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Detection of Antiseptic Resistant Genes and Biofilm Formation in Multidrug Resistant *Staphylococcus aureus* in Baghdad Hospitals

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Abstract: *Staphylococcus aureus*, a leading cause of serious human infections in both healthcare and community settings.Isolates belonging to the *S. aureus* were isolated from different clinical sources from different hospitals in Baghdad For the period from 1/9/2019 to 30/11/2019.The antimicrobial susceptibility test was performed by the disk diffusion method. Results showed that all isolates resistant to ceftazidime , and for pencillin G were 95% Likewise, the resistance to erythromycin, azithromycin, tetracycline, and ciprofloxacin were 46%, 44%, 37%, and 20%, respectively,The biofilm formation was assayed by the microtiter plate. Of the 50 isolates, 44 (88%) were biofilm producer.The minimum inhibitory concentrations (MIC) for Benzalkonium chloride was less than 2 µg/ml and for chloroxylenol ranged from 900_1950 µg/ml and povidone-ioden10% reach to 12500 µg/ml. Polymerase Chain Reaction using to detection of genes *norA* , *qacA*/*B1* , *qacA*/*B2* and *smr*, the results showed that *norA* gene found in 97.5 % of isolates, *qacA*/*B1* gene found in 2.5%, and *qacA*/*B2* in 5% , While the *smr* gene was not present in any Isolates.

Keywords: *Staphylococcus aureus*, Quaternary Ammonium Compunds, antiseptic resistance genes.

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Introduction

Staphylococcus aureus strains have disseminated on a global scale and are associated with adverse patient outcomes, increased hospital stays, a leading cause of serious human infections in both healthcare and community settings (1). Multi drug resistance and biofilm-forming capacity may contribute to the success of *S. aureus* as a human pathogen in both health care (2). This Gram-positive bacterium has been able to develop resistance to almost all classes of antibiotics, starting in the 1950s, followed by resistance to methicillin a decade later due to the activity of PBP2a protein (3).

The increasing use of antiseptic compounds creates selective pressure cause emergence of antiseptic resistance among *Staphylococcus aureus* .Resistance mechanism of antiseptic is driven mainly by multi drug resistant (MDR) efflux protein, Antiseptic efflux pumps possessed by *S.aureus* belong mainly to major facilitator superfamily (MFS) and small multi drug resistance family (SMR) and harbored by plasmid- or chromosomally based located(4)(5). Staphylococcal efflux determinants can be specialized to export one type of drug such as tetk efflux pump or transport vast array of unrelated

compound (antiseptics, dyes, and antibiotics) included mainly chromosomal *norA* efflux protein(6) . Plasmid encoded proteins Quaternary Ammonium Compunds (*qacA*) and (*qacB*) (7), and Small Multidrug resistance family (*smr*) (8) Effluxes machinery belong to MFS and SMR

family that uses secondary transporting system depend on ion moving (H+, K+) in electrochemical concentration gradients called proton motive force as energy source involved in transporting substrate from inside bacterial cell to surrounding environment to reduc intracellular concentration of toxic compound(9)(10).This study aims to detect genes contribute to the resistance of *S. aureus* bacteria to antiseptics.

Material and method

Bacterial Isolates

A total of 50 *Staphylococcus aureus* were collected from some hospitals in Baghdad city between September 2019 and November 2019, the isolates were identified using conventional tests and vitek 2 system.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates were tested by using Kirby-Bauer disk diffusion method following CLSI (11) guidelines, using commercially available 6mlm discs (Bioanalyse /Turkey) The susceptibility of the isolates was determined against 9 antibacterial agents, They include: ceftazidime , pencillin G , Erythromycin, Azithromycin, Tetracycline, ciprofloxacin ,amikacin, doxycycline and imipenem, on Mueller Hinton agar Plate, using overnight culture at a 0.5 McFarland standard followed by incubation at 37 ^{o}C for 18 to 24 h.

Detection of Biofilm Formation

S. aureus isolates were screened for their ability to form biofilm by microtiter plate according to the method described by (12) and(13). Twenty micro liter of bacterial suspension from an overnight culture was used to inoculate microtiter wells containing 180µL of Brain Heart Infusion (BHI) broth with 1% glucose. Control wells contained 200µL of BHI broth with 1% glucose. The covered microtiter plate was sealed with parafilm during incubation at 37°C for twenty-four hours. Unattached bacterial cells were removed by washing the wells three times with PBS (pH 7.2), they were dried at room temperature for fifteen minutes, and then 200µL of crystal violet (0.1%) was added to the wells for fifteen minutes . After removing the crystal violet solution, the wells were washed three times with PBS (pH 7.2) to remove the unbounded dye, and were allowed to dry at room temperature, then they were extracted twice with 200µL of 33% Glacial acetic acid, the absorbance of each well was measured 630nm using ELISA reader. The adherence capabilities of the test isolates were classified into four categories; three standard deviations (SDs) above the mean OD of the negative control (broth only) was considered as the cut-off optical density (ODc). Isolates were classified as follows: if $OD \leq ODc$, the bacteria were non-adherent; if $ODc < OD \leq 2 \times ODc$, the bacteria were weakly adherent; if $2 \times ODc < OD \leq 4 \times ODc$, the bacteria were moderately adherent; if $4 \times ODc < OD$, the bacteria were strongly adherent.

Minimum Inhibitory Concentration (MIC) to antiseptic

The minimum inhibitory concentrations (MICs) of benzylkonium chloride , chloroxylenol and povidone-ioden 10% were determined by the reference broth microdilution procedure recommended by the CLSI. At the end of incubation, the MIC was defined as the lowest concentration of antibiotics that produced no growth (14).

Detection of *qacA/B1*, *qacA/B2*, *smr* and *norA* in *S.aureus*

Isolates were fully grown in TSB (HiMedia,India) at 37°C for 24 h, then DNA was extracted using the genomic DNA extraction kit (WizPrep™g DNA mini Kit, South Korea). *S. aureus* isolates were undergone to detect *qacA/B1*, *qacA/B2*, *smr* and *norA* genes by multiplex PCR and by using specific primer pairs which designed for this study Table (1).The PCR reaction was carried out in 25 µl .After that the amplification was performed in a thermal cycler. PCR products were analyzed on 1% (w/v) agarose gels by electrophoresis (Merck, Germany).Bands were visualized under UV photographed. Ethidium bromide (Merck, Germany) as a stain was added to the agarose gel.

Table (1): Primers sequences and their product size

Target gene	5-3(Primer sequence)		Product size (bp)	Reference
qacA/B1	AATCCCACCTACTAAAGCAG	F1	630	(15)
	GCTGCATTTATGACAATGTTTG	R1		
qacA/B2	GCAGAAAGTGCAGAGTTCG	F2	361	(16)
	CCAGTCCAATCATGCCTG	R2		
smr	GCCATAAGTACTGAAGTTATTGGA	F	157	
	GACTACGGTTGTTAAGACTAAACCT	R		
norA	GGCGGTATATTTGGGGCACT	F	314	(17)
	ACGCACCTGCGATTAAAGGA	R		

Results and Discussion

In this study, 50 of *S. aureus* isolates were collected from hospitalized patients in Baghdad. These isolates were collected from a different of clinical specimens. All isolates subjected to 9 antimicrobial agents. in current study showed a varied levels of resistance rate to antibiotic, with a highest resistance rate reached to ceftazidime were 100%,and the resistance to pencillin G 95% However, it was contrary to what he mentioned (18), and resistance to Erythromycin were 46%, resistance Azithromycin 42%,It converged with the results of (19), which reached 55% ,It also violated (20) by stating that the resistance to Erythromycin was 97%, As for Tetracycline were 36%,but not compatible with it was stated by (21) that the ratio of resistance to isolates reached 100% ،resistance ciprofloxacin were 24%,while the bacteria did not show resistance to the amikacin As the ratio of resistance 10% and doxycycline 6% and impinem 2% (Table 2). It was almost identical to what it came with (22), as the ratio of resistance of its isolates to the antagonist was 0%, while (18) indicated that the ratio of resistance to bacterial isolates was 22.2% as a result of a contrast to the study results.

Table (2): Susceptibility of the 50 isolates of *S.aureus* to Antibiotics.

Antibiotic		Resistance%
ceftazidime	CZM	100
pencillin G	P	95
Erythromycin	E	46
Azithromycin	AZM	42
Tetracycline	TE	36
Ciprofloxacin	Cip	24
Amikacin	AK	10
doxycycline	DO	6
impinem	imp	2

The results of microtitre plate method showed that 88% of *S.aureus* isolates were biofilm producer , according to Figure 1.

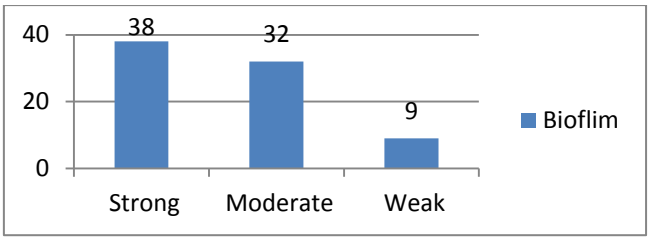

Figure (1): Formability of the biofilm i n study isolates.

High levels of antibiotic resistance are associated with strains producing the biofilm compared to strains that do not produce the biofilm. The reason may be that it is difficult to penetrate the biofilm with antibiotics, a slow rate of bacterial growth and the presence of antibiotic decomposition mechanisms (23).

The MICs of of benzylkonium chloride ,chloroxylenol and povidone-ioden 10% which are common used in hospitals were determined by using macrodilution broth method. The results showed that the Benzalkonium Chloride (Skinsept) disinfectant was more effective than the other disinfectants used, as the values of the lowest inhibitory concentration (MIC) were less than (2) µg / ml, As for the results shown by Chloroxylenol (Settol), they had a less effective inhibitory effect than Skinsept, as the MIC was from (3900_900) µg / ml, The result was agree with what was referred by (24) and the MIC values ranged from (2500_2000) µg / ml, As for the results of a Povidine-Iodine 10%disinfectant, the MIC ranged from (25000_12500) µ g / ml. The results agreed with (25), which ranged between MIC and MBC value, respectively (12500,6250) µg / ml ,The cause of resistance to antiseptics chemical to the indiscriminate use of disinfectants Which works to gain isolates bacteria causing mutations In cellular metabolism, leading to changes in the structure of the cell wall of the bacterial cell especially the changes that happen to Protein chanals, it prevents substances from entering the cell (26)

Polymerase Chain Reaction (PCR) was used to test for the presence of genes *norA* , *qacA*/*B1* , *qacA/B2* , *smr*, the results showed that *norA* gene found in 39 isolates (97.5 %)of isolates, Stream pumps are one of the mechanisms of resistance, (27) indicated that one of the reasons for

increasing resistance to antibiotics and disinfectants is the continuous occurrence of mutations in the genes that encode the *norA* flow pumps. (28) have shown that among the samples collected from all hospitals Japan The occurrence of the *norA* gene in *S.aureus* isolates was 51%., The results also showed that *qacA/B1* gene found in 1 isolate 2.5%. And *qacA/B2* in 2 isolate 5% ,as shown in Figure (2).While the *smr* gene was not present in any isolates, In a study conducted in Malaysia in 2012 to detect genes of antiseptics in the *S. aureus* strain, genes that were resistant to *qacA / B* and *smr* were identified and found by 83.3% and 1.6%, respectively (29).

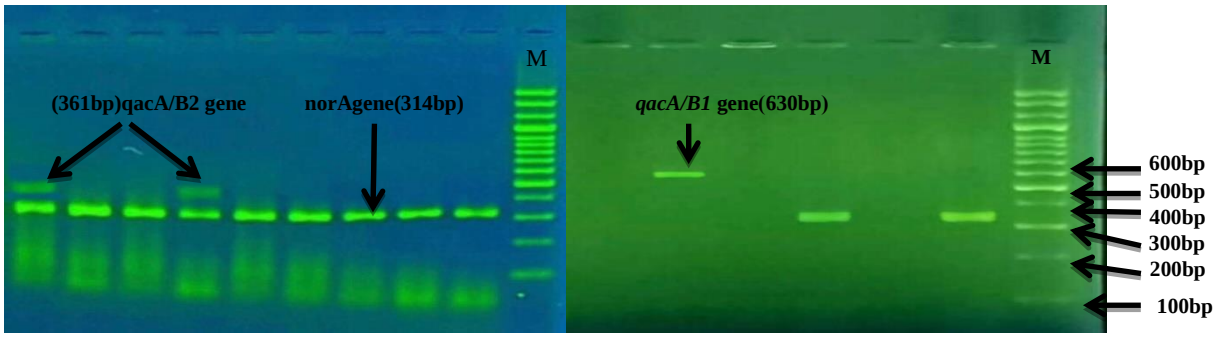

Figure (2): The electrophoresis of the Multiplex PCR reaction for the *norA* , *qacA / B2* and *qacA / B1* genes at a concentration of Agarose 2%, M represents standard Ladder DNA.

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Conclusions

The results suggest that long-term use of multipurpose solutions containing quaternary ammonium compounds may select for carriage of organisms harboring QAC genes, their increased prevalence in isolates from hospitals.

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