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Prevalence of tetracycline resistance genes in Staphylococcus aureus isolated from different clinical sources in Diyala, Iraq

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***Abstract*---**Antibiotic resistance showed by many bacterial species such as *Staphylococcus aureus* is a great global problem. This paper is included the prevalence percentage of some tetracycline's resistance genes in isolates of *S.aureus* collected from different sources. Seventy five different samples were collected from different sources, including wound, burn, nasal, blood, and urine samples from several hospitals of Baquba city and the samples cultured on two culture agar Blood Agar and Mannitol Salt Agar for studying of colony properties. Gram stain and some biochemical tests such as (coagulase, oxidase, catalase) were done for this purpose. The molecular method was dependent on PCR in the detection of specific genes, 16SrRNA, to diagnose the staphylococcus genus. The results of 16SrRNA PCR detection revealed that fifteen isolates were identified as *S. aureus*. All the isolates were subjected to polymerase chain reaction to amplify four common tetracycline resistance determinants, *tetK*, *tet38* ,*tetL*, *tetM*,. A number of 15 isolates (100%) were found to be positive for *tet*K genes , Out of 15 resistant *S.aureus* isolates, 15 (100%) were found to produce *tet38* genes,while 5(33.3%) isolates have *tet*L genes , 8(53.3%) carried *tet*M genes.. Detection of gene expression for isolate treated with Ag Nanoparticles. Detection of gene expression for isolate treated with AgNPS Nanoparticals .The results showed that gene expression of *tet38* gene was low Expression after treatment with AgNPs.

Keywords---S. aureus, antibiotic resistance, tetracycline, AgNPs, gene expression.

Introduction

Staphylococcus aureus is a gram-positive bacterium that has a greater impact on animal and human health by causing various diseases. (Mahendra *et al.*,2020) *S.aureus* is an opportunistic bacteria and can adapt to challenging environments and resist most immune defenses leading to disease occurrence. *S. aureus* causes several illnesses, especially in soft tissue and skin, but sometimes causes toxin-mediated disease and systemic disease (Hardey *et al.*, 2020). The growth of *S. aureus* infections is usually correlated with immunocompromised and hospitalization conditions (Costa *et al.*,2018). *S. aureus* showing resistance to antibiotics and causes significant public health, but some other bacteria, like some species of *Enterococcus,* exhibit intrinsic Resistance to many antibiotics because of their natural Resistance. *S. aureus* increased potential to acquired antibiotics resistance (Klare *et al.*,2003). There are some compounds that have effects on the antimicrobial resistance cases, such as thioridazine, which have an impact on β-lactam antimicrobials such as dicloxacillin that are used for treating the cases with MRSA (Wassmann *et al.*,2018). Genes of antibiotic resistance of S. *aureus* have including many genes such as *tet* resistance genes (*tetL*, *tetK*, *tetM*, *tetO*) and *erm* genes (*ermA*, *ermB*, *ermC*). These genes could be detected by PCR techniques (Li *et al.*,2019). Tetracyclines are broad-spectrum antibiotics used in the treatment and prevention of bacterial infections in both humans and animals. During the last years, Resistance to tetracycline is spreading at high rates and causes the common use of tetracyclines in animals and humans at high doses (Roberts *et al.*,2016). Tetracyclines inhibit bacterial 30S ribosome from attaching with the aminoacyl-tRNA, inhibiting protein synthesis. Tetracycline's Resistance is associated with plasmid genes responsible for proteins that protect ribosomes from antibiotics and active efflux pumps. the *tet38* gene was carried on the chromosome and can create a native efflux pump (Chunhui *et al.*,2018). The silver nanoparticles exhibit important physicochemical properties NPs have been used as an alternative method for treating various antibiotic-resistant bacterial infections and may solve the problem of multidrug-resistant bacteria (Wang *et al.*,2017). SNPs action mechanism against bacteria is doesn't clear. Still, it demonstrated great effective and lethal activity of SNPs against bacteria, especially the resistance bacteria, because of the reactivity ability of SNPs and its the bio compounds that produced such as enzymes, DNA, sugar, and proteins that produce genetic mutation and structural changes to the cell wall of bacteria and causing inhibition of bacterial growth leading to the cell death. Therefore, during the last decades, synthesizing and developing SNPs are increasing as alternative antibiotics materials (Hamida *et al.*,2020).

Materials and Methods

Specimens Collection

From the beginning of December 2019 to the end of August 2020, wound, burn, nasal, and blood urine samples were collected from cases in many Baquba

city/Iraq hospitals. The samples were cultured immediately after collection for diagnosis.

S. aureus identification and isolation

Gram staining and Colony morphology were applied for the identification of *S. aureus* isolates. Samples of nasal swabs are cultured on MSA media and Blood agar, then incubated overnight at (37) C. the blood agar is used to determine the degree of hemolytic activity. The MSA is used to determine the degree of Mannitol fermenting. The isolates with positive Mannitol fermenting were cultured on nutrient agar and keep overnight at (37) C. on blood agar, the colony showed Pin-point in size and have golden yellow in color and round, opaque, convex in shape and smooth-glistening colonies with diameter 2-3 mm were indicative of *S. aureus*. The coagulase, oxidase, and Catalase testes are done as biochemical tests for bacterial confirmation. Finally, The isolates were identified by PCR by using the 16SrRNA gene. Microscopic examination was performed for Gram-stained samples to confirm their Gram positivity.

Preparation solution of Ag Nanoparticles(AgNPs)

It was suspended with deionized distilled water by an Ultrasonic homogenizer at room temperature for 30 min in the concentration of the AgNPs was 50-60 nm, Purity 99.9 % .

Determination of AgNPs MIC values

AgNPs MIC values of the *S.aureus* strains were determined by the micro-dilution method by prepared a serial dilution of AgNPs in the range of 5.07-5200 ìg/ml.

Extraction of DNA and PCR

From the isolates, DNA was extracted by specific Kits; the purification is done according to company instructions (Promga USA). Using the extracted DNA in PCR analysis, that was done for detection of the gene (16S rRNA) that associated with *S. aureus,* then *S. aureus* isolates submitted to detection of some the genes such as *tetK,tetM,tetL,tet38* gene.

Table (1): The used primers in the study for detection of Tetracycline genes

Primer of *tet* genes				
Primer	Nucleotide sequence (5'-3')	Pcr product bp	Annealing temp°C	Reference
tetK	F-5-GTAGCGACAATAGGTAATAGT3` R-5`-GTAGTGACAATAAACCTCCTA-3`	360	55	Khoramrooz *et al.*,2017
tetL	F- 5`-AAATTGTTTCGGGTCGGTAA-3` R- 5`-	537	55	Khoramrooz *et al.*,2017

	ATTCCCCCACAAAGAACTCC-3`			
tetM	F-5`-AGTGGAGCGATTACAGAA-3` R-5`-CATATGTCCTGGCGTGTCTA-3`	159	55	Khoramrooz *et al.*,2017
Primer for *tet38* gene expression				
16SrRNA Reference gene	F- 5`-GGTCTTGCTGTCACTTATAGATGG-3` R-5`-CGGAAGATTCCCTACTGCTG-3`	164	60	Alves *et al.*,2018
tet38	F-5`-AGTTGGCAAGCGACATTAGC-3` R- 5`-GTCTCTGCAGCAGCTAAACC-3`	212	55	Antiabong,*et al.*,2017

F: Forward primer, R: Reverse primer, bp: Base pair.

Components for monoplex PCR reaction were prepared in a total volume of 20µl, including 10µl of Go Taq®Green Master mix (2X), 1µl for each primer (10 pmol), 3µl of DNA template, and 5µl of nuclease-free water. The amplification reaction was carried out using PCR thermal cycler that was setting as the initial denaturation for five minutes at 95°C, (each cycle were half minutes at (95) C° , thirty seconds at (55) C and thirty seconds at (72) °C), and the final extension needs to five minutes for the finish at (72) °C. The PCR products were submitted to electrophoresis to determine the fragments' size using a safe stain, then watched under a UV device**.**

RNA extraction

The total genomic RNA extracted using an RNA extraction kit (Promga, USA) from target four isolates from different clinical sources to measure the *tet38* gene expression level before and after treatment AgNPs in highly precise conditions and avoid any contamination especially RNase and the protection came from using of TRIzoL (guanidinthiocynate) with the ready kit. The quantity and purity measured by Nanodrop spectrophotometer device.

Determination of gene expression

Quantitative Real-time PCR (q RT-PCR) was used for the detection of gene expression of *tet38* gene and House-keeping gene, which was *16srRNA* as a control. Primers were prepared according to the company provided. The measurement of gene expression of the two genes in the resistant isolate was done before and after treatment AgNPs. The concentrations of AgNPs used in the treatment were in the dose under the MIC value to allow the bacterial growth with induction of Resistance

Statistical Analysis

SAS (2012) software was used to analysis of the statistical to find the effecting of different factors that were used in the study. LSD test (ANOVA) was used to

compare the group means. The Chi-square test was used to compare the percentage at (0.05 and 0.01 P).

Results and Discussion

Seventy-five clinical isolates of gram (+) bacteria are detected as *S. aureus* that collected from different clinical cases burn 24(32%) ,Wounds 18(24%) ,blood 15(20%), Nasal carriage 11 (14.66%), urine 7(9.33%).

Detection 16S rRNA gene by conventional PCR

The PCR results in Fig (1) showed that all 15 *S.aureus* contain *16S rRNA* gene (164bp). This gene used as the housekeeping gene exists in identified by the previous identification methods, which confirmed the accuracy of this study test and methodology used for identification of this genus. Several studies have used PCR for the detection of 16SrRNA by PCR method more rapidly and reliably. Although the 16SrRNA procedure differentiated all tested, clinical isolates identified 100%. Therefore, if an unknown organism needs to be identified, 16SrRNA sequencing is the method of choice because of the availability of universal primers

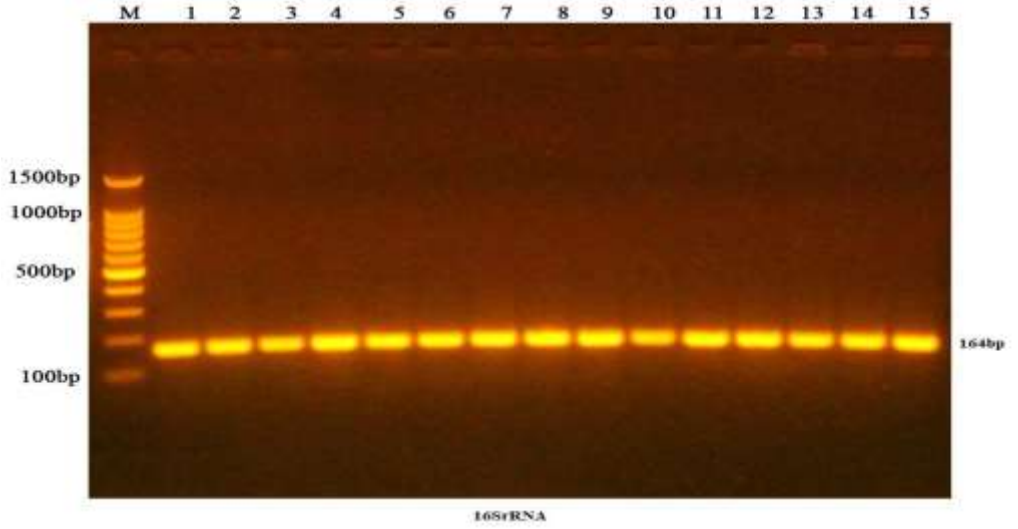
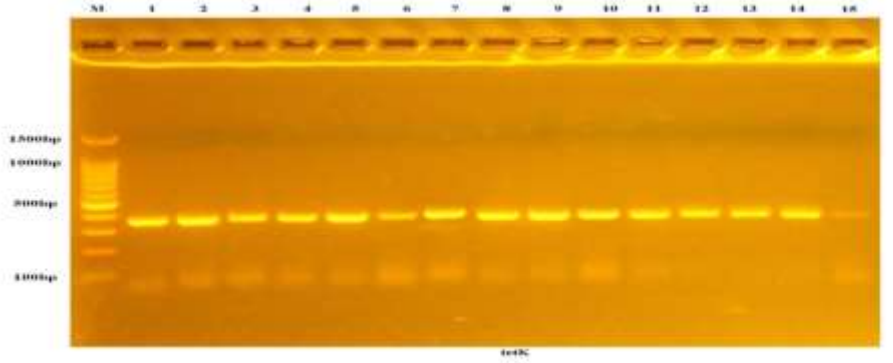


Fig (1): 16SrRNA gene of *S. aureus* samples were emigrated by electrophoresis on 1.5% agarose gel after stained by Eth. Br. Stain M refers to ladder marker. Lines 1-15 referred to (164) bp PCR products

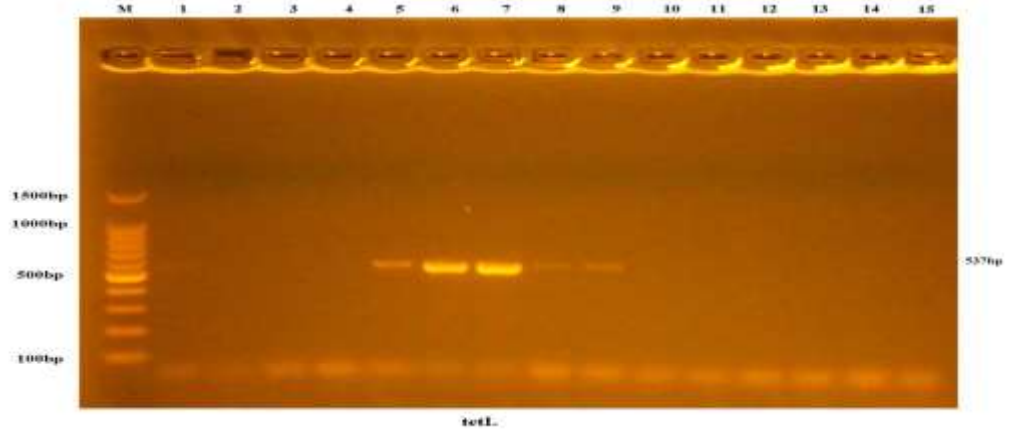
Detection of *tet* genes

The bacterial DNA of *S. aureus* was amplified to detect *tet* genes using PCR technique in a monoplex pattern with the utilization of specific primers. The Expression of *tet* gene was confirmed by agarose gel electrophoresis and visualized on a UV transilluminator**.** According to our results, by using PCR, 15 isolates (100%) were found to be positive for gene *tet*K , while 8 (53.3%) have *tet* M genes, 5(33.3%) positive for *tet*L genes. Fig.2,3 and 4 .This results disagree with

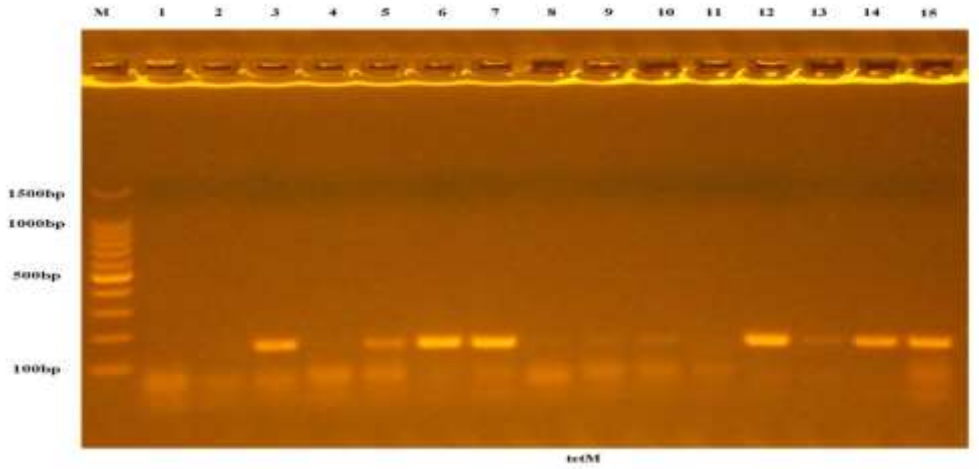
study of (Arabzadeh *et al.*,2018) that recorded the frequency of *tetK* was (62%) .The *tetK* gene protects bacteria from tetracycline by a resistance mechanism known as tetracycline efflux. This mechanism prevents the accumulation of tetracycline within bacterial cells by the synthesis of a cytoplasmic membrane protein which pumps tetracycline out of the cell at a quicker rate than it enters. This may explain why *tetK* gene does not impart resistance to minocycline.(Chopra and Roberts,2001) . The results showed as appeared in figure(3) that 5 isolates of *S.aureus* possessed gene *tetL* with frequency of (33.3%) These results disagree with the study of Emaneini,*et al* .,(2014) were recorded the prevalence of *tetL* was (17.2%) on clinical *S.aureus* isolates . the current study disagree with (Jones et al, 2006) who found 13.0 % of *tetM* genes in isolates .this study agree with Khoramrooz *et al.*,(2017) who found that *tet*M genes were detected in (56.9%) of isolates. another study by Arabzadeh *et al.*,(2018) that found that the occurrence of *tetL* and t*etM* genes were 3.33%,20% respectively and this results disagree with current study .*Staphylococcus aureus* has a wide distribution of *tetK* and *tetM* genes



Fig(2): *tetK* gene of *S. aureus* was emigrated by electrophoresis on 1.5% agarose gel after stain by Eth. Br. M refers to ladder marker. Lines 1-15 refer to (360) bp PCR products.



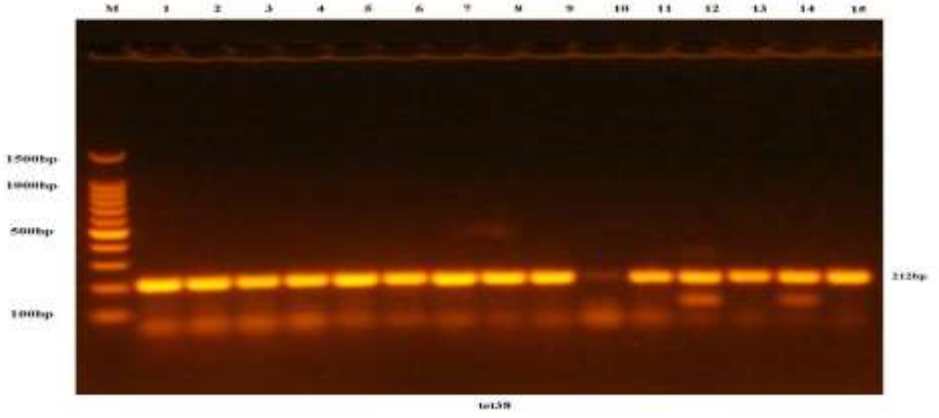
Fig(3): *tetL* gene of *S. aureus* were emigrated by electrophoresis on 1.5% agarose gel then after staining by Eth. Br. M refers to ladder marker. Lines 1-15 refer to (537) bp PCR products



Fig(4): *tetM* gene of *S. aureus* was emigrated by electrophoresis on 1.5% agarose gel after being stained by Eth. Br. M refers to ladder marker. Lines 1-15 refer to (159) bp PCR products

Molecular characterization *tet38* gene

This study was carried out in order to detect *tet38* genes in 15 resistant *S.aureus* isolates. *tet38* was screened by PCR technique for isolates. The results of gel electrophoresis for PCR product by using specific primers for this gene showed that 15 (100%) isolates were positive for *tet38* gene, as shown in Fig(5). Our rate of *tet38* gene was agreed with the study (Antiabong *et al.*,2017) was noted *tet38* gene appeared in 98.7%% among *S.aureus* isolates, There have, however, been no data assessing the direct association of chromosomally encoded efflux pump overexpression with treatment response *in vivo* in a Gram-positive pathogen that lacks the outer membrane barrier that can enhance the effect of efflux pumps.



Fig(5): tet38 gene of S. aureus was emigrated by electrophoresis on 1.5% agarose gel after being stained by Eth. Br. M refers to ladder marker. Lines 1-15 refer to (212) bp PCR products

Table 3: The Ct Value of *tet38* gene before and after Treatment with AgNPs

No. of isolates n=4	Source of isolates	Ct value of *tet38* gene before being treated with Ag NPs.	Ct value of *tet38* gene after treated with Ag NPs	(P-value
SA12	Burn	17.31	16.76	0.583 NS
SA13	Blood	19.29	18.89	0.577 NS
SA14	Wound	16.80	17.04	0.502 NS
SA15	Urine	16.01	17.77	0.541 NS
NS: Non-Significant.				

Table 4: Ct values and fold of gene expression of *tet38* and 16srRNA genes of *S.aureus* that was treated with AgNPs

Groups	No. of isolates	Mean ct of refrence16srRNA	Mean ct of *tet38*	Δ Ct	Δ Δct	(Folding) = 2-□□Ct	Average of folding
Untreated	SA12	14.29	17.31	3.03	0.00	1.00	1
	SA13	14.74	19.29	4.55	0.00	1.00	
	SA14	14.44	16.80	2.36	0.00	1.00	
	SA15	12.87	16.01	3.14	0.00	1.00	
Treated with AgNPS	SA12	12.60	16.76	4.16	1.13	0.46	0.54
	SA13	13.28	18.89	5.61	1.06	0.48	
	SA14	13.86	17.04	3.18	0.82	0.57	
	SA15	14.00	17.77	3.77	0.63	0.65	

Real-time PCR quantification of *tet38*gene Expression

The RT-PCR reaction was done by using (4) out (15) of *S.aureus* isolates. The different clinical cases are (one sample of burn, one sample of blood, one sample of the wound, one sample of urine). The Ct value of *tet38* gene in the present study (Tables 3 and 4) show the pattern of the amplification of the gene. The Expression was compared with 16S rRNA gene expression to measure the gene expression of the *tet38* genes and compare with the housekeeping gene. The assay was performed three times for each sample, and the mean of three values was considered as the quantity of given gene expression for that sample. The Δ Δct was used to determine gene expression. The Δ Δct was gained by subtracting **Δct** of sample from **Δct** of the reference gene. The results were presented when the isolates were treated with AgNPs using a concentration that was below the dose of sub MIC for each sample. The range of Ct values for the gene under this study was from16.76 to 18.89**.** The sub-MIC of AgNPS nanoparticles effectiveness on Expression of both *tet38b*and *16srRNA* genes in selected target isolates are shown in Tables 3 and 4 . Treated with AgNPS that led to the decrease in the value of gene expression in two isolates of *tet38* that was nearly average (0.54) (Table 4) .

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