

Clindamycin Resistance in PVL Positive Isolates of *Staphylococcus aureus*, Belgaum, North Karnataka (India)

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Abstract: - The widespread use of Macrolide Lincosamide Streptogramin B (MLSB) antibiotics has led to an increase in the resistant strains. Although Clindamycin is effective against MRSA but its use in the presence of Erythromycin can lead to induction of cross-resistance among members of the MLSB group which could limit the effectiveness of this drug. Furthermore, at times, some strains are also harbored with PVL genes which are encoded by two contiguous and co-transcribed genes viz., *lukF-PV* and *lukS-PV*. The results of a study conducted to find the prevalence of inducible Clindamycin resistance in PVL positive isolates of *Staphylococcus aureus* from Belgaum, Karnataka, India. Method: More than 120 isolates of *S.aureus* were collected from hospital in Belgaum during the period 2010-2012, out of which, 72 isolates confirming *S.aureus* status were kept for further study at RMRC (ICMR), Belgaum. They were characterized as resistant/sensitive using various conventional, molecular and automated techniques. PVL genes *LukS* and *LukF* in these strains were detected by PCR. Phenotypic inducible resistance to clindamycin was detected by Double Disk Diffusion test. Result: Out of 72 isolates, 46 were MRSA and 26 were MSSA. In all, 50 isolates (69.44%) showed the presence of PVL genes. Out of these, 14 PVL positive *S. aureus* isolates (26.38%) showed iMLSB phenotype which included 13 MRSA and 1 MSSA. Conclusion- The cause of concern is the 13 isolates which are MRSA, PVL positive and show inducible clindamycin resistance. Such a combination can be a treatment challenge to the clinicians who are left with very few options.

Keywords: - Macrolide Lincosamide Streptogramin B (MLSB), PVL genes, PCR, iMLSB, cMLSB, Vitek 2 compact automated system

I. INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community acquired infections [1]. Macrolide Lincosamide Streptogramin B (MLSB) antibiotics are frequently being used to treat such infections. However, their widespread use has led to an increase in the number of *Staphylococcus* strains resistant to MLSB antibiotics [2]. Clindamycin, a Lincosamide antibiotic, is among the limited choice of antimicrobials effective against MRSA and has long been an option for treating both MSSA and MRSA infections. However, there is concern about use of this antibiotic in the presence of Erythromycin resistance because of the possibility of induction of cross-resistance among members of the Macrolide, Lincosamide and Streptogramin B (MLSB group) which could limit the effectiveness of this drug [3].

PVL proteins, one of the most important and extensively investigated proteins, belong to the 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. Epidemiological and clinical data [6] 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 [7]. PVL is leukotoxic by pore induction for human polymorphonuclear cells and macrophages [8] and its presence in *S. aureus* appears to be associated with increased disease severity, ranging from cutaneous infection to chronic osteomyelitis and severe necrotizing pneumonia which could be fatal [9]. PVL production has also been linked with furuncles, cutaneous abscess and severe necrotic skin infections in school children [10] and in certain communities [11].

Data describing MLSB prevalence or clinical predictors of the presence of inducible MLSB (iMLSB) among PVL positive *S.aureus* isolates is limited in India. In the present study, we aimed to characterize iMLSB resistance in isolates of *Staphylococcus aureus* isolates, both MRSA and MSSA, taken randomly at hospitals in Belgaum catering to 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.

II. MATERIAL AND METHODS

More than 120 isolates of *S.aureus* were collected after obtaining due ethical clearance from microbiology laboratory of a hospital in Belgaum that receives samples from various outdoor and indoor patient departments, out of which 72 isolates confirming *S.aureus* status were kept for the further research study during the period 2010-2012.

(A) Collection and Identification:

S.aureus isolated from various clinical samples at microbiology departments of hospitals included in this study were collected and their identities were reconfirmed by standard microbiological tests viz most notably the gram stain, catalase, coagulase tests, beta haemolysis and manitol fermentation (*Note: Isolates absolute positive for above five tests were kept for the study and rest were left aside. Some isolates showing negative for mannitol fermentation but positive for other four tests were also not included in study*).

(B) Detection and confirmation of MRSA:

All isolates were subjected to phenotypic Cefoxitin, 30µg (Hi-Media, India) following CLSI guidelines [12] for disc-diffusion testing. The zone of inhibition was measured after 24 hrs incubation at 37°C and isolates were labeled as susceptible at ≥ 22 mm and resistant at ≤ 1 mm zone of inhibition. ATCC 43300 and ATCC 25923 were included in every batch as standard strains for MRSA and MSSA respectively.

Confirmation of MRSA was done by carrying out Multiplex PCR for the detection of *mecA* and *femB* genes which is a gold standard for confirming the identity of MRSA [13]. DNA was extracted from overnight cultures of *S.aureus* by CTAB-NaCl method [14] and was checked for quality and quantity using Nanodrop Spectrophotometer at 260/280 nm as well as visually by horizontal gel electrophoresis in 1% agarose. 1µl containing 60 ng of the extracted DNA was added to 24µl of PCR amplification mix consisting of 16 µl of double distilled autoclaved water, 2.5µl of 10X Taq buffer (Tris with 15 mM MgCl₂), 1µl of 2.5mM dNTP mix (Merck, India), 0.5 µl of 3U Taq polymerase (Merck, India), and 0.5mM of each primer given in the Table: I. Amplifications were carried out using thermal cycler 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 72°C for 2 min. The PCR products were subjected to agarose gel electrophoresis using gel red dye (Biogenei, Bangalore) and documented.

(C) Detection of *luk-F (PV)* and *luk-S (PV)* genes:

1 µl containing 60ng of extracted *S.aureus* DNA was added to 24µl of PCR amplification mix consisting of 18 µl of double distilled autoclaved water, 2.5µl of 10X Taq buffer (Tris with 15 mM MgCl₂), 1µl of 2.5 mM dNTP mix (Merck, India), 0.5 µl of 3U Taq polymerase (Merck, India) and 0.5mM of each primer given in the Table: I. Amplifications were carried out using thermal cycler with PCR conditions that consisted of initial denaturation at 94°C for 99 sec 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 sec 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 gel and documented.

(D) Phenotypic inducible resistance to clindamycin by Double Disk Diffusion test:

Isolates were placed on a Muller Hinton agar as per [12]. Clindamycin and Erythromycin disks, containing 2 µg and 15µg each respectively were placed in the center of the plate separated by a distance of 15 cm between the edges. Plates were incubated at 37° C for 24 hr. Inducible resistance to Clindamycin was defined as blunting of the clear circular area of no growth around the Clindamycin disk on the side adjacent to the Erythromycin disk and was designated as D - test positive. Absence of a blunted zone of inhibition was designated as D - test negative. Four different phenotypes were interpreted as follows [15, 16].

1. **Sensitive phenotype (S):** *S.aureus* isolates showing sensitivity to both Clindamycin (Zone size > 21mm) and Erythromycin (Zone size > 23mm).
2. **Constitutive MLSB phenotype (cMLSB):** *S.aureus* isolates showing resistance to both Erythromycin (Zone size < 13 mm) and Clindamycin (Zone size < 14mm) with circular shape of zone of inhibition if any around Clindamycin.
3. **Moderately Sensitive phenotype (MS):** *S.aureus* isolates showing resistance to Erythromycin (Zone size < 13 mm) and sensitivity to Clindamycin with a circular zone of inhibition (Zone size > 21mm) around Clindamycin disc (D-negative).
4. **Inducible MLSB phenotype (iMLSB):** *S.aureus* isolates showing resistance to Erythromycin (zone size < 13 mm) and sensitivity to Clindamycin giving D - shaped zone of inhibition (Zone size > 21mm) around Clindamycin disc (D-positive).

III. RESULTS

All *S.aureus* isolates were subjected to conventional tests and PCR based *mecA* and *femB* genes detection to confirm their identity and to detect their sensitivity to Methicillin. Out of 72 isolates, 46 were MRSA and 26 were MSSA (Table: II) by conventional and PCR based analysis any discrepancies were cleared by Vitek 2 compact automated system (BioMerieux, France. Software version: 05.02) as referred in our paper [17].

The results of PCR based detection of *luk F*-PV and *luk S*-P are shown in Table: II. Out of 72 isolates, 50 isolates (69.44%) showed the presence of PVL genes which included 36 MRSA and 14 MSSA. 22 isolates (30.56%) showed the absence of PVL genes which included 10 MRSA and 12 MSSA.

All the 72 isolates were put for MLSB phenotype detection by Double Disc Diffusion method using Erythromycin 15 µg and Clindamycin 2 µg and results are shown in the Table : III. The results showed 28 isolates (38.88%) to be Sensitive phenotype (S) and 44 to be resistant to Erythromycin. Out of 44 resistant isolates, 25 isolates (34.72%) were found to be Moderately Sensitive (MS), 19 isolates (26.39%) to be inducible MLS_B phenotype (iMLSB) and none to be constitutive MLSB phenotype (cMLSB).

Inducible Clindamycin resistance in isolates of *Staphylococcus aureus* was characterized with respect to Methicillin susceptibility and PVL positivity (Table: IV). Out of 28 Sensitive isolates, 11 were PVL positive MRSA, 8 were PVL positive MSSA, 2 were PVL negative MRSA and 7 were PVL negative MSSA. Out of 25 Moderately Sensitive isolates, 12 were PVL positive MRSA, 5 were PVL positive MSSA, 4 were PVL negative MRSA and 5 were PVL negative MSSA. Out of 19 inducible MLS_B isolates, 13 were PVL positive MRSA, 1 was PVL positive MSSA, 4 were PVL negative MRSA and 1 was PVL negative MSSA.

IV. DISCUSSION

In the present study, *mec A* positivity was found in 46 out of 72 isolates (63.89%) and 26 showed absence of *mec A* gene (36.11%). From India, MRSA prevalence has been reported to be different from different regions, being 17.2% from Madhya Pradesh [18], 54.5% from Utter Pradesh [19], 31.8% from Tamil Naidu [20], 52.23% from Sikkim [21] and 46% from Punjab [22]. Alvarez-Uria and Reddy (2012) reported 64.8% MRSA in community acquired *S.aureus* and 70.7% in hospital acquired *S.aureus* from Bathalapalli, Andhra Pradesh [23].

The prevalence of PVL gene in *S. aureus* has been observed to be very high in present study, being present in 50 out of 72 isolates (69.4%). About a decade ago, the prevalence of PVL gene in *S. aureus* was less than 5% in France [24]. But now its prevalence is increasing worldwide with reports showing 11.6% from Singapore, 35% from Cape Verde Islands, 14.3% from Bangladesh, 37.6% from Saudi Arabia [25,26,27,28].

In the present study, 78.2% of MRSA (36 of 46 MRSA isolates) and 53.8% of MSSA (14 out of 26 MSSA isolates) were found to be PVL positive. D'Souza *et al.* (2010) reported 64% PVL positive MRSA from Mumbai [29]. However, proportion of PVL positive MRSA is comparatively lower in other countries. The higher prevalence of PVL-MRSA as compared to PVL-MSSA in the present study may be due to the overuse of antibiotics in India causing selective pressure for development of resistant strains along with the virulence factor (PVL). Furthermore, although the proportion of PVL-MSSA is comparatively lower than PVL-MRSA but it is not too low to be ignored, and need even more attention because these PVL positive MSSA can in future get converted to MRSA [7]. Aires-de-Sousa *et al.* (2006) suggested that acquisition of PVL genes from different phages must be of a greater advantage to *S.aureus* than antibiotic resistance gene [26].

Detection of MLSB phenotype in 72 *S. aureus* isolates by Double Disc diffusion method showed 25 isolates (34.72%) to be Moderately Sensitive, 28 (38.89%) to be Sensitive, 19 (26.39%) to be iMLSB and none to be cMLSB. Therefore, there was total absence of cMLSB in the region of study as was also reported from Vellore (Tamil Naidu) by Angel *et al.* (2008) [30]. On the other hand, several reports from other parts of India reveal the presence of both iMLSB and cMLSB such as 24.89% iMLSB and 18.26% cMLSB from Bangalore, Karnataka [31], 21% iMLSB and 26.5% cMLSB from Delhi [32], 3.6% cMLSB and 14.5% iMLSB [15], 26.43% cMLSB and 3.57% iMLSB from Nagpur, Maharashtra [33], 43% iMLSB and 12% cMLSB from Varda, Gujrat [34]. In metropolitan cities like Delhi and Bangalore, high percentage of constitutive MLSB resistance has been suggested to be due to crowded settings, population pressure, less hygiene in community as well as hospitals settings or overuse of the drug and conversion of inducible phenotype to constitutive phenotype during treatment [32, 35, 31]. Moreover, Clindamycin resistance rates have been seen to vary with geographic region and Methicillin susceptibility [36,32, 31].

In the present study, 14 PVL positive *S. aureus* isolates (26.38%) showed iMLSB phenotype which included 13 MRSA and 1 MSSA. Actually the cause of concern are the 13 PVL positive MRSA isolates which also showed inducible Clindamycin resistance. Such strains can be a treatment challenge to the clinicians because when MRSA strains carry additional resistant genes (genes for Clindamycin or PVL genes), only few therapeutic options are left for treatment. PVL positive patients with prolonged stay in hospitals and after 3 weeks of antibiotic completion can become resistant to Vancomycin or Linezolid and have to be immediately

put on alternate antibiotics such as Teicoplanin and Rifampicin [37]. Empirical Clindamycin treatment for suspected Staphylococcal infections is not recommended in areas where the proportion of MRSA isolates exceeds 10% to 15% [38]. Among Staphylococcal infections due to isolates exhibiting inducible Clindamycin resistance, the risk of treatment failure during Clindamycin therapy is increased when there are high bacterial inoculums [39, 40].

It is, therefore, concluded that patients with PVL positive MRSA infections along with inducible Clindamycin resistance can be a treatment challenge to the clinicians and should be put on antibiotic therapy carefully, so that they should not get resistant to the antibiotic.

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Table I: Details of oligonucleotide primers used in PCR.

Primer	Target gene	Sequence (5'-3')	Amplicon size	Ref.
mec A1 mec A2	<i>mec-A</i>	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310 bp	[41]
fem B1 fem B2	<i>fem-B</i>	TTA CAG AGT TAA CTG TTA CC ATA CAA ATC CAG CAC GCT CT	651bp	[41]
luk-PV-1 luk-PV-2	<i>luk-S(PV)</i> and <i>luk-F(PV)</i>	ATC ATT AGG TAA AAT GTC TGG ACA TGA GCA TCA AGT GTA TTG GAT AGC AAA AGC	433bp	[24]

Table II. Depicting the frequency of PVL genes in MRSA and MSSA

Total isolates(n)= (72)	MRSA (46)	PVL gene positive (36)
		PVL gene negative (10)
	MSSA (26)	PVL gene positive (14)
		PVL gene negative (12)

Table III: Different phenotypes of Clindamycin resistance in S.aureusisolates

S/N	Susceptibility Pattern	Phenotype	Total	Percentage
1	ER-S, CL-S	S	28	38.88%
2	ER-R, CL-R	cMLS _B	00	00.00%
3	ER-R, CL-S; D ⁻	MS	25	34.72%
4	ER-R, CL-S; D ⁺	iMLS _B	19	26.38%

Key Words: ER: Erythromycin, CL: Clindamycin, cMLS_B: Constitutive resistance to Clindamycin, iMLS_B: Inducible Clindamycin resistance, MS: Moderately Sensitive, D⁻: negative double diffusion test, D⁺: positive double diffusion test, S: Sensitive, R: Resistant

Table IV: Distribution of PVL positive isolates in different *S. aureus* phenotypes of Clindamycin resistance.

S.N.	Phenotype	No. of isolates	MRSA		MSSA	
			PVL +	PVL-	PVL +	PVL-
1	S	28	11	2	8	7
2	cMLS _B	00	00	00	00	00
3	MS	25	12	4	5	4
4	iMLS _B	19	13	4	1	1
	Total	72	36	10	14	12

KeyWords : ER: Erythromycin, CL: Clindamycin, cMLS_B: Constitutive resistance to clindamycin, iMLS_B: Inducible clindamycin resistance, MS: Moderately Sensitive, D⁻: negative to Double Diffusion test, D⁺: positive to Double Diffusion test, S: Sensitive, R: Resistant.

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