

Multiplex System: Identification of Vancomycin (*Vana*) And Methicillin (*Meca*) Resistance Genes In *Staphylococcus Aureus*

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Abstract: Introduction: Emergence of Methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Staphylococcus aureus* (VRSA) strain from different regions of the world poses a grave concern to human health. Antibiotic resistance in *S. aureus* is mainly because of the genetic factors which modify or disrupt their target site on bacteria. **Methods:** This study is focused to identify the vancomycin and methicillin resistance gene in antibiotic sensitive and resistant *S. aureus*. The pure cultures of *S. aureus* were isolated, subjected to morphological and biochemical characterization. Antibiotic susceptibility testing was done to check the resistance pattern. DNA isolation was followed by genotyping of the antibiotic resistance genes (VanA and MecA) and housekeeping gene (AroE) was done through multiplex PCR method. All the strains showed the colonial, microscopic and biochemical characteristics (catalase and coagulase positive) specific for *S. aureus*. **Results:** Majority of the strains were resistant to cefixime (80 %) and least resistance was observed with fusidic acid (0%), while resistance frequency of the remaining antibiotics falls between them. All the strains showed the presence of housekeeping AroE gene with frequency of VanA is 2% and MecA is 24% which coincides with the findings of antibiotic resistance testing. For VanA, there might be other resistance genes of vancomycin cassette which confer the resistance against it. **Conclusion:** The study will help to discriminate the vancomycin and methicillin sensitive and resistance strains of *S. aureus* based on their respective genetic factors and help to validate the underlying mechanism in the acquisition of antibiotic resistance.

Keywords: *Staphylococcus aureus*, Resistance genes, Antibiotic Resistance, Multiplex PCR

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Introduction

Staphylococcus aureus is a facultative anaerobe which is a causative agent of various range of mild to life threatening infectious diseases. The bacteria can travel through the bloodstream and infect most of the sites in the body which include soft tissue infections, nasal infections,



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bloodstream infections, endocarditis, osteomyelitis, lungs infection, bacteremia, food poisoning and toxic shock syndrome [1, 2].

According to world health organization (WHO), emergence and rapid spread of resistant pathogens have compromised the treatment of common infections. The WHO classified 32 antibiotics to address the priority pathogens and 26 of them are considered essential in treating common and severe clinical infections, focusing on low-income and middle-income settings. Methicillin resistant *Staphylococcus aureus* (MRSA) infections have become a major problem worldwide as it is a common source of blood stream infections with the observed median rate of 12.11% reported by Global Antimicrobial Resistance and Use Surveillance System (GLASS) [3, 4]. In south Asia, Pakistan has been found to have higher frequency in antibiotic resistance which is a rising global concern [1].

The consequential implications of microbial resistance among individuals are; irresponsive to the treatment, prolonged illnesses and associated hospital stay hence, increasing the risk of other invasive treatment modalities [5]. Several studies have demonstrated and proposed the innate and acquired resistance mechanisms of bacteria for antibiotics. Innate resistance is inherited and constitutive. Acquired resistance can be achieved through several mechanisms from alteration of antibiotics to the modifications of cell membrane permeability. The resistance mechanisms (either acquired or inherent) are classified by the presence of resistance genes. These resistance genes are mainly responsible for antibiotic resistance in most of the bacteria like *VanA* for vancomycin resistance, *mecA* for methicillin resistance, *tetM* for tetracycline resistance. They can be transferred horizontally through co-infection or inherently present with conditional suppressed state in bacteria [5-8].

Antibiotics that were used to treat infections no longer work effectively against those pathogens. In developing countries, molecular characterization and identification of antibiotic resistant genes is still a gap in this regard. Therefore, the aim of this study is to identify the vancomycin and methicillin resistance genes responsible for the development of antibiotic resistance among *Staphylococcus aureus* isolates from Pakistan.

Materials and Methods:

Isolation and Identification of Bacterial strains:

A total of sixty-five isolated strains of *Staphylococcus aureus* were taken from the culture bank of Department of Biosciences, SZABIST and The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi. Isolated cultures were revived and sub-cultured on Manitol Salt Agar (MSA) via the four-way streak plate method.

Morphological characterization was done through media depigmentation (from red to yellow), colonial morphology and gram staining [9, 10]. Biochemically strains were distinguished through the biochemical tests specific for *S. aureus* which include the production catalase (oxygen production) and coagulase (blood coagulation) [11-14].

Antibiotic Susceptibility test:

Antibiotic susceptibility testing was done against different antibiotics through Kirby-bauer disc diffusion in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines over the microbial lawn grown on Muller Hinton Agar (MH) [15, 16]. The antibiotics includes methicillin, streptomycin, levofloxacin, vancomycin, oxacillin, linezolid, daptomycin, ciprofloxacin, fusidic acid, cefepime, cefixime, amikacin; after incubation zone of inhibition was measures and compared with standard zone of inhibition used for particular concentrations of antibiotics as mentioned in table no. 2.

DNA extraction:



Isolation of the DNA of resistant and sensitive bacterial strains was done through lysis buffer method [17]. Overnight grown bacterial cells were lysed via lysis buffer followed by protein separation and DNA precipitation. Dehydrated extracted DNA was reconstituted in Tris EDTA (TE) buffer and stored at -20°C.

Genotyping of Resistance Genes:

Resistance genes were genotyped through multiplex PCR strategy. Reported primers for Vancomycin (Van A) and Methicillin (Mec A) resistance genes and Aro E the housekeeping gene was taken as an internal control [18]. Forward and reverse primer sequences of the targeted genes and their respective product sizes as mentioned in table 1. The optimized PCR conditions included initial denaturation at 94°C for 5 minutes followed by the 15 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds and elongation at 72°C for 45 seconds. The remaining 25 cycles followed the denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and elongation at 72°C for 45 seconds. The final elongation was extended for 7 minutes at 72°C to complete the remaining reactions. This whole program was applied on the reaction mix of 30µl which contain 200ng of DNA, 15 µl Master mix (ThermoFisher Scientific®), 0.4mM for all the forward and reverse primers, additional 1U taq polymerase and the remaining volume was make up to 30 µl by the PCR grade water. The amplified products observed through agarose gel electrophoresis.

Table no. 1: Primer sequences of the targeted genes

Sr. No.	Gene Names	Primers	Primer Sequences	Product Size	Reference
1.	<i>Aro E</i>	Forward	3' ATCGGAAATCCTATTTACATTTC 5'	340 bp	[18]
		Reverse	3' GGTGTTGTATTAATAACGATATC 5'		
2.	<i>Van A</i>	Forward	3' GGCAAGTCAGGTGAAGATG 5'	180 bp	[19]
		Reverse	3' ATCAAGCGGTCAATCAGTTC 5'		
3.	<i>Mec A</i>	Forward	3' AGTGGAGCGATTACAGAA 5'	215 bp	[19]
		Reverse	3' CATATGTCCTGGCGTGTCTA 5'		

Results:

Morphological Characterization

Morphologically all the strains were gram positive (grape like clusters) and showed colonial characteristics of *Staphylococcus* genus which are circular convex golden yellow colonies with the diameter of 0.5-1.5 um on MSA. All the strains were able to change the color of MSA from red to yellow because of acid production [20-23].

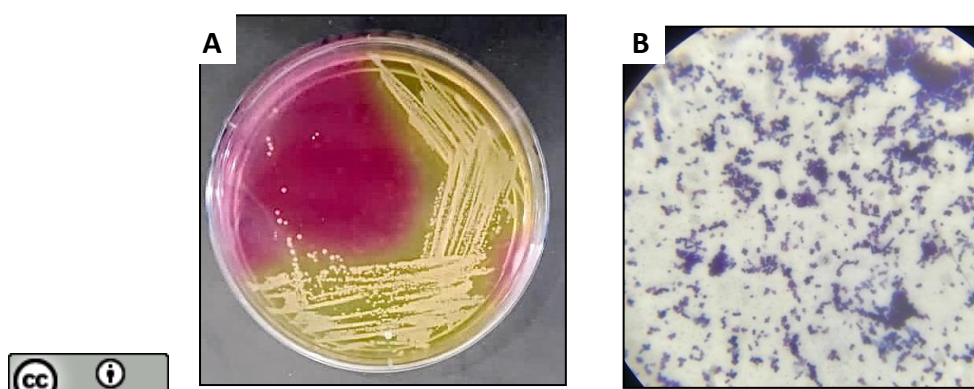


Figure. 1: (A) Colonial characterization: Golden yellow colonies with depigmented MSA, (B) Gram Staining: Grape like clusters

Biochemical Characterization

Enzyme based tests were used to biochemically characterize the bacteria [11, 14]. Catalase and coagulase test are the specific tests used for the identification of *S. aureus*. All the strains were found to produce catalase (characterized by the formation of oxygen bubbles) and coagulase (characterized by the formation of blood clots) as shown in figure no. 2 and 3.

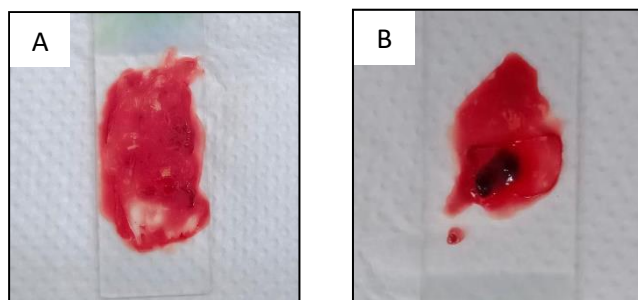


Figure 2: (A) Negative control for coagulase production, (B) Coagulase production by the *Staphylococcus aureus* as shown by the formation of blood clot.

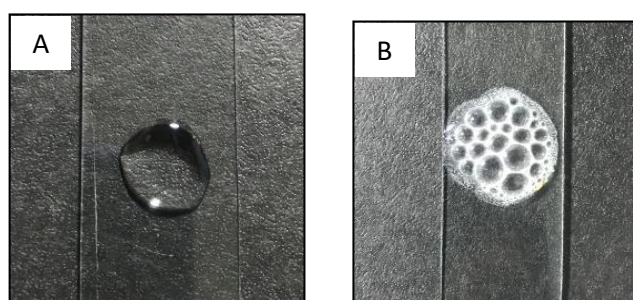


Figure 3: (A) Negative control for catalase production, (B) Catalase production by the *Staphylococcus aureus* as shown by the formation of oxygen bubbles.

Antibiotic Susceptibility Testing:

The frequency distribution of antibiotic susceptible and resistant strains is mentioned in table no. 2.

Table 2: Frequency Distribution of Antibiotic Resistant and Sensitive Strains

Antibiotics	Concentrations	Reference S-zones (mm)	Bacterial Strain	
			Resistant strains (%)	Sensitive strains (%)
Methicillin	5ug	≥17	25 %	75 %
Vancomycin	30ug	≥15	2 %	98 %
Streptomycin	10ug	≥15	5 %	95 %
Levofloxacin	30ug	≥17	2 %	98 %
Vancomycin	30ug	≥17	4 %	96 %
Oxacillin	5ug	≥13	3 %	97 %



Linezolid	30ug	≥ 16	8 %	92 %
Daptomycin	30ug	≥ 16	15 %	85 %
Ciprofloxacin	30ug	≥ 21	1 %	99 %
Fusidic acid	10ug	≥ 16	0 %	100 %
Cefepime	30ug	≥ 16	40 %	60 %
Cefixime	30ug	≥ 19	80 %	20 %

Genotyping of the Resistance Genes:

Antibiotic resistance genes were genotyped by the multiplex PCR strategy. Bacteria usually gain resistance against antibiotics by the activation of resistance genes or acquiring the resistance genes from other bacteria through co-infection. Resistance genes targeted and their amplified product sizes are 340 bp for Aro E, 180 bp for Van A and 215 bp for Mec A as shown in figure 4. The results of the genotyping of the resistance genes are shown in table 4.

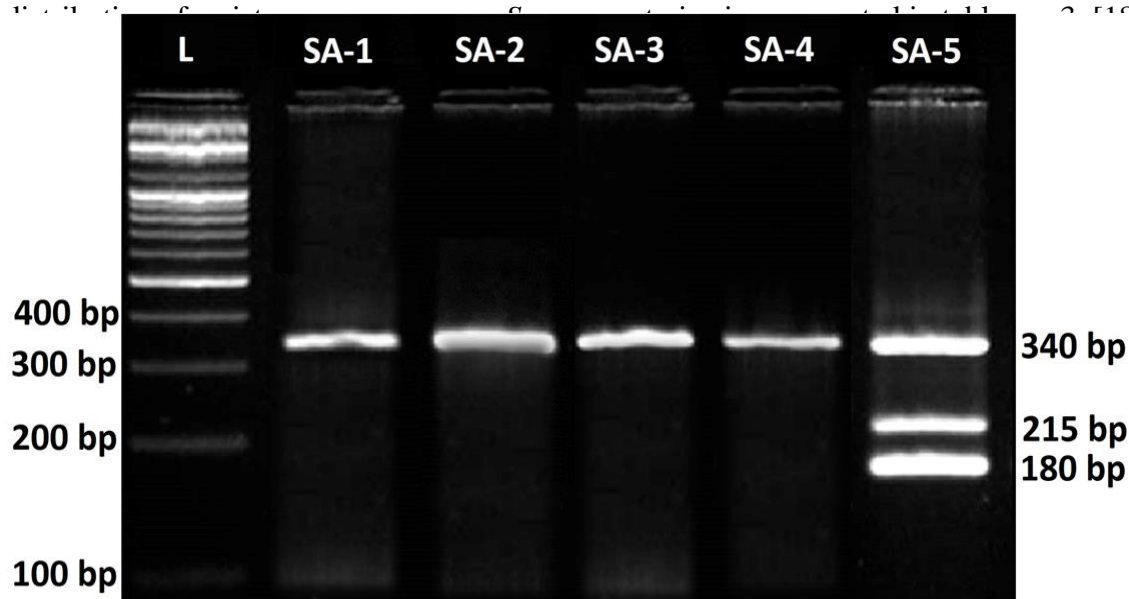


Figure 4; Lane 1: 1kb DNA Ladder, Lane 2 – 5: *Aro E* gene amplification, Lane 6: *Aro E*, *Van A* and *Mec A* gene amplification

Discussion:

Isolation and characterization of pathogenic strains are critical for generating the necessary information to identify, track, and intervene the disease outbreaks. This study identifies a multiplex approach for the genetic characterization of antibiotic resistance genes (*VanA* and *MecA*) with housekeeping gene (*AroE*) among the clinical isolates of *S. aureus*. The molecular characterization of *S. aureus* in various parts of the world has been done by RT-PCR in a considerable number of investigations, which revealed the existence of global clones, indicating epidemiological and geographical relatedness. For epidemiological understanding and public health decision-making, the capacity to correctly identify *S. aureus* strains is critical [1, 24]. Through antibiogram, the vancomycin resistant strains were classified by the formation of zone of inhibition (must not be smaller than 15mm). The *VanA* gene (vancomycin resistance) is the most prevalent and commonly found in *Enterococcus faecium* and *Enterococcus faecalis*. *Staphylococcus aureus* strains are not inherently resistant to vancomycin. Their resistance is mainly dependent on the presence of *VanA* gene, which they acquire through horizontally transferred plasmid from the co-infection of *Enterococcus sp.* hence, cause the emergence of Vancomycin Resistant *Staphylococcus aureus* (VRSA) [1, 25, 26]. As depicted in table 2 and 3, the respective genotypes (figure 4) of *VanA* and *mecA* among the isolates coincide with the



findings of antibiograms in terms of the percentage of the resistant and sensitive strains. The methicillin resistance is caused by the synthesis of modified penicillin- binding protein (PBP2a) with a lower affinity among most Beta- Lactam antibiotics. The PBP2a is encoded by the *mecA* gene, which is found on the Staphylococcal cassette chromosome of a mobile genetic element. Hence *mecA* is the driving force which showed no inhibition zone at all in antibiotic susceptibility testing [1, 27-30] Tetracycline resistance is mediated by ribosome protection mediated by *tetM* or *tetO* determinants present on either a transposon or the chromosome in *Staphylococcus species*. The *tetM* gene is thought to provide resistance to all tetracycline-group medicines, making it resistant [28, 30]. Other antibiotics have shown variable results regarding the sensitivity and resistance of *S. aureus* against them. Each targeted gene was represented by discrete bands of distinct sizes in a multiplex PCR set up. Two antibiotic resistance genes *VanA* and *MecA* and one housekeeping gene *AroE* were used. Housekeeping gene was used as an internal control to reduce the ambiguity and validate the multiplex investigations. This multiplex molecular genotyping strategy makes clinical diagnosis of *S. aureus* rapid, easy, practical, and dependable. Molecular typing is an early and precise detection method as, antibiogram may generate ambiguous results but genetic analysis may increase the clarity. This method also aids in the prevention of the spread of antibiotic-resistant strains of the organism and simplifies the formulation of antibiotic therapies [31-33].

Conclusion:

Antibiotic resistant bacterial strains are among the serious health concerns worldwide. Antibiotic susceptibility testing is unable to uncover the underlying mechanism and genes conferring the resistance. Identification of the genetic factors responsible for the antibiotic resistance may help in determining the underlying mechanism. Moreover, the multiplex PCR molecular genotyping strategy may contribute in the development of reliable, feasible, rapid, and simple approach for the detection of methicillin and vancomycin resistant strains of *S. aureus*. This will refrain the wide range spread of microbial spread and contribute to designing the effective antibiotic therapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals or human samples were used in this study.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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