

Original Research Article

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Prevalence of MRSA and Biofilm Associated Gene among Clinical Isolates of *Staphylococci*

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ABSTRACT

Staphylococcus spp is a major cause of nosocomial and community-acquired infections and represents a significant burden on the healthcare system. Establishment of a mature biofilm, play an important role in the persistence of chronic infections and also decreases the susceptibility to antimicrobials and immune defenses, making these infections difficult to eradicate. MRSA and MR-CONS has become a major public health problem in both hospitals and communities. PVL has been reported to be an important marker for the highly pathogenic community acquired *S. aureus* infections. A rapid detection of these MRSA is very important for its treatment. Hence, this study was done to determine the prevalence of *mec A*, *fem A* and *pvl* gene using triplex PCR and its relation to phenotypic and genotypic presence of biofilm formation. We collected 73 clinical isolates of *Staphylococcus spp* from the tertiary care center. AST was performed and interpreted according to CLSI guidelines 2017. TCP method was used to detect the biofilm formation. Cefoxitin disk method was performed for detection of methicillin resistant followed by triplex PCR for the detection of *mec A*, *fem A* and *pvl* gene. A simplex PCR was done for detection biofilm encoding gene such as *icaAD*. Of the 73 isolates, 49 isolates were identified as *S. aureus*, 18 and 6 were *S. epidermidis* and *S. saprophyticus* respectively. The majority of the isolate was from pus and urine. By AST, highest resistance was observed for penicillin followed by erythromycin and co-trimoxazole. 51 isolates were considered as methicillin resistance by phenotypic method and by PCR. *fem A* was seen in 49 isolates and *pvl* was detected in 27 and 9 isolates of MRSA and MSSA respectively. 58 isolates were considered as strong biofilm producers and 15 were non-biofilm producers by TCP method. PCR detected *icaAD* in 31 isolates.

Keywords

Staphylococcus,
MRSA, Biofilm,
mecA gene, CoNS

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Introduction

Antibiotic resistance and biofilm-forming capacity of the *Staphylococcus spp* contribute as a major pathogen in both healthcare and community settings. Biofilms are defined as communities of bacteria encased in a self-

synthesized extracellular polymeric matrix that attaches to a biotic or abiotic surface. Biofilm-forming staphylococci including *S. aureus* and *S. epidermidis*, have been one of the major cause of chronic polymer-associated infection. Infections involving biofilm-forming bacteria are extremely difficult to

eradicate because biofilms impair antibiotic penetration and prevent normal immune responses (Arciola *et al.*, 2012). The expression of biofilm is mostly influenced by acquisition of the methicillin resistance gene *mecA*. Biofilm formation in *Staphylococci* is encoded by *ica* ABCD operon and *ica AD* is the most prevalent gene among biofilm forming *Staphylococci*.

femA is a chromosomally encoded ubiquitous gene, which is involved in the formation of the peptidoglycan pentaglycine interpeptide linkages. It can be used as a marker for the differentiation of *S. aureus* from the coagulase negative *Staphylococci* (Francois *et al.*, 2003).

The presence of panton valentine leukocidin (PVL) toxin is the genotypic marker that differentiate community acquired methicillin-resistant *S. aureus* (CA-MRSA) strains from hospital-acquired (HA)- MRSA.

The *S. aureus* isolates with *PVL* are rapidly spreading and they cause serious skin and soft tissue infections such as pyomyositis, abscesses, breast abscesses, necrotizing fasciitis and pneumonia in otherwise healthy individuals (Gülmez *et al.*, 2012).

It has been known that methicillin-resistant *S. aureus* (MRSA) cause many device-related infections and other chronic infections grow in biofilms or on these devices. Some studies have shown that it is very difficult to treat biofilm-forming staphylococcal infections with antibiotics.

Moreover, MRSA is the most common cause of nosocomial infections in intensive care units. However, the prevalence of CA-MRSA strains in healthcare settings is increasing. Here, we studied the biofilm-forming ability of MRSA and analyzed the relationship between phenotypic and their molecular detection in clinical isolates of *S. aureus*.

Materials and Methods

A total of 73 retrospective clinical isolates of *Staphylococci* were collected between June 2015 to August 2016 from a tertiary care center in Kancheepuram, from various clinical specimens such as pus (65%), urine (25%) and blood (10%). The isolates were identified by standard methods.

Antibiotic sensitivity testing was carried out by Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) for the following antibiotics such as Penicillin (10 units), Clindamycin (2µg), Co-trimoxazole (1.25/23.75µg), Erythromycin (15µg), Tetracycline (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Vancomycin (30 µg) and interpreted according to the CLSI 2017 guidelines. *S. aureus* ATCC 25923 was used as quality control.

Screening for methicillin resistance (Cefoxitin disc diffusion)

Cefoxitin disc (30µg Hi Media Labs) was used for screening methicillin resistance. Sensitivity testing was done according to the standard disc diffusion method. Zone of inhibition was measured and interpreted according to CLSI guidelines (CLSI, 2017). *S. aureus* MRSA ATCC 43300 was used as quality control.

Biofilm production by crystal violet method

A liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and diluted 1:100 in Trypticase Soy Broth (TSB) with 0.25% glucose and 200µL of this solution was incubated in 96-well plates at 37°C for 24 hrs. Sterile broth was used as negative control. After incubation, the contents of the each well were removed by tapping. The wells were washed four times with 200µL of phosphate buffer saline (pH 7.2). The wells were fixed by 200µL of 2% sodium acetate and kept at

room temperature for 30mins, then stained by 200µL of 0.1% crystal violet. Excess stain was removed by using sterile water and attached dye was solubilized with 95% ethanol and the optical density (OD) of the adherent biofilm was read at 570nm by using ELISA reader (Biotek Epoch). The experiment was performed in triplicate (Hassan *et al.*, 2011). The isolates of biofilm production were categorized into non/Weak, moderate and strong producers (Table 1).

Optical density cut-off value (OD_c) = average OD of negative control + 3x standard deviation (SD) of negative control.

Molecular methods

DNA extraction- boiling lysis method

DNA extraction was carried out by boiling lysis-method. In this method, pure discrete colonies from sheep blood agar were transferred into a microcentrifuge tube containing 1ml of LB broth and incubated overnight at 37°C. Then it was centrifuged at 10,000 rpm for 10 minutes in Remi cooling centrifuge. The pellets were washed in 1mL TE buffer and centrifuged for another 2 minutes. The pellets were resuspended in 200 µl of DNase-free water (Qiagen, Germany) and the suspension was boiled at 100°C for 10mins in the dry bath. After boiling, the tube was kept overnight in the deep freezer (-20°C) and then centrifuged at 10000 rpm for 10 minutes. The supernatant which contained the extracted bacterial DNA was stored at -20°C and used in the PCR assay.

Detections of MRSA/ MRCoNS

A Rapid multiplex PCR (femA, mecA and pvl) was performed for the simultaneous detection and differentiation of MRSA and MR-CoNS (Abimanyu *et al.*, 2013). PCR was carried out in a 25µl reaction mix containing 200µM of dNTPs, 1X PCR buffer (TrisHCl

[10mM]; KCl [50mM]; MgCl₂ [1.5mM] and 0.1U Taq DNA polymerase (New England Biolabs, Inc, U.K), primers (10pmol each) and 5ng of template DNA. The details of the primers are given in table 1. Amplification was done using Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling conditions: one cycle of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30s, annealing for 30s at 60°C and extension at 72°C for 30s followed by a final extension at 72°C for 7mins.

Detection of biofilm-associated genes (icaAD)

Biofilm-associated genes (icaAD) was amplified by simplex PCR method using primers such as F: TAGTAATCACAGCCAACATCTT and R: AAACAAACTCATCCATCCGAAT which gives an amplicon size of 496bp (Araujo *et al.*, 2006). *S. epidermidis* 35984 was used as positive control. PCR was carried out in a 25µl reaction mix containing 200µM of dNTPs, 1X PCR buffer (TrisHCl [10mM]; KCl [50mM]; MgCl₂ [1.5mM] and 0.1U Taq DNA polymerase (New England Biolabs, Inc, U.K), primers (10pmol each) and 5ng of template DNA. Amplification was done using Mastercycler (Eppendorf, Hamburg, Germany) with the following cycling conditions: one cycle of initial denaturation at 94°C for 4mins, 30 cycles of denaturation at 94°C for 30s, annealing for 30s at 55°C and extension at 72°C for 1min followed by a final extension at 72°C for 5mins.

Results and Discussion

Of 73 isolates, the majority of isolates were obtained from pus (65%) followed by urine (25%). By phenotypic method, 49 (67.1%) isolates were identified as *S. aureus* and the remaining 24 (33.8%) isolates were identified as *S. epidermidis* (18 isolates) and *S.*

saprophyticus (6 isolates). Among 73 isolates, highest resistance was observed for penicillin (n=43, 59%) followed by erythromycin (n=35, 50%) and cotrimoxazole (n=35, 50%) (Figure 1). All the isolates were found to be susceptible to gentamicin and vancomycin.

Out of 73 staphylococcal isolates, *femA* was amplified in 49 (67%) isolates of *S. aureus*, whereas no amplification of *femA* was seen in remaining 24 (32.8%) isolates of *S. epidermidis* and *S. saprophyticus*. Triplex PCR detected *mecA* in 36 (49.3%) isolates of *S. aureus*; 13 (17.8%) and 2 (2.7%) isolates of *S. epidermidis* and *S. saprophyticus* respectively (Table 2 and Figure 2). Out of 36

MRSA isolates, 27 (75 %) were found to be positive for *pvl* gene which showed the community associated MRSA and the remaining 9 (25%) isolates were hospital associated MRSA. Among MSSA, 9/13 (69.2%) were *pvl* positive, CA-MSSA.

Out of 73 isolates, 58 (55.1%) isolates were considered as strong, 63 (32.1%) isolates as moderate and 25 (12.7%) isolates as non-biofilm producers (Table 3). Simplex PCR of *icaAD* gene showed positive in 35/49 (71.4%), 10/18 (55.5%) and 3/6 (50%) isolates of *S. aureus*, *S. epidermidis* and *S. Saprophyticus* respectively (Figure 3).

Table.1 Detections of MRSA/ MRCoNS (Al-Talib *et al.*, 2009)

Target genes	Primer sequences 5' → 3'	Expected Amplicon size
<i>femA</i>	F: CGATCCATATTTACCATATCA R: ATCACGCTCTTCGTTTAGTT	450 bp
<i>mecA</i>	F: ACGAGTAGATGCTCAATATAA R: CTTAGTTCTTTAGCGATTGC	293 bp
<i>lukS (PVL)</i>	F: CAGGAGGTAATGGTTCATTT R: ATGTCCAGACATTTTACCTAA	151 bp

Table.2 Distribution of *mecA* and *pvl* gene in isolates of *Staphylococci*

Genes	No. of isolates showed positive <i>S. aureus</i>	No. of isolates showed positive <i>S. epidermidis</i>	No. of isolates showed positive <i>S. saprophyticus</i>
<i>femA</i>	49 (67%)	0	0
<i>mecA</i>	36 (49.3%)	13 (17.8%)	2 (2.7%)
Total no. of isolates	49	18	6

Table.3 Distribution of biofilm formation in isolates of *Staphylococci*

Biofilm formation	No. of isolates <i>S.aureus</i>	No. of isolates <i>S. epidermidis</i>	No. of isolates <i>S. saprophyticus</i>
Strong	42 (85.7%)	13 (72.2%)	3 (50%)
Moderate	2 (4%)	0	2 (33.3%)
Non biofilm producers	5 (10%)	5 (27.7%)	1 (16.6%)
Total	49	18	6

Fig.1 Antimicrobial susceptibility pattern of clinical isolates of *Staphylococci*. P- Penicillin; E- Erythromycin; CD- Clindamycin; COT-Cotrimoxazole; GEN- Gentamycin; TET-Tetracycline; CIP-Ciprofloxacin; VA-Vancomycin

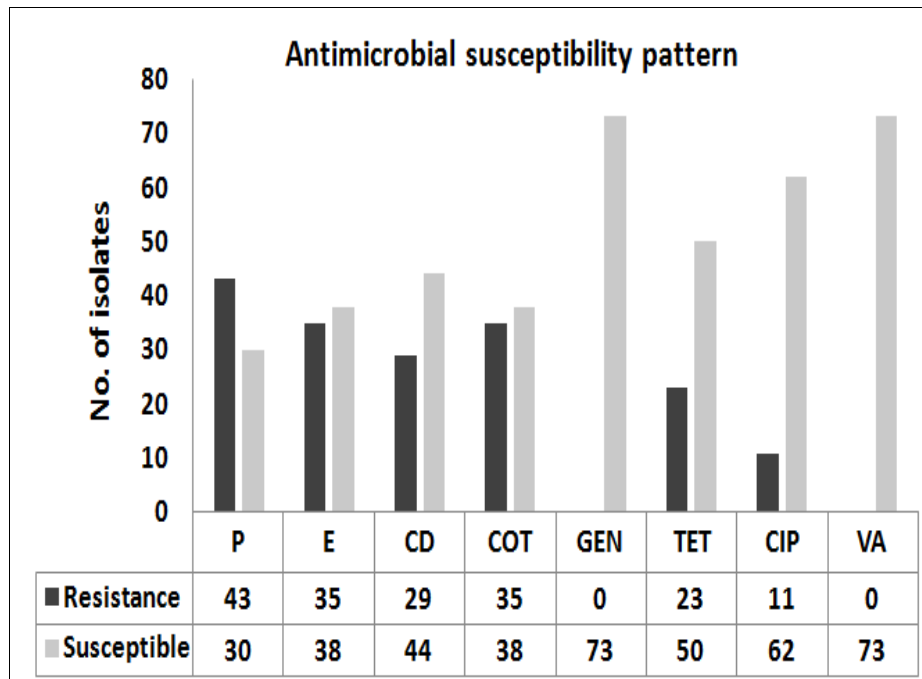
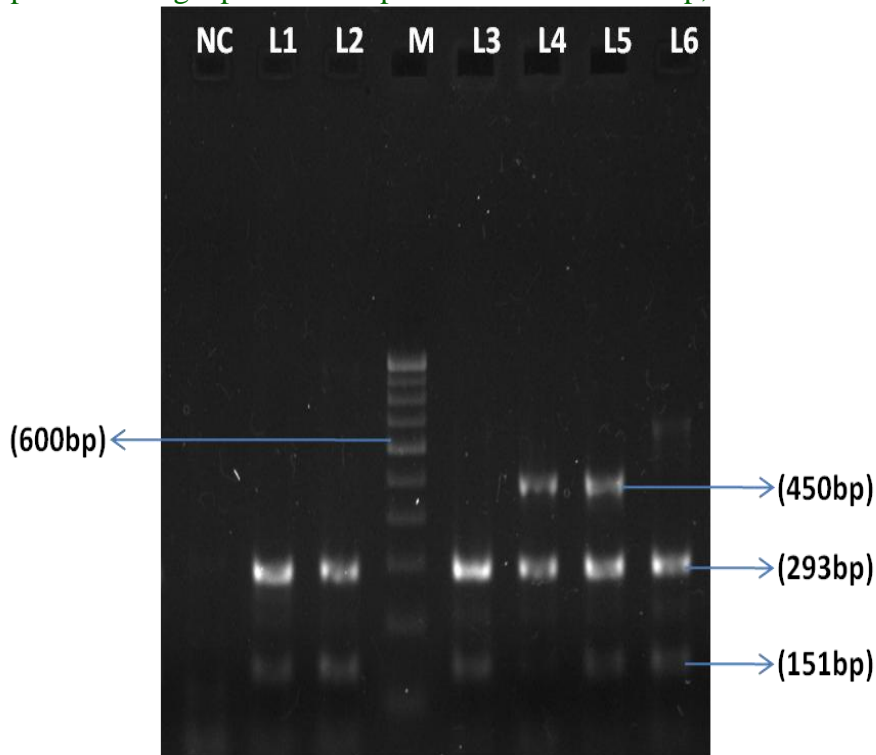
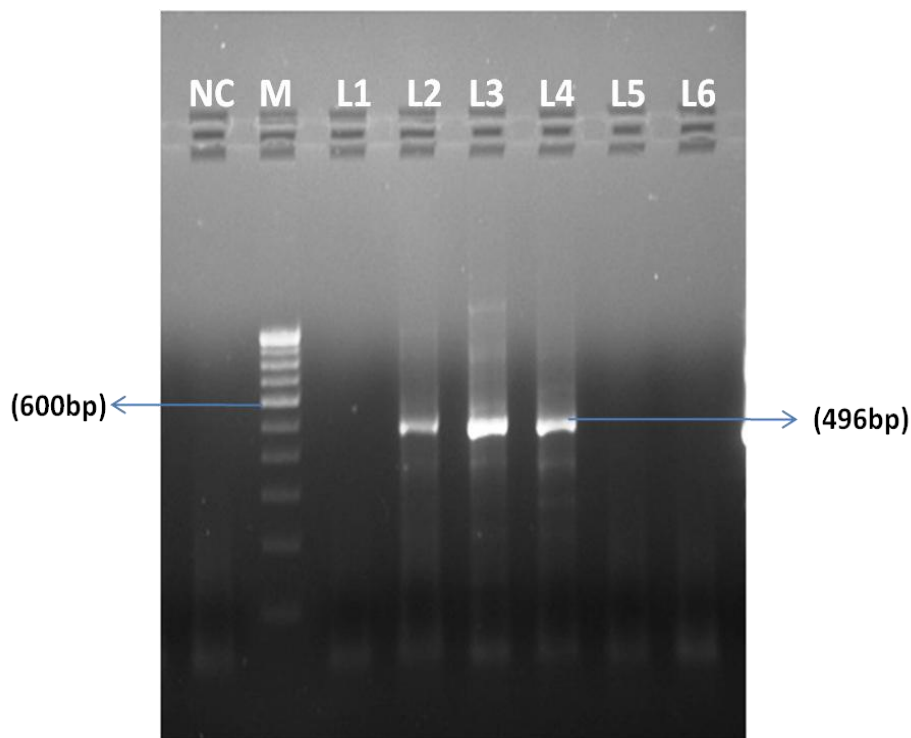


Fig.2 Representative gel picture of triplex PCR. fem A- 151bp, mec A- 293bp, pvl-450bp



NC: Negative control; L1-L6: samples; M: 100bp DNA Marker

Fig.3 Representative gel picture of ica AD (496bp)



NC: Negative control; L1-L6: samples; M: 100bp DNA Marke

Global emergence of MRSA and MRCONS is serious public health problem and challenge to clinicians. A number of factors contribute to the pathogenicity and drug resistance of *S. aureus* and *CONS*. In the present study, highest resistance was observed for penicillin, a similar finding of penicillin resistance (87.9%) was observed by Duran *et al.*, 2012.

The prevalence of MRSA in human infections is quite high. In Asian countries, the prevalence rate of MRSA in hospitals has been found to be 41% in India, 42% in Pakistan, 18% in Philippines, 38% in Malaysia, 50-70% in Korea, 53-83% in Taiwan and 70% in Hong Kong and Japan (Mehndiratta and Bhalla, 2014). For the past few decades, the prevalence of MRS in Indian hospitals is continuously increasing and varies from region to region. In the present study, 36/49 (73.4%) of *S. aureus* was found to be methicillin resistant by phenotypic method and all of them were *mecA* positive thereby

confirming the methicillin resistance. The prevalence of MR-CoNS among clinical isolates was found to be 58.3% (14/24). Previous studies from other parts of the world by Koksai *et al.*, (2009), Garza-González *et al.*, (2010) and Talebi *et al.*, (2016) have reported 67.5%, 69% and 60% MR-CoNS respectively, which was higher than our study. In our study, *S. epidermidis* was the most predominant species (24.4%) from clinical isolates followed by *S. saprophyticus* (8.2%).

Panton–Valentine Leukocidin (PVL) appears to be epidemiologically associated with certain CA-MRSA strains. We evaluated the presence of PVL toxin as a marker for identifying the CA-MRSA isolates and found positive in 27/36 (75%) and 9/13 (69.2%) isolates of MRSA and MSSA respectively. Similar studies also showed the association of *pvl* gene in CA MRSA than HA MRSA (Eshwara, 2013; Bouchiat, 2015; Bhatta *et al.*,

2016). Among the *ica* genes, *icaAD* are more in *S. aureus* and CONS. Thus in the present study, these genes are screened and detected in 35/49 (71.4%), 10/18 (55.5%) and 3/6 (50%) isolates of *S. aureus*, *S. epidermidis* and *S. saprophyticus* respectively. This finding is in contrast to other studies which showed 84.13% and 100% prevalence in *S. aureus* (Shanmugararaj *et al.*, 2012; Salman *et al.*, 2012).

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