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ORIGINAL ARTICLE

Status of Methicillin Resistant *Staphylococcus aureus* Infections and Evaluation of PVL Producing Strains in Belgaum, South India

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Abstract:

Background: Panton Valentine Leukocidin (PVL) toxin, responsible for increased virulence and more severe infections can be found in both Methicillin-sensitive and Methicillin-resistant strains of *Staphylococcus aureus* (MSSA and MRSA). *Aims & Objectives:* To generate baseline data on the extent of MRSA infections and to estimate the frequency of PVL-positive *S.aureus* in Belgaum, South India. *Material & Methods:* 70 clinical isolates of *S.aureus* were obtained from various laboratories in Belgaum city. These isolates were identified, phenotypically characterized as MRSA/MSSA by disc diffusion method using oxacillin discs (1 µg) and genetically by multiplex PCR for *mecA* and *fem B* genes. PCR was subsequently carried out on all isolates to detect *LukS-PV* and *LukF-PV* genes, the markers for potential producers of PVL toxin. *Results:* 27 out of 70 isolates (38.6%) were confirmed as MRSA by PCR for *mecA*. The prevalence of PVL gene was 85.1% and 48.8% in MRSA and MSSA respectively. The overall prevalence of PVL positive *S.aureus* was 62.85%. *Conclusion:* Our study showed high percentage of PVL positive MRSA and MSSA, higher than the most reports worldwide. In the backdrop of bacterial strains gaining multiple drug resistance, our study warrants further epidemiological studies in hospitals and community levels in the region.

Key Words: MRSA, PVL, PCR.

Introduction:

Staphylococcus aureus, frequently a member of the normal skin flora and nasal cavity often causes abscesses, infections of wound, skin, soft tissue, osteomyelitis, endocarditis, pneumonia etc. It may also cause staphylococcal scalded skin syndrome, a severe disease in infants or the toxic shock syndrome.

Methicillin resistant *S. aureus* (MRSA) is one of a member of greatly feared strains of *S. aureus* which have become resistant to many antibiotics. MRSA strains have been associated with nosocomial or hospital acquired infections (HA-MRSA infections) world over and have emerged as an important cause of community acquired infections (CA-MRSA) as well [1]. Resistance to methicillin is mostly determined by the presence of *mecA* gene encoding altered penicillin binding protein which shows low affinity to β-lactam antibiotics [2].

The pathogenicity of *S. aureus* infections is related to various bacterial surface components (e.g. capsular polysaccharide and protein A),

including those recognizing adhesive matrix molecules (e.g. clumping factor and fibronectin binding protein) and to extracellular enzymes and toxins (e.g. coagulase, staphylokinase, hyaluronidase haemolysins, enterotoxins, toxic shock syndrome toxin, exfoliatins and Panton Valentine Leukocidin or PVL etc.) [3].

PVL is one of the most important and extensively investigated proteins that belongs to the recently described family of synergohymenotropic toxins [4]. These toxins damage membranes of host defense cells by synergistic action of two non-associated classes of secretory proteins designated as Luk-S and Luk-F, which are encoded by two contiguous and co-transcribed genes *viz.*, *LukF-PV* and *LukS-PV* [5] of bacteriophages inserted in the bacterial chromosome. PVL is leukotoxic by pore induction for human polymorphonuclear cells and macrophages [6]. Epidemiological and clinical data [7] provide compelling evidence that the high virulence potential of community acquired MRSA is associated with genes like *lukF-PV* and *lukS-PV* (PVL), but direct evidence that PVL plays a role in pathogenesis has been limited [8]. The presence of PVL in *S. aureus* appears to be associated with increased disease severity, ranging from cutaneous infection requiring surgical drainage to severe chronic osteomyelitis, and severe necrotizing pneumonia which could be fatal [9]. PVL production has been linked with furuncles, cutaneous abscess and severe necrotic skin infections in school children [10] and in certain communities [11]. The PVL toxin being an important virulence factor led us to investigate the frequency of PVL producing *S.aureus* in Belgaum in Northwest Karnataka, (India) which has several tertiary care hospitals & caters to a large number of patients from North Karnataka, Southern Maharashtra and Goa. Indoor as well as outdoor patients were included together in the study with the intention to cover cases of both community acquired as well as hospital acquired infections for obtaining a more generalized picture.

Material and Methods:

Samples:

A total of 70 isolates of *S. aureus* were obtained from microbiology laboratory of a tertiary care centre in Belgaum that receives samples from various diagnostic laboratories, primary and secondary care centres as well as outdoor and indoor patient departments (OPD and IPD) of attached hospitals.

Identification:

Samples identified as *S.aureus* by standard microbiological methods at microbiology departments of respective hospitals were collected and reconfirmed as *S.aureus* at Regional Medical Research Centre (ICMR), Belgaum by conventional biochemical tests using standard protocols followed by identification of a few representative strains by Vitek 2 Compact automated microbial identification system using Gram positive (GP) cards (Bio Merieux, USA).

MRSA and MSSA assays and antimicrobial sensitivity tests:

All isolates were subjected to phenotypic antimicrobial susceptibility tests by Kirby Bauer disc diffusion method using Oxacillin discs, 1µg (Hi-Media, India) following CLSI guidelines [14]. The zone of inhibition was

determined after 24 h of incubation at 35°C (susceptible ≥13 mm; intermediate 11-12 mm; and resistant ≤10 mm). Standard MRSA strain (ATCC43300) and MSSA strain (ATCC25923) were included in each batch. A few random strains were subjected to automated antibiotic sensitivity testing by Vitek 2 Compact system (Bio Merieux, USA) as well, for confirmation.

DNA extraction for PCR:

Bacterial DNA was extracted from overnight cultures of *S.aureus* by CTAB-NaCl method [15]. The quality and quantity of isolated DNA was determined using Nanodrop 1000 spectrophotometer (JH BioSciences, USA) at 260/280 nm as well as visually by horizontal gel electrophoresis in 1% agarose.

Multiplex PCR for detection of *mecA* and *fem B* genes:

Multiplex PCR for the detection of *mecA* and *femB* genes was carried out following Unal *et al*., 1995 [16]. 1µl of 60 ng of the extracted DNA was added to 24 µl of PCR amplification mix consisting of 16 µl of doubled distilled autoclaved water, 2.5 µl of 10X Taq buffer (Tris with 15 mM $MgCl_2$), 1µl of 2.5mM dNTP mix (Merck, India), 0.5 µl of 3U Taq polymerase (Merck, India), and 0.5mM of each primer. The details of primers (Sigma, India) used for the detection of *mecA* and *femB* gene are given in Table 1. Amplifications were carried out using thermal cycler (iCycler, BioRad Inc., USA) with PCR 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 72C for 2 min. 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).

PCR for detection *of luk-F (PV)* and *luk-S (PV)* genes:

1 µl containing 60 ng of extracted *S.aureus* DNA was added to 24 µl of PCR amplification mix consisting of 18 µl of doubled distilled autoclaved water, 2.5 µl of 10X Taq buffer (Tris with 15 mM $MgCl_2$), 1µl of 2.5 mM dNTP mix (Merck, India), 0.5 µl of 3U Taq polymerase (Merck, India) and 0.5 mM of each primer (Table 1). Amplifications were carried out using thermal cycler (iCycler, BioRad Inc.,

Table 1: Details of oligonucleotide primers used in PCR.

Primer	Target gene	Sequence (5'-3')	Amplicon size	Ref.
Mec A1	*mecA*	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	Jonas *et al.*
Mec A2		GTA GAA ATG ACT GAA CGT CCG ATA A		1999 [33]
Fem B1	*femB*	TTA CAG AGT TAA CTG TTA CC	651bp	Jonas *et al.*
Fem B2		ATA CAA ATC CAG CAC GCT CT		1999 [33]
Luk-PV-1	*Luk-S(PV)*	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	433bp	Lina *et al.*
Luk-PV-2	*& luk-F(PV)*	GCA TCA AGT GTA TTG GAT AGC AAA AGC		1999 [27]

USA) with PCR conditions that consisted of initial denaturation at 94°C for 99 min followed by 10 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s. and extension at 72°C for 90 s. followed by 25 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s and extension at 72°C for 90 s, with final extension at 72°C for 5 min. The PCR products were electrophoresed in 1% agarose gels and documented.

Results:

Out of the 70 isolates of *S. aureus*, 31 were found to be resistant to Oxacillin (1 µg) by Kirby Bauer disc diffusion method (Table 2) while 30 were sensitive and 9 were classified as having intermediate resistance (inhibition zone size between 11 to 12 mm) as per CLSI guidelines.

The *mecA* gene, considered as a genotypic marker for MRSA was detected by PCR in 27 isolates that included 20 Oxacillin (1 µg) resistant strains, 2 strains that showed intermediate resistance and 5 strains that were sensitive to Oxacillin (Table 2).

Table 2: Results of Oxacillin susceptibility test in disc diffusion and *mecA* gene PCR for differentiation of MRSA from MSSA (n=70).

		PCR (*mec*A)		Total
		+ ve	- ve	
Oxacillin (1 µg)	Resistant	20	11	31
	Intermediate	2	7	9
	Sensitive	5	25	30
	TOTAL	27	43	70

Among the 70 isolates, 19 (27.14 %) did not possess the *femB* marker out of which 6 (22.22%) were *mecA* positive (MRSA) and 13 (30.23%) were *mecA* negative (MSSA).

PCR for the PVL marker *lukS- lukF-PV* revealed that 23 out of 27 MRSA (*mecA* positive) isolates harbored this gene (85.1%) while it was 21 out of 43 (48.8%) in MSSA (*mecA* negative). Overall PVL positivity was 62.85 % (Table 3).

Table 3: PCR results for *LukPV* (PVL) positivity among MRSA and MSSA (n=70).

	MRSA *(mecA* +ve*)*			MSSA *(mecA* –ve*)*		
	femB +ve	*femB* –ve	PVL positivity	*femB* +ve	*femB* –ve	PVL positivity
LukPV (PVL) +ve	19	4	23/27 (85.1%)	19	2	21/43 (48.8%)
LukPV (PVL) –ve	2	2		11	11	
Total	21	6		30	13	

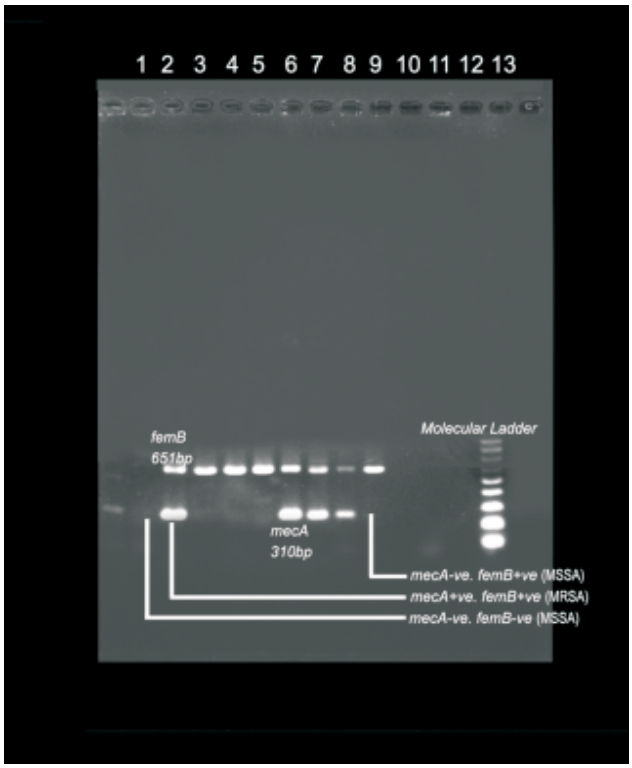

Fig. 1: Agarose gel showing amplification of *mecA* and *femB* in multiplex PCR. Lane1: MSSA (ATCC25923); Lane2: MRSA (ATCC43300); Lanes 3 to 12: Clinical isolates; Lane13: 100bp DNA ladder.

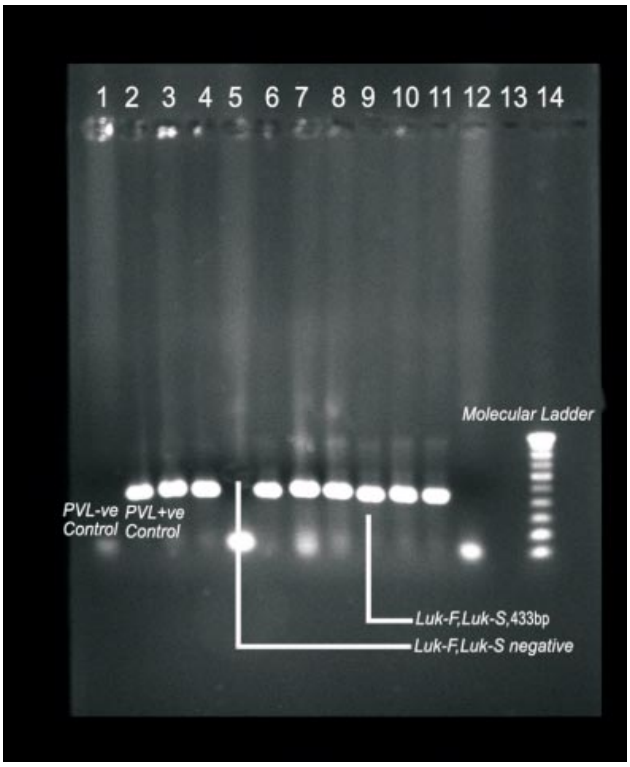

Figure 2: Agarose gel showing amplification of *LukF*-PV, l*ukS*-PV. Lane 1: PVL negative *S.aureus* (control); Lane 2: PVL positive *S.aureus* (reference strain). Lanes 3 to 13: Clinical isolates. Lane14: 100bp DNA ladder.

Discussion:

Infections due to *S.aureus* are very common and MRSA continues to be a serious and formidable challenge to health care providers as their prevalence is reported to be increasing exponentially [17]. In the past, MRSA infections were reported mostly from hospitalized patients but now they are encountered in community settings as well. Understanding the types of *S. aureus* infections, their pattern and distribution, as well as the factors associated with their spread are of paramount importance for its management and control.

Conventional phenotypic method of identification of MRSA by Oxacillin discs was compared with the PCR based molecular method based on detection of the presence of *mecA* gene, considered predominant molecular marker for MRSA, although there are other rarer non-*mecA* mediated mechanisms of developing Methicillin resistance [18,19]. Objectivity of PCR helped to ascertain the affiliations of 9 isolates that showed intermediate resistance to Oxacillin out of which 2 isolates were adjudged MRSA because of possessing *mecA* gene and 7 isolates as MSSA due to absence of this marker. PCR for *mecA* there-

fore was found to be more useful in determination of MRSA and MSSA status of *S. aureus* isolates. Among the 7 *mecA* negative isolates showing intermediate resistance to Oxacillin, the possibility of a few strains having non-*mecA* mediated Methicillin resistance cannot be ruled out. The 11 isolates which were *mecA* negative but Oxacillin resistant were due to the absence of *mecA* gene but its phenotypic expression was most likely due to non-*mecA* mediated Methicillin resistance as described earlier [20].

In our study, 27 of the 70 isolates (38.6%) were found to be MRSA by *mecA* positivity in PCR. The prevalence of colonization or infection by MRSA continues to increase [21] with considerable variation between countries [22] or hospitals in the same area [23]. MRSA infections in India have been recorded between 18.4 and 33.3% among hospitalized patients [24] in general while is stated to be an under reported entity in community settings [25]. In our study, overall 38.6 % *mecA* positivity, although not the highest reported from the country, can still be considered on the higher side in this region and is therefore a cause for concern.

Kobayashi *et al.* (1994) reported that though *femB* genes are detectable only in *S. aureus,* an absence of *femB* gene does not mean that the isolate is not *S. aureus*. They showed up to 3% of *S. aureus* isolates in their study were negative for *femB.* In our study, we found 19 *femB* negative isolates (27%) out of which, 6 were *mecA* positive (MRSA), and among the remaining 13 *mecA* negative (MSSA), 2 were PVL positive. Therefore 8 isolates, despite being *femB* negative, were either positive for *mecA* or PVL. Eleven isolates were negative for *femB*, *mecA* and PVL despite being reconfirmed as *S.aureus* phenotypically. We therefore conclude that *femB* negative *S.aureus* may be present in much higher numbers in clinical specimens than previously reported. In agreement with the studies of Kobayashi *et al*. (1994) [26], *we* observed that *femB* negativity was more in MSSA (30.23 %) than in MRSA (22.22 %).

In our study we found 85.1% MRSA isolates were positive for PVL gene while it was 48.8% in MSSA. The high percentage of PVL producing MRSA as well as MSSA strains is interesting and can be due to the carriage of large parts of SCC *mec* including the *luk-F* and *luk-S* genes to other resistant strains of *S.aureus* by various ways of recombination. However nearly half of MSSA (48.8%) strains producing PVL gene is also a cause for concern. According to some reports, the prevalence of PVL positive *S. aureus* is less than 5% in France, 4.9% in UK, 8.1% in Saudi Arabia, 14.3% in Bangladesh and 35% in Cape Verde Islands [27-31]. From India, D'Souza *et al*. (2010) reported 64% PVL positive MRSA from Mumbai [32], which is quite high, compared to other countries and in our study 62.85% PVL positive *S.aureus* was seen. The high prevalence of PVL may also be due to misuse of antibiotics in the country causing selective pressure for development of resistant strains along with the virulence factor i.e. PVL.

The 62.85% PVL positive *S.aureus* isolates found in our study is much higher than most reports worldwide. In the backdrop of bacterial strains gaining resistance to wide spectrum of antibiotics recently, the high incidence of MRSA coupled with relatively higher proportions of potential PVL producers, both in MRSA and MSSA, warrants setting up a surveillance

mechanism for *S.aureus* infections at hospital and community levels in the region. Our findings also underscore the need for genetic studies on *S.aureus* infections in India and other parts of the world as well.

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