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Research Article

ASSOCIATION OF VIRULENCE FACTOR (PANTON–VALENTINE LEUKOCIDIN) WITH *MECA* GENE IN *STAPHYLOCOCCUS AUREUS* ISOLATES IN TERTIARY CARE HOSPITAL

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ABSTRACT

Introduction: The pathogenicity of *Staphylococcus aureus* depends on various bacterial surface components and extracellular proteins. *S. aureus* expresses a variety of virulence factors, including Panton–Valentine leukocidin (pvl). pvl is a cytotoxin produced by *S. aureus* that causes leukocyte destruction and tissue necrosis. Despite the presumed importance of *pvl* as a virulence factor, few data are available on its prevalence among *S. aureus* isolates in our area.

Objectives: This study was aimed to determine the association between *mecA* gene and virulence genes such as *pvl* gene in strains of *S. aureus* and to determine the prevalence of the *pvl* gene in *S. aureus* isolates using the polymerase chain reaction (PCR) technique.

Methods: A total of 200 non-repeated, confirmed clinical isolates of *S. aureus* were used from various departments. Cefoxitin (30 ug) disc diffusion method was used as phenotypic method for detection of methicillin-resistant *S. aureus* (MRSA). We used PCR amplification to test for the *pvl* and *mecA* gene in *S. aureus* isolates.

Results: Of 200 strains of *S. aureus* isolated in our hospital, 60 (30%) were identified as MRSA based on cefoxitin disc diffusion method. These same 30 isolates were confirmed for *mecA* gene by PCR. All strains had *mecA* gene. All *mecA* positive strains of *S. aureus* were tested for pvl gene. Of 200 *S. aureus*, 123 (61.5%) strains were pvl positive. Among which 33 (55%) were pvl positive MRSA and 90 (64.28%) pvl positive methicillin-susceptible *S. aureus* (MSSA) strains.

Conclusion: The prevalence of the *pvl* among the MRSA isolates was found relatively higher in number among pus samples which indicate a possible key role of *pvl* in pathogenesis of pyogenic infections, especially skin and soft tissue infections in community setting.

Keywords: Panton–Valentine leukocidin, Cefoxitin disc diffusion method, *mecA, Staphylococcus aureus,* Methicillin-resistant *S. aureus,* methicillin-susceptible *S. aureus.*

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INTRODUCTION

Staphylococcus aureus is the most virulent *Staphylococcus* species. *S. aureus* can cause variety of infections, ranging from minor skin infections to post-operative wound infections. One major obstacle for the treatment of *S. aureus* infections is the development of antibiotic resistance in the isolates. This resistance phenomenon originated with penicillin, the first broad-spectrum antibiotic, which was discovered in the 1940s. Its adaptive power to antibiotics has resulted in the emergence of methicillin-resistant *S. aureus* (MRSA) in the beginning of the 1960s. Methicillin resistance is mediated by an acquired penicillin-binding protein, PBP2a, a peptidoglycan transpeptidase encoded by the *mecA* gene that has low affinity for beta-lactams. Thus, when the four native peptidoglycan synthetases (penicillin-binding proteins 1, 2, 3, and 4) are bound and inactivated by beta-lactams, PBP2a can still affect cell wall synthesis. *mecA* is harbored on the *Staphylococcal* chromosomal cassette mec (SCCmec), a genetic element that integrates site-specifically into the *S. aureus* chromosome [1].

MRSA strains are particularly serious and potentially lethal pathogens that possess virulence mechanisms including toxins, adhesions, enzymes, and immunomodulators [2]. Virulence genes play very important roles in bacterial pathogenesis [3] and *S. aureus* could not be an exception to this. It was in view of this that some of the virulence genes such as Panton–Valentine leukocidin (*pvl*), exfoliating toxin A, and toxic shock syndrome were screened for with the hope one of the virulence genes could be incriminated in *S. aureus* infections.

Initially, MRSA infections were observed in hospitalized patients and those with chronic illnesses. These types of infections are caused by strain of *S. aureus* labeled as hospital-acquired MRSA (HA-MRSA). In 1990s, another type of MRSA strain was emerged that primarily causes skin and soft tissue infections in healthy people. It is called community-acquired MRSA (CA-MRSA) [4].

Molecular characterization of SCC*mec* types of MRSA is very essential for studying the epidemiology of MRSA. This problem is further confounded by the recent spread of CA-MRSA and the identification of vancomycin-resistant MRSA. In addition to the limited treatment options, *S. aureus* strains acquire and express numerous virulence determinants that continue to increase its ability to cause a wide spectrum of human disease [5,6].

pvl is a pore-forming toxin secreted by some *S. aureus* strains which destroy leukocytes by creating pores in the mitochondrial membrane and associated with skin and soft tissue infections. *pvl* is a virulence factor associated with severe MRSA infections for which routine detection is time-consuming and dependent on the culture environment [7].

Moreover, the *pvl* is encoded by a bi-cistronic operon known as *lukF-PV/ lukS-PV* gene. Epidemiologic and clinical data [8,9] provide compelling evidence that the high virulence potential of CA-MRSA is associated with the genes *lukS-PV and lukF-PV* encoding the subunits of the *pvl* and has also been used as an additional marker alongside SCC*mec* IV and V to classify MRSA into CA-MRSA, though not all CA-MRSA carry the *pvl* genes.

Molecular typing techniques have been used with increasing frequency in studies of the epidemiology of MRSA and also for rapid detection of MRSA clones and virulent genes also [10-12].

Community-acquired and hospital-acquired MRSA both of these contain *mecA* gene. As the *mec A* gene is essential for acquiring resistance of MRSA strains, the *pvl* gene is an additional factor which is seen in community-acquired strains [13]. Identification of *mecA* positive strains can be used as a guide for separating infected patients from others in hospital environment to prevent gene transfer among clinical strains and also the distribution of virulent factors.

Having knowledge about the prevalence of MRSA and their virulence factors is useful for treatment and control of community- and hospital-acquired *S. aureus* infections.

Aim and objectives

To determine the association between *mecA* gene and *pvl* gene in strains of *S. aureus* isolated from hospitalized patients for grouping the MRSA isolates into community- and hospital-acquired MRSA genetically and to determine the prevalence of the *pvl* gene in *S. aureus* isolates using the polymerase chain reaction (PCR) technique.

METHODS

This study was the prospective type of study, carried out at Bharati Vidyapeeth Medical and Dental college, Sangli, Krishna Institute of Medical Science, Karad and Regional medical research center, ICMR, Regional center, Belgaum.

Inclusion criteria

A total of 200 non-repeated clinical isolates of *S. aureus* as per statistical calculation from various clinical specimens such as blood, pus, surgical site, wounds, sputum, tracheal aspirates, and urine were included in this study. Isolates were identified as *S. aureus* based on morphology, colony characteristics, and biochemical reactions as per the standard protocol. All clinical isolates of *S. aureus* confirmed by phenotypic tests as per Clinical Laboratory Standard Institute (CLSI) guideline [14,15]. Antibiotic sensitivity testing of isolates of *S. aureus* to various antimicrobial discs was carried out using Kirby-Bauer disc diffusion method. All antimicrobial discs were obtained from Hi-Media Laboratories Pvt. Ltd. Mumbai, India.

Detection of MRSA

Phenotypic method: Cefoxitin (30 ug) disc diffusion method

All testing was done according to the CLSI as well as the manufacturer's recommendations. All confirmed *S. aureus* strains were tested for methicillin resistance by the Kirby-Bauer disc diffusion method using cefoxitin disc (30 µg) diffusion method. Zone diameters ≤19 mm were reported as methicillin-resistant and ≥22 mm was considered as methicillin sensitive [16].

Genotypic identification of MRSA (*MecA*-based PCR)

PCR for the detection of *mecA* gene is done at molecular laboratory of ICMR Regional center, Belgaum, by following method of Unal *et al.* [17]. Primer sequences used for *mecA* detection were as follows:

- 5'- AAT CTT TGT CGG TAC ACG ATATTC TTC ACG -3'
- 5'-CGT AATGAG ATT TCA GTA GAT AAT ACA ACA -3' (Amplicon size 310bp).

Bacterial DNA was extracted from overnight cultures of *S. aureus* by CTAB- NaCl method [18].

The quality and quantity of isolated DNA was determined using Nanodrop 1000 spectrophotometer (JH Biosciences, USA. Model: ND1000) at 260/280 nm as well as visually by horizontal gel electrophoresis in 1% agarose. Briefly, 1 µl of 60 ng of the extracted DNA was added to 24 µl of PCR amplification mix consisting of 16 µl of double-distilled autoclaved water, 2.5 µl of ×10 Taq buffer, 1 µl of 2.5 mM dNTP mix (Merck, India), 0.5 µl of 3U/µl Taq polymerase (Merck, India), and 0.5 mM of each primer. The *mecA* gene was amplified using the primers (Sigma, India) as described by Jonas *et al*., 1999 [19]. Amplifications were carried out in a thermal cycler (iCycler, BioRad Inc., USA) with conditions that consisted of 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 2 min. Amplicons of 310 bp were consistent with *mecA* gene amplification. The PCR products were subjected to agarose gel electrophoresis using gel red dye and images were acquired using Alpha Imager gel documentation system (JH biosciences, USA. Model: D E 400).

S. aureus ATCC 25923 (*mecA* negative) and ATCC 43300 (*mecA* positive) were used as controls for all phenotypic tests and genotypic test. The sensitivity and specificity of each test were calculated using the PCR results as a gold standard test.

Detection of *pvl* genes

PCR was performed on all the isolates to detect *pvl* gene. PCR for the detection of *pvl* gene was carried out using Cabrera *et al*. method [20]. Primer sequences used for *pvl* detection were as follows:

- luk-PV-1, 5'- ATC ATT AGG TAA AAT GTC TGG ACA TGA-3'
- luk-PV-2, 5' - GCA TCA AGT GTA TTG GAT AGC AAA AGC-3' (Amplicon size 433bp).

Each primer had a concentration of 1 µM while the *Taq* mix made up of the following: 10 mM of MgCl2, 0.2 mM of dNTP mix, and 1 U of *Taq* polymerase (NEB, USA). For detection of *PVL* gene, amplification was carried out with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for 35 cycles of amplification. The expected product size was 433 bp.

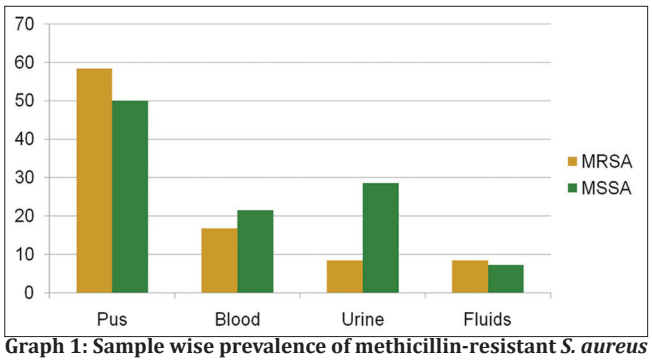
RESULTS

A total of 200 isolates of *S. aureus* from various clinical specimens were subjected to MRSA screening as well as to check their antibiotic sensitivity pattern. Maximum (61%) isolates were from pus sample and least from urine (5.4%). The prevalence of MRSA was different among various clinical specimens. Frequency of isolating MRSA was maximum from wound swab and pus (56.60%) specimens, followed by blood, fluid, and urine specimens (Graph 1).

Among all clinical samples, pus from wound infections was in highest number from which *pvl* positive MRSA was isolated.

In this study, out of 60 MRSA, 45 strains were CA-MRSA and 15 strains were HA-MRSA. Of 140 methicillin-susceptible *S. aureus* (MSSA) strains, 100 strains were CA-MSSA and 40 strains were HA-MSSA (Table 1).

Of 200 strains of *S. aureus* isolated in our hospital, 60 (30%) were identified as MRSA based on cefoxitin disc diffusion method. These same isolates were confirmed for *mecA* gene by PCR. All strains had *mecA* gene. All strains were tested for *pvl* gene. Of which total 123 (61.5%) strains were *pvl* positive. Among which 33 (55%) strains had both *pvl* and *mecA* gene and 90 (64.28%) strains had only *pvl* but not *mecA* gene (Table 2).



Graph 1: Sample wise prevalence of methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus*

Table 1: Prevalence of community-associated and hospital-associated strains of *S. aureus*

Type of strain	Community-associated strains	Hospital-associated strains	Total
MRSA (*mecA*+ve)	45	15	60
MSSA (*mecA* -ve)	100	40	140
Total	145	55	200

MRSA: Methicillin-resistant *S. aureus*, MSSA: Methicillin-susceptible *S. aureus*, *S. aureus: Staphylococcus aureus*

Table 2: The percentage of *pvl* gene among MRSA and MSSA isolates

Type of strains	*pvl*+ve	*pvl* –ve	Total	p value
MRSA (*mecA*+ve)	33 (55)	27 (45)	60	p>0.5
MSSA (*mecA* -ve)	90 (64.28)	50 (35.71)	140	p<0.001
Total	123 (61.5)	77 (38.5)	200	p<0.005

MRSA: Methicillin-resistant *S. aureus*, MSSA: Methicillin-susceptible *S. aureus*, *S. aureus: Staphylococcus aureus*

DISCUSSION

According to the results of this study, 30% of isolates identified as MRSA which were positive for *mecA* gene. Indian network for surveillance of antimicrobial resistance (INSAR) group, a multihospital-based study in various parts of India shown that the overall MRSA prevalence in India was 42% in 2008 and 40% in 2009 [21].

Our study correlates with Indian study by Mehta *et al*. reported 31.8%, Vidya *et al*. reported 29.1%, and Kumari *et al*. from Nepal reported 26.14% prevalence of MRSA in their study [22-24]. Our MRSA prevalence is less than study by Dr. S. Kulakarni *et al*. reported higher incidence of 70.3% [25].

The prevalence of MRSA was different among various clinical specimens. Frequency of isolating MRSA was maximum from wound swab and pus (56.60%) specimens, followed by blood, fluid, and urine specimens. Our result correlates with study of Hossein *et al*. [26].

The occurrence of *pvl* gene in previous studies has been reported from 2% to 35% [27,28]. The *pvl* positive strains lead to infections with different clinical appearance even in immunocompramised patients lead to necrotic pneumonia, which its mortality can be as much as 75% [29]. Therefore, frequent monitoring of this pathogen, its antibiotic susceptibility and determining their virulence factors are of great importance in control and treatment of infections.

pvl was considered as important marker for differentiation of HA-MRSA and CA-MRSA. In this study, out of 145 community-associate *S. aureus* isolates, 108 strains had *pvl*. However, some studies have shown positive association of *pvl* genes among HA-MRSA isolates. *pvl* is more commonly seen in CA-MRSA as compared to HA-MRSA.

A study conducted by Okon *et al*., there was no colocalization of *pvl* and *mecA* genes on the genome of all the *S. aureus* strains examined, i.e., it was only possible to have *pvl* or *mecA* genes but not both genes on the chromosome of the *S. aureus* isolates [30].

Reports from various countries show the increasing prevalence of *pvl* among MRSA isolates. Kaur *et al*. from India, have reported overall 62.85% of *pvl* prevalence among MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%) which indicates a higher prevalence among MRSA than our findings [31]. A similar study by D'Souza *et al*. from Mumbai, India, reported prevalence of 64% *pvl* positive isolates among MRSA [32].

A number of investigations have provided evidence that the prevalence of the *pvl* gene is high among MSSA [33].

Our findings suggested that though the prevalence of the *pvl* toxin varied in different geographical regions, in our region, the MRSA strains may be important reservoirs of the *pvl* toxin, which was now being slowly acquired by the MSSA strains.

CONCLUSION

We did not find any significant association of *pvl* gene with *mecA* gene. *pvl* was more associated with community-associated strains of MRSA and MSSA. The prevalence of the *pvl* among the MRSA isolates was found relatively higher in number among pus samples which indicate a possible key role of *pvl* in pathogenesis of pyogenic infections, especially skin and soft tissue infections in community setting. We can be concluded that the presence of *pvl* can be used as a reliable marker for CA-MRSA.

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