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ANTIBIOTIC RESISTANCE AND STAPHYLOCOCCAL SUPER ANTIGENIC DETERMINANTS IN METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS ISOLATED FROM ANTERIOR NARES OF DENTAL STUDENTS

Jebadass Jasmine Vinshia[1,2], Kesavaram Padmavathy[3]*, Jimson Sudha[4], Baskaran Sathyapriya[5]

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ABSTRACT

Background: *Staphylococcus aureus* primarily inhabits the human anterior nares. Asymptomatic Methicillin-resistant *Staphylococcus aureus* (MRSA) nasal carriage in healthcare professionals makes them potential MRSA reservoirs. Since identification and decolonization would promote cross-contamination reduction and curb communal transmission, this study was designed to identify MRSA nasal carriers among dental students and detect staphylococcal super antigenic determinants among them. **Methods:** *Staphylococci* isolates (n=52) from dental students(n=42) were investigated in the study. Following initial microbial speciation and antimicrobial susceptibility determination using standard identification methods, the MRSA strains were identified phenotypically using cefoxitin disc (30 µg). Genes encoding the virulence determinants, namely, TSST (test), enterotoxins (sea and seb), and cytotoxin (pvl), were looked for by PCR. **Results:** Only 15.09% of isolates were identified as *Staphylococcus aureus*, and all of them (n=8) were scored as MRSA using the cefoxitin disc diffusion method. None of the tested isolates showed the presence of virulence determinants in standard molecular techniques. **Conclusion:** Lower prevalence of S. *aureus*, MRSA, coupled with the absence of virulence determinants in the strains, suggests strategies for better surveillance. Tracking virulence-causing genes in nasal carrier S. *aureus* strains could enhance efforts to prevent infection outbreaks.

[1]*Bharath Institute of Higher Education & Research, Chennai, India*
[2]*Department of Microbiology, Rajas Dental College & Hospital, Kavalkinaru, Tirunelveli, India*
[3]*Department of Microbiology, Research Laboratory for Oral and Systemic Health, Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education & Research, Chennai, India*
[4]*Department of Oral Pathology and Microbiology, Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education & Research, Chennai, India*
[5]*Department of Anatomy, Sree Balaji Dental College & Hospital, Bharath Institute of Higher Education & Research, Chennai, India*

For Correspondence: **padmabakianath@gmail.com**

INTRODUCTION

Staphylococcus aureus, a typical microbiota of humans, can occasionally act as an opportunistic pathogen causing less severe cases of infections of the skin and mucous membranes, can affect the respiratory system and cause pneumonia, and can also cause fatal infections like endocarditis and osteomyelitis [1,2]. *S. aureus, a* pathogen of global priority is known to colonize almost many parts of the human body such as the skin and vagina, the anterior nares seem to be the most frequent ecological niche [1,3]. Although several protensive researches revealed that around 50% of the population harbor *S. aureus* in their anterior nares, studies conducted in recent years made it clear that carrying this bacteria in the nasal cavity leads to the emergence of Community-acquired and Hospital-acquired Staphylococcal infections [4-8]. Healthcare workers bearing S.*aureus* in their anterior nares plays a key role in the epidemiology of the infections. Determination of the potential with which *Staphylococcus aureus* acquires resistant genes is the key part of the process of framing infection control protocols. The emergence of Methicillin resistant *S. aureus* (MRSA) strains that carry *mecA* gene and are also found to exhibit concomitant resistance to broad-spectrum beta-lactam antibiotics [6,8].

The global emergence of MRSA strains can be attributed to the heightened utilization of methicillin (a semi-synthetic penicillin introduced in 1959) to address beta-lactamase producing penicillin-resistant strains of S. *aureus* [9]. Initiation of MRSA infection was predisposed by factors such as admission to intensive care units(ICUs), recent hospitalization, extended antibacterial medications, and surgery. Till date only minimal statistical information concerning the prevalence of nasal carriage of MRSA among the HCWs and its subsequent virulence potential among the community is available in the literature [11,12]. An accelerated virulence potential exhibited by *S.aureus* is primarily due to the expression of virulence factors. *S. aureus* possesses various virulence factors, including hemolysins, Panton-Valentine leukocidin (PVL), exfoliative toxins (ETs), toxic shock syndrome toxin 1 (TSST-1), and staphylococcal enterotoxins (SEs) [6,13]. The release of PVL is responsible for severe infections such as necrotizing pneumonia, diffused cellulitis, and systemic conditions like osteomyelitis [6,14]. Furthermore, toxins such as TSST-1 and staphylococcal enterotoxins are part of the superantigen (Sag) family. TSST-1 can lead to severe conditions like toxic shock syndrome, while staphylococcal enterotoxins cause staphylococcal food poisoning. Additionally, exfoliative toxins (ETs) are responsible for causing staphylococcal scalded skin syndrome. Nevertheless, the results of previous studies have stated the presence of about 20 divergent antigens in S.*aureus* and also estimated that nearly 80% of the strains carry a minimum of single virulence determinant [6, 15].

The ability of *Staphylococcus aureus* to cause a horde of fatal infections is most likely due to the secretion of wide range of virulence determinants such as enterotoxins, cellular adhesion proteins, staphylococcal superantigens such as proteins involved in bypassing immune response, and presence of various other virulence factors [1,10,16,17]. The possible solution to detect the transmission potential of S.*aureus* strains in the healthcare system depends upon understanding its key virulence determinants. To strengthen infection control policies, investigate suspected outbreaks, and prevent nosocomial transmission, the application of genomic analysis techniques, including molecular typing methods for S. *aureus* isolates, is crucial [3,18,19]. The study aimed to assess the nasal carriage frequency of methicillin-resistant *Staphylococcus aureus*, and to investigate the expression of key virulence determinants, including the toxic shock syndrome toxin gene (*tst*), enterotoxin genes (*sea* and *seb*), and cytotoxin-encoding gene (*pvl*) in S. *aureus* carrier isolates.

MATERIALS AND METHODS

This Cross-sectional study was conducted between October – March, 2021. The study protocol was examined and authorized by the Institutes Ethics Committee, Sree Balaji Dental College & Hospital, Chennai, India (Ref No: SBDCH/IEC/06/2021/1). Forty-two students (male n=18, female n=24) pursuing dental under-graduation or post-graduation at a private dental College Hospital in Chennai, India, were included as the study participants. The following criteria were used for the selection of the study participants. Inclusion criteria: Participants of both genders, diabetic and non-diabetic subjects. Exclusion criteria: Participants with a recent history of a respiratory illness, recently had nasal surgery, subjects with skin and mucous membrane infections, and those under medication over the past two months. Sterile pre-moistened cotton swabs were used to collect nasal swabs from both anterior nares of the participants and then those aseptically collected swabs were processed using standard microbiology identification methods.

Preliminary analysis

MacConkey agar (Partially selective and differential medium) was inoculated with the aseptically collected nasal swabs and incubated aerobically at 37°C for 24hrs. After incubation, small, pink colored colonies grown on MacConkey agar suggestive of *Staphylococcus* species were sub-cultured on Mannitol salt agar. A total of 52 staphylococci were isolated from the samples. Pink colored colonies on MacConkey agar were scored as *Staphylococcus aureus* while yellow-colored colonies were scored as Coagulase-negative *Staphylococcus* species [Fig 1]. The *S. aureus* isolates were subjected to biochemical tests, O-F glucose fermentation and production of enzymes, catalase, and oxidase, coagulase tests (both slide and tube).

Determination of antibiotic resistance pattern

The Kirby Bauer disc diffusion method, following CLSI guidelines (2020), was performed to determine the susceptibility to antibiotics such as levofloxacin, ciprofloxacin, cotrimoxazole, erythromycin, clindamycin, tetracycline, tigecycline, linezolid, teicoplanin, and gentamicin. High-Level Mupirocin disc was used to assess mupirocin resistance [Fig 2], while the agar screening method was adopted to determine vancomycin (6µg/mL) resistance. Cefoxitin disc was a surrogate marker for detecting methicillin resistance, while inducible clindamycin resistance was assessed with the D-test. A standard control strain, *S. aureus* ATCC 25923 was included [19].

DNA extraction

Briefly, 2-3 bacterial colonies were suspended in lysis solution containing 50 µL lysostaphin (150 mg/ml), 50 µL lysozyme (10 mg/ml)and 10 µL RNase (10 mg/ml)(Sigma-Aldrich Chemical Co., St. Louis, MO). The tubes were incubated for 40 min at 37^0C. Subsequently, 150 µL of tris buffer (0.1 M/pH 8.0) and 50 µL of proteinase K (20 mg/ml) was added and was heated at 60°C for 10 minutes in a water bath, followed by re-incubation at 95°C for an additional 10 minutes. The microfuge tubes were briefly centrifuged for 30sec at 10,000 g and the supernatant was kept frozen at -80°C until PCR [20,21].

Detection of virulence determinants

The infection potential of *Staphylococcus aureus* isolates to initiate, and promote transmission was analyzed by screening for the existence of virulence-associated genes encrypting the staphylococcal superantigens like the toxic shock syndrome toxin gene (*tst*), the staphylococcal enterotoxin genes (*sea* and *seb*) and the cytotoxin gene(*pvl*) by PCR using previously described primers [21]. Known clinical isolates of *S. aureus viz.*, IGB_SA_362, IGB_SA_365, IGB_SA_367, IGB_SA_124 were included as positive controls for virulence genes, *tst, sea, seb* and *pvl* respectively.

Statistical analysis:

Fisher's exact test (Two tailed) was adopted to assess the statistical significance using GraphPad software. P value was set at 0.05 to assess significance.

RESULT

A total of 52 Staphylococci were isolated from the swabs collected from 42 study participants. Of the 52 Staphylococcal isolates, only 8(15.4%) were confirmed as *S. aureus*, and all the *S. aureus*(n=8) were scored as MRSA using the conventional cefoxitin Discs. All the MRSA isolates (100%) were found be susceptible to the glycopeptides, (vancomycin and teicoplanin) while only 25% of the MRSA isolates were susceptible to the fluoroquinolones (ciprofloxacin, levofloxacin) (Fig 1).

Statistical analysis of the susceptibility rates between the antibiotic classes revealed a significant difference between glycopeptide susceptibility (100%) Vs fluoroquinolone susceptibility (25%) (p=0.007) while, no statistical significance was observed between the susceptibility rates of clindamycin (100%) *Vs* erythromycin (62.5%) (p=0.2) and vancomycin/ teicoplanin (100%) *Vs* linezolid (87.5%) (p=1.0). Its noteworthy that, none of the MRSA isolates that were screened for virulence determinants (*tst, sea, seb*, and *pvl*), were found to harbor these virulence genes. [Fig 3,4]. Nevertheless, only the known positive control sample showed a significant amplification of the virulence determinants.

DISCUSSION

Staphylococcus aureus can exist as part of the normal flora on the skin and mucous membranes, yet it also has the potential to cause severe and invasive infections, including pneumonia, bacteremia, and toxic shock syndrome [6]. Colonization with S. *aureus*, primarily in the anterior nares, the bacterium's most common ecological habitat, can be a significant predisposing factor for various systemic diseases, posing a potential threat to the human population [3]. Nasal colonisation has been linked to various infections, including hospital and community-acquired Staphylococcal infections [7,8].

Figure 1: Colony morphology of *S. aureus* (yellow colonies) and Coagulase negative staphylococci (pink colonies) on MSA plate

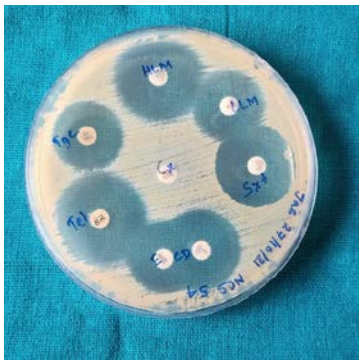

Figure 2: AST plate showing Cefoxitin resistance and susceptibility to High-level mupirocin.

Staphylococcus aureus expresses various cell-associated and additional virulence determinants, including hemolysins, Panton-Valentine leukocidin (PVL), exfoliative toxins (ETs), toxic shock syndrome toxin 1 (TSST-1), and staphylococcal enterotoxins (SEs) that elevates destructive cellular mechanisms such as attachment to the cell structures, cellular invasion, bacterial proliferation and a poor immune status directly enhancing disease progression [13].A continuous hike in the occurrence of MRSA induced infections led to an urgent need for the invention of newer, faster, and dependable identification systems and typing methodologies. [22]. This study investigated *Staphylococcus aureus* isolates from nasal carriers among healthcare professionals by determining their antibiotic resistance patterns [Graph 1], and the presence of virulence genes such as *tst*, *sea*, *seb* and *pvl* among the tested pathogens.

Fifteen percent of the study participants screened in the present study were found to be nasal carriers of *S.aureus*. The prevalence rate is different to those reported by other studies from different geographical locations, namely Iraq - 32.21% [23], China-24.7% [3], Turkey-17.3% [6], Iran-20.8% [1] and Columbia-38.5% [8]. Also, the incidence of MRSA isolates was reported as 15.4% in our study which is comparatively higher than that reported by Chen *et al*., 2017 (0.3%) [3], Dagi *et al*., 2015 (2.9%) [6], and Mustafa *et al*., 2023[22] & Perez *et al*., 2011[8] (4.8%). Nevertheless, a relatively higher incidence (25.8%) of MRSA had been reported by Nhan *et al*., 2011 [13]. Table 1 represents a comparative analysis of the antibiotic susceptibility patterns of the *S. aureus* isolates investigated in the relative studies.

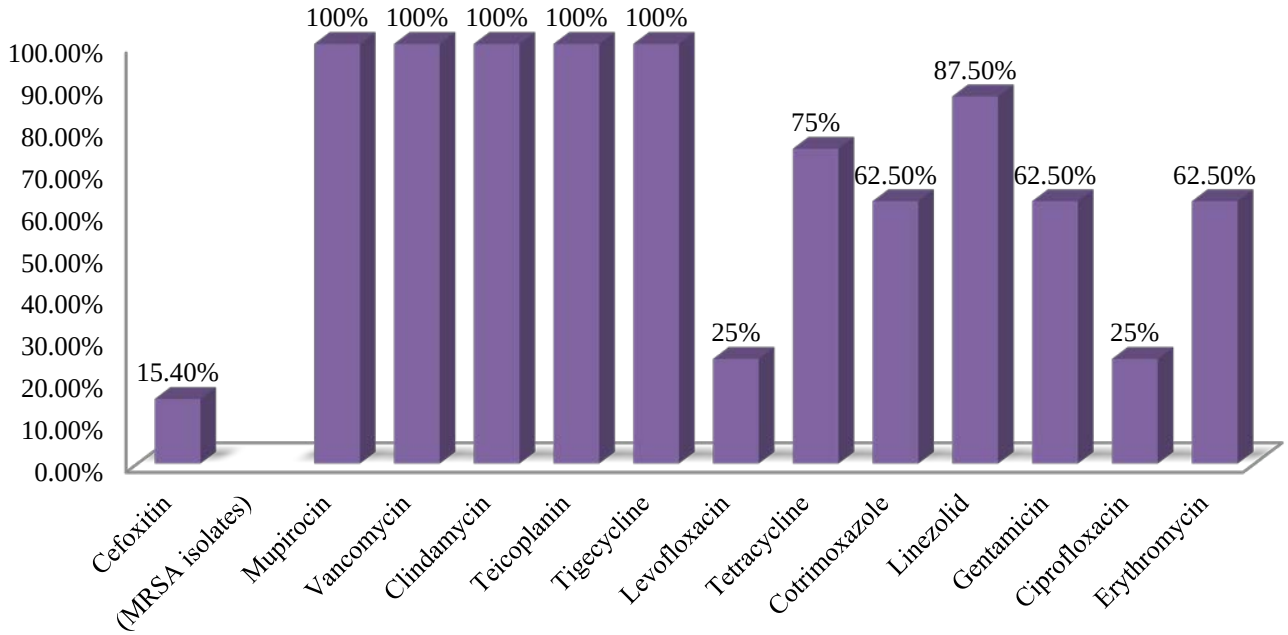

Figure 1: Antibiotic susceptibility profile of the *S. aureus* isolates

Table 2: Comparative analysis of AST patterns

Antibiotics	**This Study**	**Chen *et al*., 2017**	**Dagi *et al*., 2015**	**Mustafa *et al*., 2023**
Cefoxitin (30 µg) (Resistant - MRSA)	15.4%	1.4%	2.9%	31(48%)
Mupirocin (200 µg)	100% S	NP	96% S	84% S
Vancomycin (6 µg)	100% S	100% S	100% S	88% S
Clindamycin (2 µg)	100% S	86.3% S	3.12% S	48% S
Teicoplanin (30 µg)	100% S	100% S	NP	88% S
Tigecycline (15 µg)	100% S	NP	NP	100% S
Levofloxacin (5 µg)	25% S	100% S	98% S	NP
Tetracycline (30 µg)	75% S	82.2% S	96% S	52% S
Cotrimoxazole (25 µg)	62.5% S	< 10% R	NP	NP
Linezolid (30 µg)	87.5% S	-	100% S	92% S
Gentamicin (30 µg)	62.5% S	< 10% R	98% S	88% S
Ciprofloxacin (5 µg)	25% S	4.7% R	98% S	60% S
Erythromycin (15 µg)	62.5% S	56.2 %	88% S	68% S

NP- Not performed, R- Resistant, S- Susceptible.

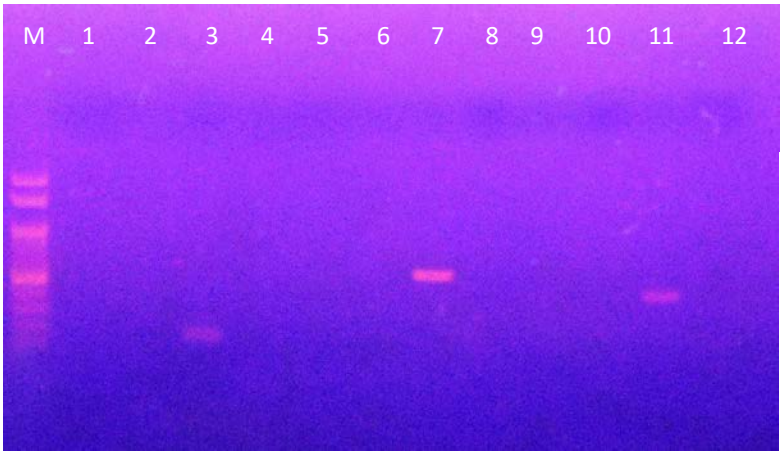

Figure 3: Gel picture of PCR for virulence genes Lanes: M: 100bp ladder, 1,2: *tst* negative, 3: *tst* Known positive control, 4: *tst* Known negative control, 5,6: *sea* negative, 7: *sea* Known positive control, 8: *sea* Known negative control, 9,10: *seb* negative, 11: *seb* Known positive control, 12: *seb* Known negative control.

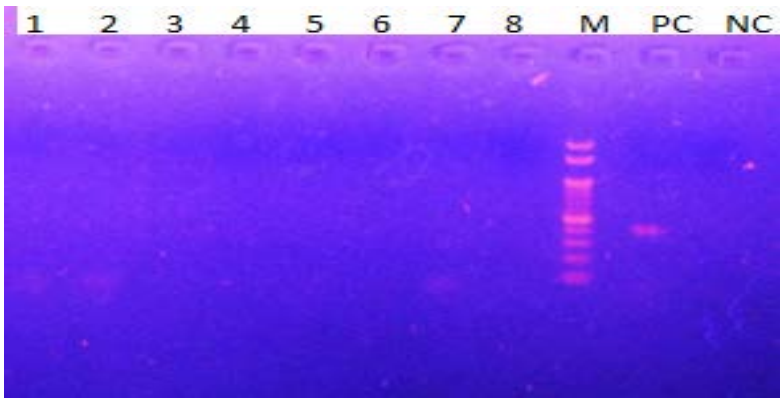

Fig 4: Gel picture of PCR for ***pvl* gene:**
Lanes 1,2,3,4,5,6,7,8: pvl negative, M: 100bp DNA ladder, PC:pvl known positive control, NC: pvl known negative control

Comparing the antibiotic susceptibility patterns of the current investigation with data from other researchers reveals that the strains have gradually developed resistance to commonly used antibiotics over time [3,6,22] however the majority of the Staphylococcal isolates examined in this study were susceptible to most of the tested antimicrobials. Previous reports have documented that, staphylococcal enterotoxin encoding genes, *sea* followed by *seb* and toxic shock syndrome toxin encoded by *tst* are the most common superantigens harbored by the nasal carrier isolates [23-25]. On the contrary, none of our study isolates were found to harbor super antigenic determinants, *tst*, *sea*, seb and *pvl*.

CONCLUSION

MRSA nasal carriers among the healthcare professionals are a cause of concern due to their ability to actively transmit the infection within the hospital and among community. The overall prevalence of S. *aureus* in the nasal cavity of dental students was recorded as 15.4% in the present study. All the MRSA isolates demonstrated susceptibility to mupirocin, vancomycin, clindamycin, teicoplanin, and tigecycline. Clindamycin susceptibility exhibited by the MRSA isolates in this study is of clinical relevance as clindamycin is documented to disrupt bacterial protein synthesis, exerts increased intracellular levels in phagocytic cells, enhances opsonization, upsurges intracellular killing, decreases bacterial adhesion to host cells and also decreases/ reduces exotoxin secretion by Staphylococci. Notably, clindamycin is known to exhibit prolonged post-antibiotic effect which may be attributed to persistence of the drug at the ribosomal binding site. Mupirocin remains the preferred decolonizing agent for carriers. None of the study isolates carried super-antigenic determinants *tst*, *sea*, *seb*, and *pvl*.

Significant findings of the study

1. In this study, 15.09% of the dental students were found to be nasal carriers of *S.aureus*.
2. None of the methicillin resistant Staphylococcus aureus isolate exhibited High-level Mupirocin (HLM) resistance.
3. Interestingly, none of the MRSA isolates expressed virulence determinants.

Importance of the study

The asymptomatic nasal carriage of MRSA strains presents a global threat, facilitating the transmission of both hospital-acquired and community infections. When coupled with the expression of pathogenic toxin-producing genes by these isolates, the intensity of infection transmission is exacerbated. Therefore, obtaining crucial data on the antibiotic resistance pattern and the expression of virulence determinants by MRSA isolates is essential for enhanced management and control of MRSA carriage among healthcare personnel.

Limitation of the study

Only a smaller number of isolates from a single, specific healthcare settings were investigated in the study, which may introduce selection bias. Thus, a larger population from varied geographical locations and exposures could be investigated in future studies to draw final conclusion.

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Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Kesavaram Padmavathy contributed in conceptualizing, data curating, statistical analysis, reviewing and editing the manuscript. Jebadass JasmineVinshia performed experimental work, collected data and performed statistical analysis, textual interpretation and drafting of the final manuscript. Jimson Sudha contributed in investigation and supervision of whole study. Baskaran Sathyapriya contributed in accessing resources and reviewing the manuscript. All authors read and approved the manuscript.

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