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Molecular Characterization of Methicillin Resistant and Extended Spectrum β-Lactamase *Staphylococcus aureus* Isolated from Burn Patients

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ABSTRACT

Antibiotic resistance has become a major world health challenge and has limited the ability of physician's treatment. Staphylococcus aureus the most notorious pathogens causes morbidity and mortality especially in burn patients. However, Staphylococcus aureus rapidly acquired resistance to multiple antibiotics. Vancomycin, a glycopeptide antibiotic remains a drug of choice for treatment of severe Methicillin Resistance S. aureus infections. This study aimed to detect the emergence of beta-lactam and glycopeptide resistance genes. 50 clinical specimens of S. aureus collected from burn patients in burn and plastic surgery units in Sulaimani-Iraq city. All specimens were confirmed to be positive for S. aureus. All the isolates were assessed for their susceptibility to different antibiotics depending on NCCL standards, followed by Extended Spectrum Beta Lactamase detection by double disk diffusion synergy test. The production of β- lactamases was evaluated in the isolated strains by several routine methods and polymerase chain reaction. Among the isolates 94% were Methicillin resistance and 34.28% were Extended Spectrum Beta Lactamase producer. PCR based molecular technique was done for the bla genes related to β- lactamase enzymes by the specific primers, as well as genes which related to reduced sensitivity to Vancomycin were detected. The results indicated that all isolated showed the PBP1, PBP2, PBP3, PBP4, trfA and trfB, graSR, vraS except the vraR gene and the prolonged therapy of

Methicillin resistance infection with teicoplanin have been associated with progress of resistance and the rise of tecoplanin resistance may be a prologue to evolving Vancomycin resistance. In conclusion, beta-lactam over taking can rise Vancomycin- Intermediate S. aureus strains leading to appearance of Vancomycin resistance although the treatment of Vancomycin resistant infections is challenging.

1. INTRODUCTION

Staphylococcus aureus being the most versatile human pathogen and one of the major causative microbe in bacterial infections, because of its impressive capacity to colonize and persistence in a range of diverse environments [1]. Inhibition of bacterial transpeptidases enzyme (penicillin-binding proteins, PBPs) that facilitate bacterial cell wall formation is the mechanism of action β- lactam antibiotics. In contrast, vancomycin is binding to the C-terminal of the cell wall precursor pentapeptide (Lipid II) and restricts its use for cell wall synthesis. To encounter the action of β-lactam drugs the bacteria produce a surrogate transpeptidase by production of a new enzyme named the penicillin-binding protein 2′ (PBP2′) with low affinity for β-lactams. [2]. When exposed to high levels of β-lactam antibiotics *Staphylococcus aureus* cope this problem by producing a cell wall of distinct muropeptide structure and the four native PBPs become inaction and their transpeptidase function is substituted by PBP2 [3]. Changes in gene expression related to resistance phenotype and to compare susceptibility of *S. aureus* when faced different environment stress such as antibiotics has been studied by microarray which provide a greater information about molecular basis for understanding the resistance mechanisms.

In response to vancomycin (VAN) and methicillin an essential protein the penicillin-binding protein 2 (PBP 2) of *Staphylococcus aureus* plays an important role [4], [5]. upon growing of *S. aureus* in the inhibition of cell wall, and cell wall active antibiotics, a set of genes the VraSR system is activated [6].

S. aureus is known for its adaptability to environmental stresses such as exposure to antibiotics by a variety of mobile genetic elements such as transposons, staphylococcal cassette chromosomes, pathogenicity islands and plasmids that aid in genetic exchange between bacteria via horizontal gene transfer (7). Efficient steps of infection control and specially to cure staphylococcal infections require a knowledge about *S. aureus* susceptibility patterns and molecular characterization of genes influenced resistance [8].

Different virulence factors that produce by strains of *S. aureus* (Table 1) which enhance their dissemination across the adjacent tissue and multiplication [9].

Table 1: Genes associated with the VISA phenotype.

Associated Genes	Functions	Reference
GraSR	stress survival, cell wall and pathogenesis control pathways also involved in modifications of cell envelope.	[10],[11]
vraSR	Up-regulation of *VraSR*, reduced susceptibility to vancomycin.	[12]
trfA/trfB	Associated with reduced susceptibility to vancomycin.	[13]
Pbp1	Essential for growth of both MSSA and MRSA	[14]
pbp4	Thickening of cell wall and reduced autolytic Activity.	[15]

2. METHODS AND MATERIALS

Collection and identification of Staphylococcus aureus

This study was conducted in Microbiology laboratory in burn and plastic surgery hospital in Sulaimani city. A total of 50 clinical samples of *Staphylococcus aureus* were collected from

burn patients. All specimens were confirmed to be positive for *Staphylococcus aureus* using selective mannitol salt agar medium, biochemical tests, and Api staph System.

Antibacterial sensitivity

The susceptibility profiles were tested by the conventional disk diffusion method to different antibiotics and aligned with NCCL standards. In addition, disc diffusion method according to CLSI were used to detect the tolerance of the strains to methicillin [16].

Staphylococcus aureus was tested for methicillin resistance by using an Oxacillin disk. Agar plates containing 2-4% NaCl were incubated at 35ºC and read after 24 hours of incubation. Organisms were deemed methicillin resistant when the zone of inhibition was 10 mm for *S. aureus*.

Detection of extended spectrum β-lactamases (ESBL)

Disc approximation method were used to detect β- lactamase producers of Staphylococci. Tested bacterium were inoculated on Muller –Hinton agar plates and left for 5-15 min at room temperature. Placed on the center of the medium Amoxicillin-Clavulanic acid disc 30 µg/ disc, then Cefotaxim 30 µg/disc, Ceftazidime 30 µg/disc and Pipracillin 100 µg/disc placed around Amoxicillin–Clavulanic acid about 3 cm far from the centric disc. Followed by incubation at 30-35°C for 24 hr. Presence of ESBL producers were confirmed by an enhanced zone of inhibition around the Amoxicillin- clavulanic acid disc with one or more of the used antibiotics.

Genomic extraction

The most resistant bacterial isolate which was MRSA was subjected to genomic DNA extraction according to the manufacture company (Tiangene) information for bacterial genomic extraction provided with the kit. For PCR application from each test sample 2.5 µL of the total extracted material was used as a template DNA.

Amplification of bla genes

primers specific for *PBP1, PBP2, PBP3, PBP4, trfAB, vraSR, and graSR* synthesized by Sigma and Cinnagen (Table 2). PCR Amplifications were performed according to manufacture instruction. The PCR products were analyzed by electrophoresis using 2 X Tris-borate buffer at 60 V for 3 hours, the gel was examined by ultraviolet illumination and photographed by a digital camera.

Table 2: PCR primers used in this study

Primer or probe	Sequence of primer (5´ - 3´)	Company
trfA forward	TCC CCC GGG CAA GTT GGT TAT AAT	Sigma
trf A reverse	CGG GAT CCG CAT CAT CAT CAG ACA	Sigma
trfB forward	TCC CCC GGG GGA CAA ATC GCA AAC	Sigma
trfB reverse	CGG GAT CCC TTG CTT GCC ACA CAC	Sigma
vraR forward	GGA TGA TCA TGA AAT GGT ACG TAT AGG	Sigma
vraR reverse	GTG CAA GAT AGA ACA CAA GCT GTT ATC	Sigma
vraS forward	TTA CAT ATG AAC CAC TAC ATT AGA AC	Sigma
vraS reverse	AAG CTT ATC GTC ATA CGA ATC CTC CT	Sigma
graSR forward	TAC ATC TAT ACG ATT ATA TC	Sigma
graSR reverse	ACA TAT GAC TAA CAT CTA TC	Sigma
Pbp1 sense	GAT ACG CGA GGA AAG ATTGC	Cinnagen
Pbp1 reverse	TTT ACG GCA TAA GAG GCC AG'	Cinnagen
Pbp2 sense	TCG AAG TAT TTT GGA AGA G'	Cinnagen
Pbp2 reverse	GTG AAT GAC TGA TTT TACG'	Cinnagen
Pbp3 sense	GTA TGA TTA CTT GTT CGG TCTC'	Cinnagen
Pbp3 reverse	CAA CCA TGC GCT ACA CAATC	Cinnagen
Pbp4 sense	GAG TAA GTT TGC TCT TCG'	Cinnagen
Pbp4 reverse	GTA CAG AAG GCA TTT CGACG	Cinnagen

3. RESULTS AND DISCUSION

Collection and confirmation of S. aureus

All of 50 isolates were derived from burned wound and tissue. Among 50 Staphylococcus aureus, 3 isolates were methicillin sensitive *S. aureus* (MSSA), the remaining 47 isolates 94% were identified as MRSA.

Coagulase production is a key character of *S. aureus* and most pathogenic and toxigenic Staphylococci are confined to this species, so the detection of coagulase is very important in routine identification of S. aureus by using either the slide or the tube coagulase test [17]. All *S. aureus* isolates were subjected to further bacteriological and biochemical analyses.

API staph system was used to differentiate the isolate and to confirm *S. aureus* identification; The results obtained were found to be in excellent agreement with earlier classical biochemical tests of coagulase positive staphylococci [18].

Antibacterial response

The sensitivity of 50 *S. aureus* isolates has been tested against 22 antibiotics (figure 1). The inhibition zones diameters were measured and aligned with the standard of CLSI (2011). The results obtained indicated that all the isolates were 100% susceptible to penicillin. In another study on chemical bombarded exposed patients in Halabja 75km southeast Suleimani, it was revealed that 100% of S. aureus isolated were resistant to penicillin [19] our observations were in agreement with these conclusions.

The results presented in this study indicated that all isolates (100 %) were resistant to Pencillin G, (94%) to Oxacillin, (88.57%) to Cotrimoxazol, (85.71%) to Erythromycin, (85.71%) to Tetracyclin, (82.85%) to Gentamycin, (82.85%) to Ciftriaxone, (80%) to Fucidic acid, (77.14%)to Doxycyclin , (71.42%) to Chloramphenicol, (68.57%) to Clindamycin, (62.85%) to Amoxillin-Claviculin, (54.28%)to Piperacillin, (54.28%) to Rifampicin, (37.14%) to Ciprofloxacin , (31.42%) to Norfloxacin, (28.57%) to Levofloxacin, (2.85%) to Nitrofurantion, also (2.85%) were resistant to Quinupristin Dalfopristin, all isolates were sensitive for Minocyclin, Vancomycin, and Teicoplanin and (34.28%) were extended spectrum β- lactamase producer.

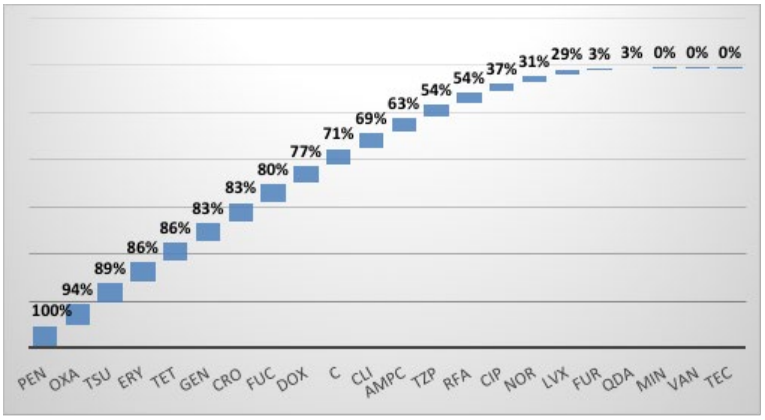

Figure 1: percentage rate of antibiotic susceptibility

PEN: Penicillin G, OXA : Oxacillin, TSU: Cotrimoxazol , ERY: Erythromycin , TET: Tetracyclin GEN: Gentamycin, CRO: Ciftriaxone , FUC: Fucidic acid , DOX: Doxycyclin C : Chloramphenicol , CLI: Clindamycin, AMPC: Amoxicillin-Clav , TZP: Piperacillin -Tazobactum, RFA: Rifampicin, CIP: Ciproflocacin , NOR : Norfloxacin , LVX : Levofloxacin ,FUR : Nitrofurantion , QDA: Quinupristin Dalfopristin , , MIN : Minocyclin , VAN : Vancomycin , TEC : Teicoplanin .

High resistance to most antibiotics noticed in this study. Spreading of epidemic strains is linked with high rate of MRSA infection in different countries [20]. The mec A gene which is found in staphylococcal cassette chromosome (SCC) in S. aureus is responsible of resistance to Methicillin [21], [22].

MecA gene transfer horizontally among different Gram positive bacteria and staphylococci species [23]. In addition, *mecA* gene has been illustrated to be responsible for the synthesis of penicillin - binding protein 2a (PBP2a). PBP2a is different from other PBPs in that its active site has been shown to block the binding of all β-lactams but it allows the transpeptidation reaction to proceed [24].

Detection of extended spectrum β-lactamases (ESBL)

Double disk diffusion method was used for the detection of extended spectrum β- lactamases. ESBL producing bacteria detected by synergistic of the inhibition zone between each of the cephalosporine disks and the disk containing clavulanate. [25].

The results showed that 34.8% isolates were positive for ESBL production (fig-2). In resistance to third-generation cephalosporins such as cefotaxime, ceftazidime, and cefepime

the extended-spectrum beta-lactamases (ESBLs), play a significant role. All the ESBL positives were MRSA which were resistance to most of the antibiotics.

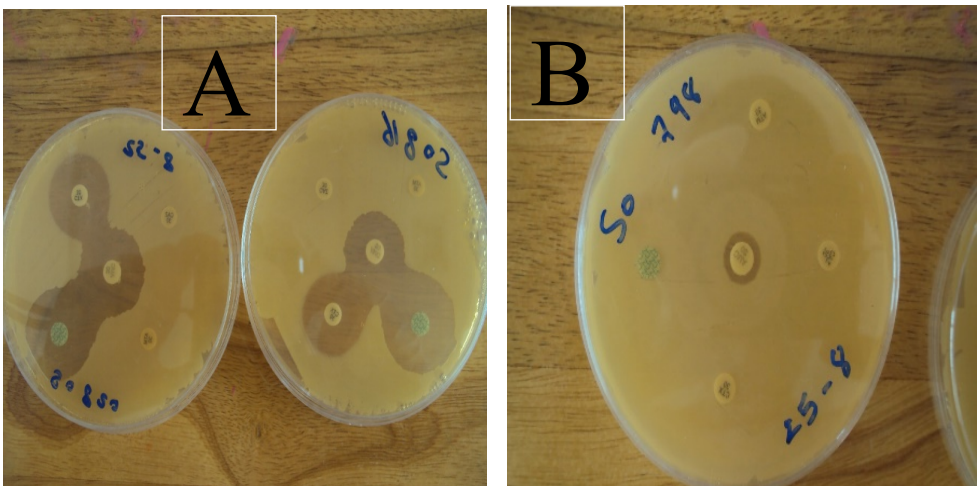

Figure 2: A:(positive result) Extended spectrum β-lactamase production by *S. aureus*
B: (negative result).

The obtained results reveals that ESBL confirmed tolerance to penicillin, and other broad spectrum cephalosporins and related oxyimino β- lactams .

4-4 Amplification of the β-lactamase determinants

The *bla* genes related to β- lactamase enzymes were assayed by PCR with the corresponding primers for the *pbp1, pbp2, pbp3, pbp4, trfA,trfB, graSR, and vraR and vraS* genes.
The results of the amplified PCR products showed that all the MRSA isolates according to multidrug resistance were positive for the β- lactamase determinants except *vraR* gene (Figures - 3). PCR amplified product gel electrophoresis of isolate of MRSA strain:

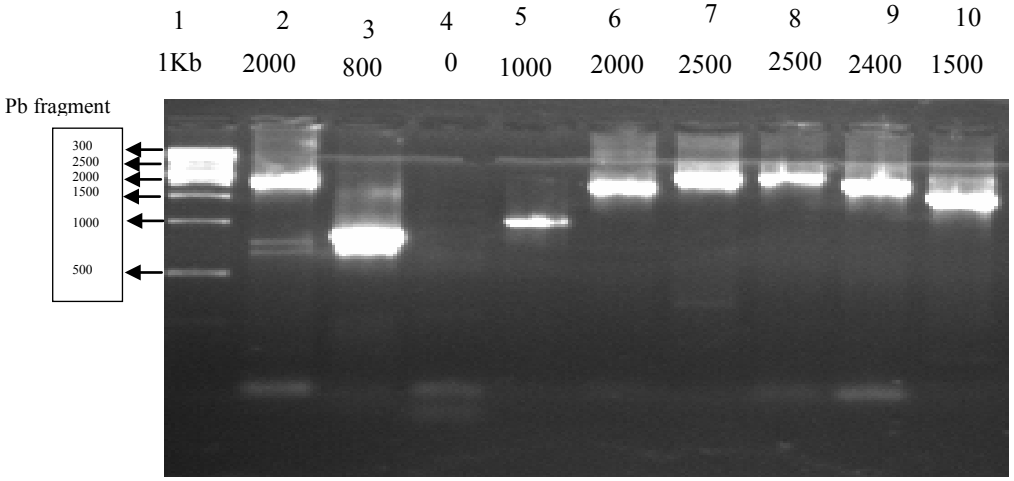

Figure 3: Gel electrophoresis of the PCR amplified products (Line 1: DNA marker, Iine2: *trfA* gene, Lines 3: *trfB* gene, line 4: *vraR* gene, line5: *vraS* gene, line 6: *graSR*, Line 7: *pbp1* gene, line8: *pbp2* gene, Line 9: *pbp3* gene and line 10: *pbp4* gene, isolates negative with the: *vraR* gene.

S. aureus Penicillin binding proteins *pbp1* to *pbp4* are enzymes concerned with the last stages of peptidoglycan biosynthesis. [26]. In addition, these *pbps* have gained a lot of attention since they are the aims of beta lactam antibiotics which bind covalently to these proteins, affecting synthesis of bacterial cell wall [27].

It was noted that over expression of *PBP4* resulted in an increase in β_lactam resistance and in greater peptidoglycan cross-linking [28]. *S. aureus* PBP4 is identical to other LMM PBPs and is arranged into the super family of penicillin-susceptible and our observation were in a good agreement with these correlations.

Inactivation of the over produced PBP4 increase β_lactam susceptibility and decrease the MIC for isolates resistant to vancomycin [29]. The majority of studies to date have approved an increase in the level of PBP4 when *S. aureus* isolates are exposed to β_lactam antibiotics [30]. It means that even in the presence of β-lactam antibiotics the active site of this enzyme can remain intact. The biochemical features of *PBP4* enable activators to modulate the activity of this enzyme to prolong the effectiveness of glycopeptide antibiotics such as vancomycin [31].

All isolated have been confirmed to harbor the *graSR* genes which are associated with oxidative stress survival, stress response, cell wall alteration and control mechanisms for pathogenesis [11].

In addition to these results, a β-lactam antibiotics enhanced two components regulatory system (VraSR) consisting of a sensor kinase (*VraS*) and a response regulator (*VraR*) that control β_lactam-dependent induction of PBP 2 transcription as well as that of other cell wall biosynthesis genes [32]. The VraSR disturbance in MRSA clinical strains, results not only the decrease of β_lactam and/or vancomycin MICs but also in the reducing transcription of PBP 2 [34]. ESBL genes are coded on plasmid and are passed from one strain to another; leading to emergence the multi drug resistance nosocomial pathogens bacteria whose infections failed to cure by widely used antibiotics.

All isolated showed the *trfA* and *trfB* gene and the continued treatment of MRSA infection with teicoplanin have been involved with resistance progress [34].

At sub inhibitory concentrations beta-lactam antibiotics can induce in vitro intermediate vancomycin resistance. The intact *trfA* locus was necessary for this induction and the prior use of beta-lactam antibiotics can weaken vancomycin potency in the curing MRSA infections [35]. The most widely used medications for the treatment of multi resistant methicillin resistant *S. aureus* are Vancomycin and teicoplanin. Resistance to Glycopeptide in *S. aureus* can be induced by mutation and selection when exposed to glycopeptides. In addition, resistance to Tecoplanin is more rapidly attained than vancomycin resistance and the emergence of tecoplanin resistance may be a precursor to expand vancomycin resistance [36].

4. CONCLUSION

In conclusion depending on the antibacterial susceptibility tests unnoticed high rate of MRSA were detected that increase the risk of nosocomial infections. The Meropenem antibiotics was the most effective antibiotics. Further studying the ESBL types and relation between ESBL types and antibacterial susceptibility test. Introducing PCR technology in to hospital laboratories for early detection of pathogens because bacteria producing ESBL should be detected rapidly so that suitable antibiotics received by patients.

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