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Molecular Detection of *medA* Virulence Gene in *Staphylococcus aureus* Isolated from Iraqi Patients

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Abstract: *Staphylococcus aureus* is associated with multi drug resistance infections and high levels of illness and the efflux pump has vital role in multi drug resistance for antimicrobial agent in *S. aureus*. (one hundred and fifteen) clinical specimens (burns, blood, Ear, Nasal, Urinary tract infection and wounds) were collected from patients attending different hospitals in Baghdad. *S. aureus* has been identified using biochemical,Vitek-2 system Furthermore, molecular methods. Molecular method was depended on The polymerase chain reaction (PCR) assay was applied to determine the major specific genes, *16S rRNA* and *medA gene* were used to diagnos *Staphylococcus* genus and *med A* gene to diagnos *S. aureus* species. Out of 115 isolates, 55 were identified by Vitek-2 system the result about 20 *S. aureus* isolate Furthermore identified *S. aureus* by PCR Just to make sure. all *S. aureus* isolates were tested toward the different class of clinically important antibiotics by using vitek 2 system. The results of resistance were as following: Penicillin G (10) unit (100%) Oxacillin (99%), Imipenem (3%). while the resistance to chloramphenicol, (72%), Azithromycin (40%), Erythromycin was (36%), Tetracycline (72%) Doxycycline is (7%) , Ceftazidime (99%), Ciprofloxacin (25**%)**. The aim of this study is to investigate the prevalence of number of chromosomal efflux pumps genes). The results indicated the presence of *16s rRNA* gene in all isolates (100 %) (from 20 isolates) The *med A* gene has been recorded the second highest prevalence present in 19 (92.71%) isolates., these results showed the role of *16S rRNA* for molecular detection *Staphylococcus* genus and using *medA* gene in *S. aureus* bacteria considered one of the most important features in the description of *Staphylococcus aureus* isolates.

Keywords: *S. aureus* , Efflux pump, *mdeA*, *16S rRNA*

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Introduction

Staphylococcus aureus is a Gram-positive non-motile coccal bacterial that can survive and replicate in harsh environments (1). Most *staphylococci* are cause disease in a variety of conditions including aerobic and anaerobic atmosphere (2). The genus currently consists of more than 80 species and subspecies, many of which are found on the skin and mucous membranes of humans (3). *Staphylococci* are important pathogens in humans, causing opportunistic infections and a wide spectrum of life-threatening systemic diseases, it causes a range of infectious diseases such as sepsis, endocarditis and pneumonia. In addition, it can cause uncomplicated skin infections impetigo, also soft tissue infections, (4,5) with or without abscess formation (6). Furthermore, *S. aureus* can cause toxin-mediated diseases such as toxic shock

syndrome, scalded skin syndrome and food poisoning (7). *S. aureus* are pathogenic bacteria that have become a great public health concern in recent years due to their ability to produce an array of virulence factors and drug-resistant variants (2,8,9). It is also the most common species in humans that produces the enzyme coagulase; therefore this property is a useful diagnostic test. (10). The bacterial *16S rRNA* gene encodes for the ribosomal RNA small subunit. It contains DNA sequences that are common to all bacteria and some that are unique to each species (11,12). Initial studies on bacterial taxonomy and phylogeny depend on the Sanger sequencing of the most common housekeeping marker which is the *16S rRNA* gene (13). Efflux pumps transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment (14). Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multipledrug resistance (MDR) (15). Efflux pumps are found in almost all bacterial species and genes encoding this class of proteins can be located on chromosomes or plasmids (16). Major facilitate super family which is only found in Gram-negative bacteria MdeA: The *MdeA* protein is a member of the MFS family of efflux pumps and composed of (479) amino acids which forms transmembrane helices. This 52 kDa protein effluxes fluoroquinolone has low affinity (17). Effluxes with low levels of sequence similarity with *MdeA* include QacA (23% similarity), EmrB of E. coli, LmrB of *Bacillus subtilis* and FarB of *Neisseria gonorrhoeae* (18). Mutations in promoter region of *MdeA* lead to the overexpression of the pump that use proton motive (19,20).

Materials and Methods

Isolation and Identification of Bacterial Isolates

A total of (115) clinical specimen from both gender with different age were collected and identification from the beginning of December 2019 to the end of August 2020, from patients in different hospitals of Baquba city. The isolates were identified by their colony characteristic, gram-stain according to Bergey's manual (14). and confirmed by the pattern of biochemical profiles using Vitek 2-GN system.

Antibiotic Susceptibility Testing

To estimate potential resistance of *S.aureus* isolates against 10 type of antibiotics from different classes, all isolates had been subjected to antibiogram test according to Clinical And Laboratory Standards Institute (CLSI,2020) (23), for Penicillin G (10) unit, Oxacillin, Imipenem, chloramphenicol, Azithromycin, Erythromycin, Tetracycline, Doxycycline, Ceftazidime, Ciprofloxaci,. Detection of *S.aureus* phenotypes based on the drug resistance patterns. Multidrug-resistant (MDR) phenotype is defined as *S. aureus*, which is resistant to

more than one antimicrobial agent in three or more antimicrobial categories. Extensively drug-resistant (XDR) phenotype is defined as *S. aureus*, which is resistant to more than one antimicrobial agent in all the antimicrobial categories, except in two or less.

Molecular Identification

DNA Extraction

Genomic DNA was isolated from bacterial growth according to the protocol of Wizard® Genomic DNA Purification Kit ABIOpure, USA (21). Quantus Fluorometer was used to detect the concentration and purity of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Polymerase Chain Reaction (PCR) Cycle of *16S rRNA and medA genes*

The DNA of isolates was targeted for *16S rRNA* and *medA* genes using primers (11, 22) listed in Table 1. A reaction mixture (20 µl) contained 3 µl of DNA, 1 µl of each primer, 10 µl of Master Mix, and 5 µl of nuclease free water. The experiment was sustained according to the following program: initial denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 30 Sec. , 55°C for 30 Sec. , 72°C for 30 Sec. and a final extension at 72°C for 7 minutes as shown in Table 2. The PCR products were analyzed using 1.5% agarose gel electrophoresis and the ethidium bromide stained bands in gel were visualized using Gel imaging system.

Table (1): Primer sequences used for PCR

Genes	Primer sequence (5'-3')	Product size (bp)	Annealing Temp. (ºC)	References
16S rRNA-F *16S rRNA*-R	GGTCTTGCTGTCACTTATAGATGG CGGAAGATTCCCTACTGCTG	164	60	(11)
medA-F *medA* –R	TATGGCGATTGTTGTTTTTACTAC AACCGTGTGCATTCATTTCTGG	174	62	(22)

Table (2): PCR program

Steps	ºC	m:s	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55-62	00:30	
Extension	72	00:30	
Final extension	72	00:30	1
Hold	10	10:00	

Results and Discussion

Identification and Isolation of *Staphylococcus aureus*

A total of 55clinical isolates of gram positive bacteria primary identified as *Staphylococcus aureus* were collected from different clinical sources Table (3). The source of these isolates were as follows: The isolation results showed that burns (40**%**), blood (55%), Ear (11.76)%, Nasal (60%). UTI (60%) and wounds (36%) isolated out of 115(100%).

Table (3): prevalence of *S. aureus* according to the sources of isolation

Source of Isolation	No. of sample	S. aureus	Percentage of S.aureus from total isolates%
UTI	25	15	60
Blood	20	11	55
Wounds infection	25	9	36
Burn infection	30	12	40
Nasal carriage	10	6	60
Ear infection	5	2	11.76
Total isolates	115	55	40
Chi-square (χ^2)	---	---	12.892 **
P-value	---	---	0.0001
** ($P \leq 0.01$).			

Antibiotics Resistance of *Staphylococcus aureus*

In recent years, *S aureus* has become resistant to both new and traditional antibiotics. Thus, treatment of antibiotic resistant bacteria represents a therapeutic problem. All isolates were subjected to antimicrobial agents and the resistance patterns according to the recommendations of CLSI,2020 (23). The results of the current study showed as shown in Table (4) that all *Staphylococcus aureus* isolates were 100% resistant to Penicillin G . These results were identical to (24,25,26) who found that *Staphylococcus aureus* resistance to penicillin and oxacillin was 100%,The cause of bacterial resistance to penicillins is due to the presence of many resistance genes or due to mutations in the genes encoding Penicillin Binding Proteins (PBPS), whereby the antagonist loses its ability to bind with the target proteins, leading to the cell's impermeability of the antagonist (27). Subjected to aminoglycoside antibiotics, erythromycins that act by inhibition of protein synthesis .showed relative activity against the tested *S. aureus* isolates (45%) of them were sensitive. (28). As for the resistance to Azithromycin, it reached 40%, the reason for resistance to anti-macrolides is due to the change in the target site as a result of mutations, as the Azithromycin works to inhibit protein synthesis by binding to the 50s ribosomal subunit, and this is due to the plasmid gene (29). The results showed that the resistance of the isolates to ceftazidime reached 99%, whereas (30). The resistance to the ceftazidime reaches 69%. The resistance is considered one of the beta-lactam group (third generation cephalosporins) antagonists that interfere with the

peptidoclycan layer affecting the formation of the bacterial cell wall and that one of the causes of bacterial resistance to the anti-mutagenic (*bla Ctx-M*) gene carried on plasmids encoding CTX enzymes M-which transforms the antibiotic into an inactive compound by breaking down the beta-lactam ring in the antagonist (31). On the other hand, the resistance of isolates to anti-Tetracyclin was 72%, The results were consistent (32). Reported, who stated that the rate of resistance to anti-Tetracyclin was 72%, but not compatible with what was reported by (33). as well as the results of the study showed that the resistance of bacteria to Doxycyline was 7% and this disagreement with a study in Iran (34) reported that 20% of *S. aureus* were resistant to doxycycline. But not in agreement with the results of the researcher (13). if the resistance rate reached 33.33%, where the resistance of the bacteria to the Doxycyline is a result of a change in the target site or as a result of genes carried on the plasmids, as the resistance to the antagonist returns to its association with the 30S ribosomal small unit, which leads to the cessation of tRNA binding (35). The results showed that the resistance of bacteria to Ciprofloxacin was 25%, the result deferent with local study done by (36). stated that the resistance rate was 72%, that resistance to anti-kionolones occurred as a result of chromosomal mutations or by changing the target site, which had an inhibitory effect on DNA replication as a result of its interference with the enzyme Topoisomerase II (DNA gyrase) encoded by the gene B / *gyrA* (37). Most of the utilized antimicrobial agents were regarded as targets for the staphylococcal efflux pump (38). Some evidence hypotheses that efflux pumps can be used by the cell as a first line defense mechanism that prevents drugs from reaching the cellular lethal concentrations (39). The diversity of fluoroquinolone antibiotics, mainly Ciprofloxacin, made the effective treatment of infections caused by *S.aureus* strains feasible, but these strains rapidly become resistant to these antimicrobial agents (40). For chloramphenicol, the result agreed with local study done by (41). where resistant to chloramphenicol was (75.2%), (42). In a study in Kenya, *S. aureus* showed resistance to chloramphenicol (84.8%), that agree with our result(72%), but (43). recorded a low percentage of resistance (11.3%) and (55%) respectively, which disagree with the results of the present study. High level of S. aureus sensitivity to aminoglycosides representing by impinem**.** The rate of resistance of isolates to anti-impinem was 3%, and this result is consistent with what was mentioned (44), as the rate of resistance of isolates to the impinem reached 0%, while (31). Indicated that the resistance rate of isolates of *Staphylococcus* bacteria reached 3%, agree with the results obtained by (45). Erythromycin This may be due to their belonging to the same group (β-lactum group) they act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls.

Table (4): antimicrobial susceptibility rate of *S. aureus* isolates against antimicrobial agents

Type of antibiotic	Typeof susceptibility	Resistance No percentage
Penicillin G (10) unit	R	100
Oxacillin	R	99
Impinem	S	3
chloramphenicol,	R	72
Azithromycin	R	40
Erythromycin	R	36
Tetracycline	R	72
Doxycycline	S	6
Ceftazidime	R	99
Ciprofloxacin	R	25

Staphylococcus aureus identifecation by detection *16S rRNA* gene

The PCR results Figure (1) showed that *16S rRNA* gene (164bp) exists in all 20 *S. aureus* Figure (1) identified by the previous identification methods, and this confirmed the accuracy of this study test and method used for identification of this genus.

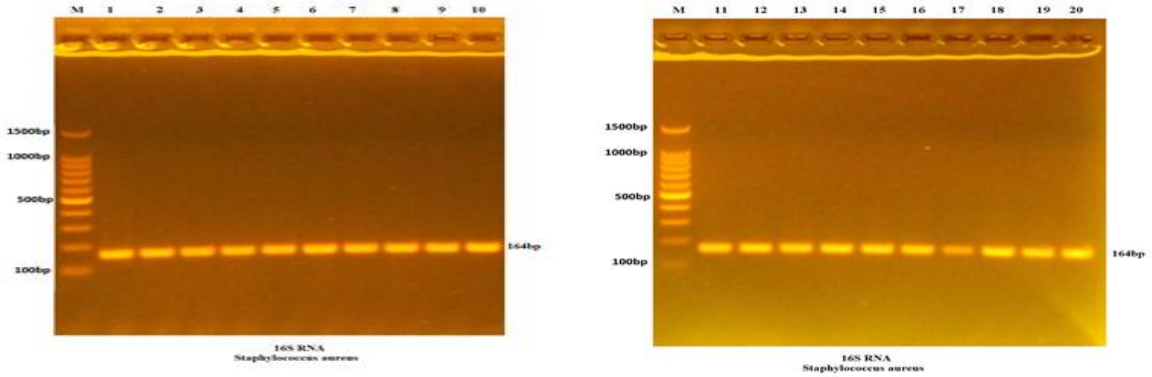

Figure (1):Gel electrophoresis of PCR products for *16S rRNA* gene in *S.aureus* isolate. Lane (M): l00bp ladder, Lane (c): Negative control, Lane (1-10): positive result with positive bands of 164 bp *S.aureus*.

The amplifying of DNAs from phylogenetically divergent bacteria by targeting conserved regions of the *16S rRNA* gene have become a powerful tool in detection and identification of bacteria (46). The present findings suggested that PCR using *16S rRNA* gene was an excellent method for detection of *S.aureus* spp. isolates. These results are in agreement with other studies which mentioned that detection and sequencing of this gene is an effective means for the identification of clinical isolates of *S.aureus* (47,48). The results of (49). revealed the ability of using *16s rRNA* and *23s rRNA* genes for molecular

identification of *staphylococcus* clinical isolates and this tool was rapid and accurate with high identification genomic rate (100 %). isolates. These results are in agreement with other studies which mentioned that detection and sequencing of this gene is an effective means for the identification of clinical isolates of *S.aureus* (47). The results of (49). revealed the ability of using *16s rRNA* and *23s rRNA* genes for molecular identification of *staphylococcus* clinical isolates and this tool was rapid and accurate with high identification genomic rate (100 %).

Detection of chromosomal efflux pump genes by Polymerase Chain Reaction (PCR)

Extracted sample has been used in order to detect the presence of genes encoding efflux pumps and determination of the prevalence of each gene among *S.aureus* clinical isolates uniplex polymerase chain reaction (PCR) for each DNA. The PCR reaction included 20 isolates for detection the chromosomal efflux pump *mdeA*. The PCR products have been confirmed by analysis of the bands on gel electrophoresis. PCR products have been confirmed by comparing their molecular weight with 100 bp DNA Ladder. The distribution of efflux pump in multidrug resistant isolates was shown in Figure (2). In Iran, out of a total of 60 multidrug resistant isolates of *S. aureus*, the *MdeA* genes were detected in 61.7% of isolates (50). A study by (51). In South Africa, a tertiary academic hospital in Pretoria city.

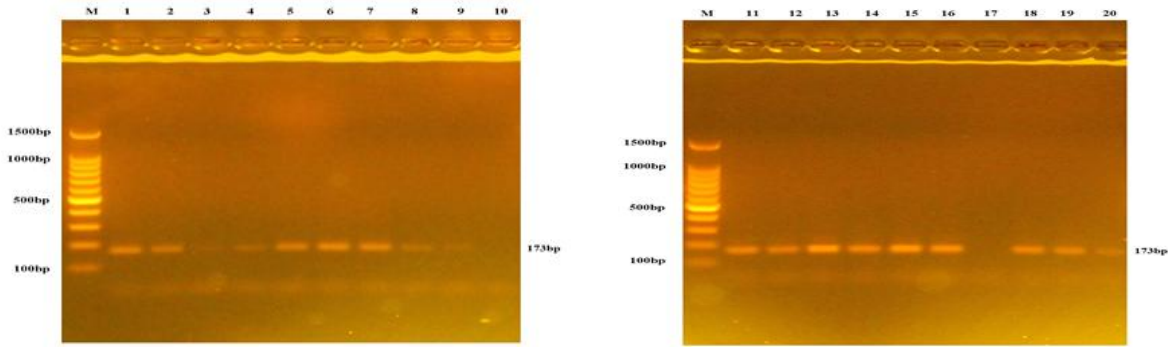

Figure (2): The usual banding patterns found with the uniplex PCR assay, PCR amplification of the mdeA gene from S. aureus, with the amplicon size 173bp, on an ethidium bromide-stained gel. Electrophoresis of DNA amplification products in a (1.5%) agarose gel was used to separate them. The electrophoresis was done for 1.5 hours at 70 volts. Lanes 1-18 represent the number of amplified PCR products (SA1,12) negative amplification of mdeA, (SA2,3, 4,5,6,7,8,9,10, 11.13,14,15,16,17,18) positive amplification of mdeA. Within the gel, the lengths of markers and PCR amplicon bands are noted.

Statistical Analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. T-test was used to significant

compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.(52).

Conclusions

Our study confirmed the role of *16S rRNA* for molecular detection *S.aureus* at the level of genus and detecting medA gene using in *S. aureus* bacteria considered one of the most important features in the descriptionof resist isolates. According to the results of the present study , most local clinical isolates of *S. aureus*, carrying the efflux pump genes and the efflux pumps system plays a vital role in multidrug resistance in clinical *S. aureus* isolates. The results of detection efflux pump genes demonstrated the presence of *mdeA* gene in all multidrug resistant local isolates of *S. aureus* and also high prevalence. This might be indicated to the significent role of these genes in the resistance mechanism.

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