceptibility tests of S. Typhi isolates were performed by disc diffusion tests and the tetracycline-resistant isolates were screened to detect the presence of tet-A, and tet-B genes by two sets of primers. The PCR products were shown by using 1.5% agarose gel in electrophoresis. Low level of resistance was observed against tetracycline 5 (4.76%). Among the 5 tetracycline-resistant Salmonella isolates, only two isolates carried tet-A gene and one of these 2 isolates carried tet-B gene also. Conclusion Resistance to Salmonella strains is arising and the two cases of tetracycline resistance are alarming.

Keywords: *Salmonella Typhi; Tetracycline; tet genes; ERIC-PCR.*

Introduction

Salmonella Typhi belongs to the subspecies *S. enterica*, and this bacterium is considered as a human-specific pathogen, which causes human typhoid fever which is an acute systemic fever disease. The risk factors of typhoid fever are like poor sanitation, contaminated food and water, as well as the disease may also be caused by direct/close contact with infected persons or carriers. The chronic carriers of *S.* Typhi spread the bacteria around with faeces for months (Hornick, 1970; Kanungo *et al*., 2008). Many of *S.* Typhi strains have been shown to have resistance to some antibiotics like tetracycline.

Tetracyclines are antibiotics that interfere with protein synthesis to inhibit bacterial development. The advent of bacterial resistance to these antibiotics has restricted their use nowadays. To date, three separate tetracycline resistance mechanisms have been identified: tetracycline efflux, ribosome defense and alteration of tetracycline.

In Gram-negative bacteria, Tetracycline efflux is happened by using protein consist of twelve transmembrane fragments (TMS).

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DOI: https://doi.org/10.14741/ijmcr/v.8.5.3

The 2nd mechanism involves ribosomal protection proteins (RPPs) which share with the GTPases in protein synthesis. As well as the third mechanism is chemically modified tetracycline by a cytoplasmic protein.

The Gram-negative bacteria' resistance generally depended on the efflux pump system that encoded by many genes like *tet-A* and *tet-B* genes which were the most frequently described. The aim of this research was to detect *tet-A* and *tet-B* genes in *Salmonella* Typhi isolates in Al-Najaf/ Iraq.

Materials and methods

Specimen collection

A total of 1743 blood samples were collected from patients attended consultation clinics in Al-Najaf hospitals. Each specimen was added to Brain heart infusion (BHI) broth and attached to the private information of the patient. The samples were collected in sterile bags, refrigerated, and immediately transmitted to the laboratory within 4 hr in the icebox (Quinn *et al*., 2004; Cherneck and Berger, 2008). Then all samples were incubated at 37°C for 5-7 days.

Isolation and identification of *S*. Typhi

The isolation and identification of *S*. Typhi from blood samples were done with the classical culture, gram stain, biochemical tests and then were confirmed by using Vitek 2 system (Al Naiemi *et al*., 2008; Christner *et al*., 2010).

Antimicrobial Susceptibility Testing (AST)

According to the guidelines recommended by the Clinical and Laboratory Standards (CLSI, 2016) and commercially available antibiotic discs (Cyprus, Belgium and Bioanalyse, Turkey), 26 of variety antibiotic discs were chosen including Tetracycline (30µg). A single colony of each confirmed *Salmonella* isolate grown on XLD agar, was inoculated into BHI broth and incubated at 37°C until designed turbidity matched.

The turbidity of BHI broth was adjusted to 0.5 McFarland standard, then sterile cotton swab was soaked in the bacterial inoculum and pressed onto the wall of the tube, and spread all over the Müller-Hinton agar (MHA) plate surface. After 15 minutes, antibiotic discs were placed on plates (15 mm at lease the distance between them) and incubated at 37°C for 18-20 hrs. The zone diameter around each disc was measured and compared with the breakpoints of CLSI to determine the susceptible (S), intermediate (I), and resistant (R) criteria.

Primers oligonucleotide design

The oligonucleotide sequences of tet-A and tet-B were obtained from Ma *et al*. study published in 2007.

Target	Primer name	Oligo sequence (5'-3')	Amplicon size (bp)
tet-A	*tetA*-F	TTGGCATTCTGCATTCACTC	**494**
	tetA-R	GTATAGCTTGCCGGAAGTCG	
tet-B	*tetB*-F	CAGTGCTGTTGTGTCATTAA	**571**
	tetB-R	GCTTGGAATACTGAGTGTAA	

DNA extraction

The DNA extraction of tetracycline-resistant *S*. Typhi isolate was performed using Promega Wizard Genomic DNA Purification Kit as in the manufacturer's instructions. The templates DNA was transferred to a sterile Eppendorf tube and stored at -20°C.

Polymerase Chain Reaction (PCR) Assay to Detection of *tet-A* and *tet-B* genes

Thermocycler Biosystem was used for PCR amplification. The specific primers and suitable Master mix were used to detect the *tet-A* and *tet-B* genes. The reactions were performed in a final volume of 25µl which containing 12.5 µl PCR Master Mix, 2 µl bacterial DNA and 1 µl each primer and 8.5 µl PCR grade water. PCR consisted of initial denaturation at 95˚C for 5 min, 35 cycles of 1 min at 94˚C, 1 min at 55˚C and 1 min at 72˚C, plus a final extension at 72˚C for 10 min.

After loading of PCR products and DNA-size ladder (GeneDireX INC, USA) in the wells, the agarose (1.5%) was transferred into an electrophoresis chamber containing enough 1x TBE buffer to covers the gel. Electrophoresis was performed at 60-70 V for 90 minutes. Then PCR products were visualized with ultraviolet light. The level of DNA bands was measured according to the ladder to determine the target product size.

ERIC-PCR fingerprinting among Tetracycline resistant isolates

ERIC-PCR is one of genomic fingerprinting methods to the characterization of microbial populations and communities. It is the amplification of genomic DNA located between enterobacterial repetitive intergenic consensus (ERIC) elements using PCR primers (ERIC-1 and ERIC-2) and the resulting similar-sized PCR products are matched to know the relationships between microbial populations and communities in outbreak infections (Zagorianou *et al.*, 2012).

Statistical Analysis

The chi-square test was performed to analyse the results to know the significance of different values in the results. If P-value is equal to or less than 0.05, it means that the result is statistically significant (Poey *et al*., 2012).

Results

Confirming the Isolates of *Salmonella*

The biochemical characterization and confirmed isolates by VITEK-2 system revealed that only 105 (6.02%) isolates belonged to *S. enterica* serotype Typhi.

Antimicrobial susceptibility analysis

All of the 105 *S*. Typhi isolates were evaluated for susceptibility to 26 different antibiotics belonged to eleven classes. A summary of susceptibility rates (according to CLSI / 2018 guidelines as resistant, intermediate resistant, and susceptible) for all antibiotics against *S*. Typhi is given in the following table.

Antibiotic disc	Percentage of isolates exhibited		
	Susceptible	Intermediate	Resistance
Aztreonam	89.7	0.0	10.3
Ceftriaxone	88.8	0.0	11.2
Ceftazidime	82.2	7.5	10.3
Cefotaxime	84.1	4.7	11.2
Cefepime	35.5	54.2	10.3
Cefoxitin	52.3	9.3	38.3
Cefixime	82.2	1.4	16.4
Amoxicillin-Clavulanate	90.7	0.9	8.4
Piperacillin/Tazobactam	35.7	38.6	25.7
Imipenem	98.1	1.9	0.0
Meropenem	100	0.0	0.0

Ciprofloxacin	55.1	44.9	0.0
Levofloxacin	100	0.0	0.0
Lomefloxacin	64.7	35.3	0.0
Nalidixic Acid	51.4	2.8	45.8
Tobramycin	86.9	11.2	1.9
Amikacin	85.3	13.2	1.5
Gentamicin	99.1	0.0	0.9
Piperacillin	57.9	29.9	12.1
Ampicillin	90.7	0.0	9.3
Ticarcillin	83.3	0.0	16.7
Chloramphenicol	98.1	1.9	0.0
Tetracycline	95.3	2.8	1.9
Colistin	99.1	0.0	0.9
Trimethoprim-sulphamethoxazole	98.1	0.0	1.9
Trimethoprim	97.2	1.9	0.9

All of the 105 *Salmonella* Typhi isolates were evaluated for susceptibility to tetracycline, only five (4.76%) isolates were resistant (2, 1.90%) and intermediate (3, 2.86%).

Prevalence of tet genes

In the present study, only two *S*. Typhi isolates (1.87%) observed resistant to tetracycline antibiotics, two types of tetracycline primers were used: *tet-A* and *tet-B* (Figure 4-11), the antimicrobial resistance phenotypes corresponded with PCR results that ST 68 observed positive results with the two types and the other (ST 21) appeared positive result with *tet-A* only (Figure 4-9, Figure 4-10 and Figure 4-11).

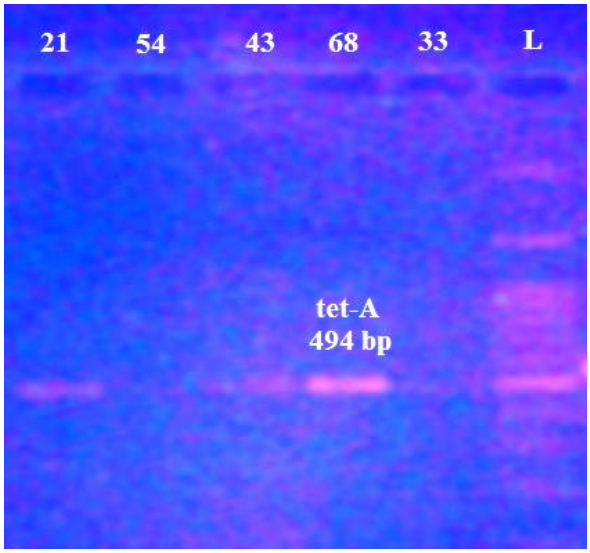


Figure (4-9): Ethidium bromide-stained agarose gel of monplex PCR amplified products from extracted DNA of *S*. Typhi isolates and amplified with *tet-A* genes primers. The electrophoresis was performed at 70 volt for 1.5 hr.
Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (68 and 21) show positive results with *tet-A* gene.
Lane (33, 43 and 54) show Negative results with *tet-A* gene.

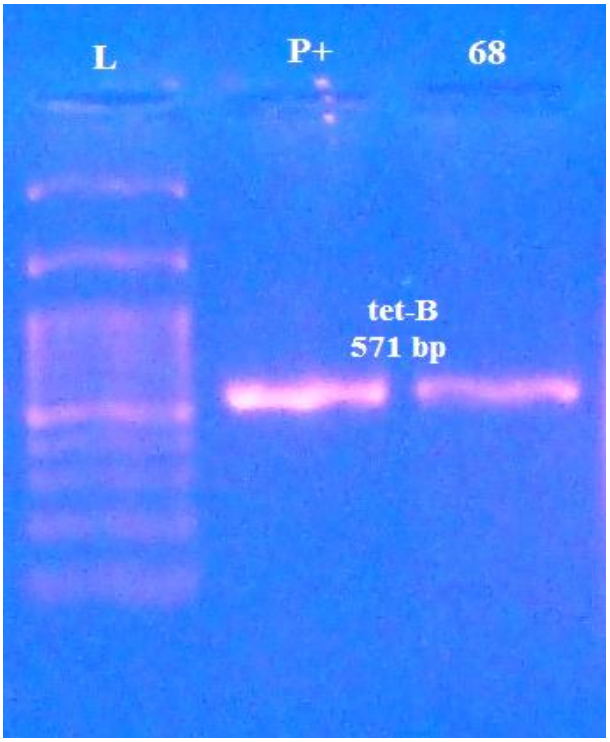
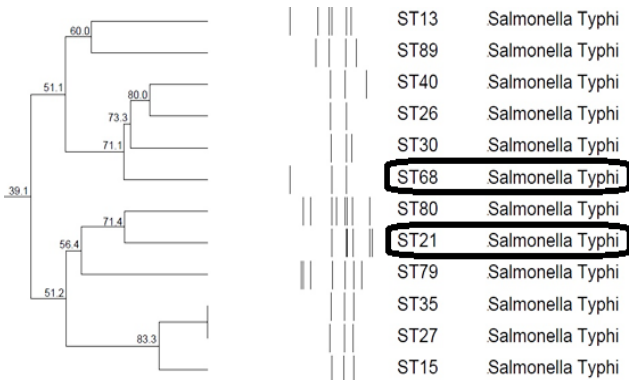


Figure (4-10): Ethidium bromide-stained agarose gel of monplex PCR amplified products from extracted DNA of *S*. Typhi isolates and amplified with *tet-B* genes primers. The electrophoresis was performed at 70 volt for 1.5 hr.
Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (68) show positive result with *tet-B* gene.
Lanes (P) show positive isolate *tet-B* gene.

Detection of epidemiological relationships between isolates by ERIC-PCR

ERIC analysis revealed different patterns among tetracycline resistance of *Salmonella* Typhi, including a distinct fingerprinting which exhibited the range of similarity was 39.1% between the ST21 and ST68 isolates.



Discussion

Antimicrobial susceptibility testing among *Salmonella* isolates

In this study, the results of antimicrobial susceptibility testing showed The most frequent resistance of the

isolates were against nalidixic acid (45.8%), Cefoxitin (38.3%), piperacillin-tazobactam (25.7%), ticarcillin (16.7%) and Cefixime (16.4%). On the other hand, this study showed the resistant of Imipenem, Meropenem, Ciprofloxacin, Levofloxacin, Lomefloxacin and Chloramphenicol were (0.0%). These results agree with the results being reported in Al-Najaf by Al-Kraety, (2017) who found that all of *S*. Typhi isolates were sensitive to imipenem, meropenem and cefixime were sensitive. The result of this study demonstrated that the isolates showed reduced susceptibility to tetracycline (1.9%), colistin (0.9%), trimethoprim (0.9%) and trimethoprim-sulphamethoxazole (1.9%).

It was noteworthy that the resistance rate of the isolates to tetracyclines was strikingly low, which may in part be due to the less frequent and less using of this antibiotic for the treatment of bacterial infections in humans and animals in Al-Najaf.

Detection of Tetracyclin resistance genes

Through the antibiotic-resistant test, it appeared that only two (1.87%) *Salmonella* Typhi isolates (Sa21 and Sa68) were resistant to tetracycline. Both isolates carried tet-A gene, but one of them (Sa68) also carried tet-B. Sa21 isolate appeared resistance to beta-lactam antibiotics (ticarcillin, cefoxitin and cefotaxime) and nalidixic acid as well as tetracyclin. While the other isolate (Sa68) showed resistance to beta-lactam antibiotics (ticarcillin, piperacillin and piperacillin/ tazobactam) as well as tetracycline. In a related study in Al-Najaf, Al-Baldawy, (2017) detected 48.71% isolates positive for the *tet-A* gene and 28.20% isolates positive for the *tet-B* gene. While in Basrah, the *tet-A* and *tet-B* genes were detected in 48.71% and 28.20% of tetracycline-resistant strains isolates respectively (Al-Mazini, 2015).

Most *Salmonella* spp have been discovered as tetracycline-resistant strains, in prior researches, carried the gene tet-A (Pezzella *et al.*, 2004; Hur *et al.*, 2011).

Tetracycline is one of the most frequently used antibiotics in animal production compared to other antibiotics that may be associated with the frequent occurrence of tet-A and tet-B in isolates of *Salmonella* Typhi (Chee-Sanford *et al.*, 2009).

ERIC-PCR fingerprinting

The dendrogram exhibited similarity ratio of less than 39.1% in two levels which mean the differences between these isolates were because of the differences in strains. A large proportion of differences or a low similarity ratio between *Salmonella* isolates indicate that these isolates have many different sources. This is of concern as the causes can be varied as well. For the first reason, the focus of WHO, FAO and WFP (WFP) are contaminated food and water, especially fast food and imported foods, which health checks and their potential in Iraq cannot control. On the other hand, Al-Najaf Al-Ashraf has a speciality that makes it a center of attraction for many, including the attraction of many pilgrims or tourists throughout the year, some of them come to tourism, including religious tourism, cultural tourism and business tourism. Also, because of safety, it has become a center for displaced people from hot cities in Iraq, where many displaced families have intended to live. In addition to the presence of many universities, colleges and religious and academic institutes, it attracts a lot of students from all countries of the world to study and research, making the region in a continuous movement of the arrival and departure of passengers from different countries of the world. Thus the process of controlling the outbreak will be more complicated, especially that each clone has its characteristics and genes (virulence and resistance). This requires the use of various treatments to obtain positive results.

Conclusion

This study showed weak resistance to tetracycline and other types of antibiotics because they are seasonal spread, so this is considered the beginning of the development of resistance genes, especially since this bacterium continued to spread even in the inappropriate seasons for it, which is the winter season, so its chance is greater for development and acquisition of plasmids in addition to genetic mutations.

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