Molecular characterization of Panton-Valentine leukocidin (PVL) toxin–encoding phages from South India

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Abstract

A total of 19 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were investigated for Panton-Valentine leukocidin (PVL) toxin, PVL gene sequence variation and PVL-encoding phages. Whole genome sequencing was performed for all isolates. Analysis of MRSA isolates (n = 19) confirmed that most MRSA (n = 11) were positive for the PVL gene and were multidrug resistant. ST772-MRSA-V was the predominant PVL-positive MRSA clone, although all of them were found to carry the ΦIND772PVL phage in the genome. This study provides insights into the evolution of a new lineage of PVL-MRSA and highlights the potential risk of the emergence of multidrug-resistant community-acquired MRSA with high virulence.

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Introduction

Panton-Valentine leukocidin (PVL) toxin is a specific virulent entity, often associated with recurrent *Staphylococcus aureus* skin and soft tissue infection (SSTIs) and necrotizing pneumonia. Mortality due to PVL-positive necrotizing pneumonia is reported to be high (40–60%) [1]. This is due to pro-inflammatory and cytotoxic effects on neutrophils, monocytes and macrophages. Incubation of the cells with low doses of PVL (0.04–0.4 μg/mL; 1–10 nM) results in inflammasome activation, induces a huge interleukin 1β release within minutes and results in cell death (apoptosis) [2]. Despite clear epidemiologic data, the function of PVL in causing pathogenesis is controversial. Some animal models and clinical studies in bone and joint infections and necrotizing pneumonia have recognized PVL as an indicator of disease severity independent of methicillin resistance [3]. However, several other clinical trials have shown that severe SSTIs caused by PVL-producing and non producing strains do not have a difference in outcomes [4].

PVL production is encoded by two cotranscribed genes, *luk*S-PV and *luk*F-PV. The PVL encoding genes (*luk*S-PV and *luk*F-PV) are bacteriophage encoded (ΦPVL, Φ108PVL, Φ7247PVL, ΦSa2958, ΦSa2MW, ΦSLT, ΦSa2USA, ΦTCH60) [5]. PVL has been epidemiologically linked with community-acquired (CA) methicillin-resistant *S. aureus* (MRSA), but some CA-MRSA strains do not carry PVL genes [6]. PVL-positive hospital-acquired (HA) MRSA strains have also been reported [7].

In India, CA-MRSA clones are genetically diverse, and three-fourth are PVL positive [8]. Sequence type (ST) 772 and ST22 were the major clones reported from India [9,10]. ST772-MRSA-V, called the Bengal Bay clone, is a multidrug-resistant (MDR), PVL-positive CA-MRSA clone initially isolated and reported from India [8]. Four studies from India have reported 16% to 64% of PVL gene prevalence in *S. aureus* [9–12]. Despite the high incidence of PVL-positive *S. aureus,* clonal lineages as well the typing of PVL-encoding phages in *S. aureus* have not been adequately reported from India.

This study was planned to investigate the following: to analyse the distribution of PVL genes in CA- and HA-MRSA; to compare antimicrobial resistance pattern of PVL-positive and -negative MRSA isolates; to analyse mutations in PVL genes; to type PVL encoding phages; and to assess the epidemiology and molecular characteristics of PVL-positive and -negative *S. aureus* isolated from bloodstream infections.

Methods

A total of 19 nonduplicate MRSA isolates collected during 2015–2016 from patients with sepsis were included in this study. Antibiotic susceptibility testing was performed by the disk diffusion method for the following antibiotics: cefoxitin (30 μg), gentamicin (10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), netilmicin (30 μg), rifampicin (5 μg), erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg) and linezolid

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TABLE 1. Antimicrobial resistant pattern, mutational analysis of PVL gene variant, PVL phage typing and molecular characteristics of PVL-positive and -negative MRSA isolates from bloodstream infection

						lukS-PVL			*lukF*-PVL							
						Nonsynonymous mutation		Synonymous mutation	Nonsynonymous mutation	Synonymous mutation						
Isolate ID	CA/HA MRSA	Source of MRSA sepsis	Antimicrobial resistance profile	Accession no	PVL gene	470 (T)	527 (G)	663 (T)	140 (C)	456 (A)	789 (G)	PVL encoding phage	SCC*mec* type	*spa* type	ST	CC
VB9939	CA	SSTI	Gen, SXT, ery, cip	MLQK00000000	+	T	A	G	C	G	A	ΦIND772PVL	V	t657	ST722	CC1
VB16578	CA	Epidural MRSA abscess	Gen, SXT, ery, cip	MLQD00000000	+	T	A	G	C	A	A	ΦIND772PVL	V	t657	ST722	CC1
VBA46389	CA	SSTI	Gen, SXT, ery, cip	MLQG00000000	+	T	A	G	T	A	A	ΦIND772PVL	V	t548	ST772	CC1
VB9352	CA	Prostatic abscess	Gen, SXT, ery, cip	LXWR00000000	+	T	A	G	C	A	A	ΦIND772PVL	V	t657	ST772	CC1
VB23686	HA	Epidural MRSA abscess	Gen, SXT, ery, cip	MANS00000000	+	T	A	G	C	A	A	ΦIND772PVL	V	t657	ST772	CC1
VB26276	HA	Necrotizing soft tissue infection	Gen, SXT, ery, cip	LWMF00000000	+	T	A	G	C	A	A	ΦIND772PVL	V	t657	ST772	CC1
VBA4283	CA	MRSA sepsis	Gen, SXT, ery, cip	Yet to receive	+	A	A	G	C	A	A	ΦIND772PVL	V	t021	ST772	CC1
VB31683	HA	Necrotizing soft tissue infection	Ery, clin, cip	MANT00000000	+	T	*A*	*G*	*C*	*A*	*A*	ΦPVL	IVc	t657	ST22 (EMRSA-15)	CC22
VBA44094	HA	Necrotizing soft tissue infection	Ery, clin, cip	MLQH00000000	+	T	*A*	*G*	*C*	*G*	*A*	ΦPVL	IVc	t474	ST22 (EMRSA-15)	CC22
VB9982	HA	Necrotizing pneumonia	Cip	MLQI00000000	+	T	*A*	*G*	*C*	*A*	*A*	ΦPVL	I	t6827	ST2371	CC22
VB20017	HA	MRSA sepsis	Cip	MLQE00000000	+	T	*A*	*G*	*C*	*G*	*A*	ΦPVL	V	t6827	ST2371	CC22
VBA43011	CA	Infective spondylodiscitis	Ery	MLQJ00000000	−	NA	NA	NA	NA	NA	NA	NA	IV	t127	ST1	CC1
VB1490	HA	SSTIs	Gen, ery, tet, clin, TRM	MLQB00000000	−	NA	NA	NA	NA	NA	NA	NA	III	t037	ST239	CC8
VBA35316	HA	Epidural MRSA abscess	Ery, gen, tet, TRM	MLQC00000000	−	NA	NA	NA	NA	NA	NA	NA	V	t2473	ST72	CC8
VBA43964	HA	Infective endocarditis	Gen, ery, tet, clin, TRM	MLQA00000000	−	NA	NA	NA	NA	NA	NA	NA	II	t4615	ST580	CC398
VB12268	CA	SSTI	Susceptible to all tested antibiotics except cefoxitin	LXWS00000000	−	NA	NA	NA	NA	NA	NA	NA	V	t657	ST672	Singleton
VBV169	CA	MRSA sepsis	Susceptible to all tested antibiotics except cefoxitin	LWMG00000000	−	NA	NA	NA	NA	NA	NA	NA	V	t657	ST672	Singleton
VBP3985	CA	MRSA sepsis	Susceptible to all tested antibiotics except cefoxitin	NBSJ00000000	−	NA	NA	NA	NA	NA	NA	NA	IVd	t304	ST6	Singleton
VB44746	CA	Necrotizing fasciitis	Susceptible to all tested antibiotics except cefoxitin	MLQF00000000	−	NA	NA	NA	NA	NA	NA	NA	IVh	t131	ST1290	Singleton

CA, community acquired; CC, clonal complex; cip, ciprofloxacin; clin, clindamycin; ery, erythromycin; gen, gentamicin; HA, hospital acquired; MRSA, methicillin-resistant *Staphylococcus aureus;* NA, not applicable; PVL, Panton-Valentine leukocidin; SCC, staphylococcal cassette chromosome; *spa,* staphylococcal protein A; SSTI, skin and soft tissue infection; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; tet, tetracycline; TRM, trimethoprim.

(30 μg). Inducible clindamycin resistance was detected by the D-zone test. Minimum inhibitory concentrations of vancomycin were determined by microbroth dilution method according to Clinical and Laboratory Standards Institute guidelines [13]. SCC*mec* typing was performed as previously described [14].

DNA isolation from pure cultures was performed using the QiAamp DNA mini Kit (Qiagen). The whole genome shotgun sequencing was performed for all the isolates using the Ion Torrent PGM system (Life Technologies) with 400 bp chemistry. The raw data generated were assembled *de novo* using AssemblerSPAdes v.5.0.0.0 embedded in Torrent suite server v.5.0.0.4. Genome sequence was annotated using PATRIC, the bacterial bioinformatics database and analysis resource (http://www.patricbrc.org) [15], and the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). Downstream analysis was performed using the Center for Genomic Epidemiology (CGE) server (http://www.cbs.dtu.dk/services) and PATRIC. Resistance gene profiles were analysed using ResFinder 2.1 from the CGE server (https://cge.cbs.dtu.dk//services/ResFinder/). ST was determined for all the isolates in the allele order of *arcc, aroe, glpf, gmk, pta, tpi* and *yqil* by comparing the sequences with the *S. aureus* database maintained at the MLST website (http://saureus.mlst.net/). PHASTER (PHAge Search Tool Enhanced Release) was used for annotation and identification of prophage sequences in the bacterial genome.

Results

Of the analysed genomes (n = 19), 11 MRSA isolates were found to be PVL positive and MDR, except two isolates. Among PVL-positive isolates, five were CA-MRSA and six were HA-MRSA. In half of the PVL-positive cases, bloodstream infection occurred as a result of SSTIs or pneumonia. All PVL-positive isolates were ciprofloxacin resistant and were often susceptible to clindamycin (D-zone negative).

The genome was analysed for single nucleotide polymorphisms (SNP) in both *luk*S-PV and *luk*F-PV genes, using the published genome of CA-MRSA strain USA300 (accession no. CP000255) as the reference sequence. Although PVL gene sequence is highly conserved, each of these genes showed nucleotide variations at three different positions. Of the identified SNPs, three were nonsynonymous (two in *luk*S-PVL and one in *luk*F-PVL). This includes phenylalanine (F) to tyrosine (Y) substitution at amino acid residue 157 (nucleotide, 470) and arginine (A) to histidine (H) substitution at amino acid residue 176 (nucleotide, 527) in *luk*S-PVL; and alanine (A) to valine (V) substitution at amino acid residue 47 (nucleotide, 140) in *luk*F-PVL. Remarkably, all PVL-positive MRSA isolates were identified as H variant (nucleotide A at 527 and histidine (H) residue at 176) (Table 1). Regardless of different MRSA clonal lineages, PVL gene variation and PVL encoding phages, the integration site (*att*L and *att*R) of these prophages seemed to be similar in all isolates.

A specific association between PVL phages and ST was observed. The Bengal Bay clone (ST772-MRSA-V) was the predominant PVL-positive MRSA clone, followed by small numbers of EMRSA clone (ST22-MRSA-IV). Among the PVL-negative MRSA isolates, clonal types were heterogeneous, including USA400 clone (ST1-MRSA-IV) and Hungarian clone (ST239-MRSA-III). It is noteworthy that the majority of PVL-positive MRSA carried the ΦIND772PVL phage in the genome, which is identified with the clonal lineage of ST722-MRSA-V and which belongs to clonal complex (CC) 1 (Table 1). Interestingly, for the first time in India, we observed ΦPVL carrying MRSA isolates belong to CC22 with the ST2371/ST22. All these ΦPVL encoding PVL toxin were identified in HA-MRSA. The phage ΦIND772PVL was found to carry PVL as well the staphylococcal enterotoxin (*sea*) gene, while ΦPVL was only identified with the PVL gene. *spa* typing showed high genetic diversity, as indicated by the presence of 11 different *spa* types (n = 19) among PVL-positive and -negative isolates.

Discussion

PVL toxin is considered an important marker for differentiation of HA- and CA-MRSA. The present study provides an insight into the microepidemiology of PVL-positive MRSA isolates from bloodstream infection. We observed that the majority of PVL-positive HA-MRSA are associated with SSTIs, and less likely with pneumonia or sepsis. MDR PVL-positive MRSA was observed in this study. Presumptive identification based on susceptibility to ciprofloxacin and gentamicin is no longer reliable in detecting PVL-positive CA-MRSA [16,17]. However, our study showed that all PVL-positive MRSA are ciprofloxacin resistant.

PVL gene variation identified in this study was similar to the previously described nonsynonymous SNPs [7,18–20]. In our study, all the PVL-positive isolates were identified as being of the H variant and capable of causing invasive disease. Besseyre et al. [21] demonstrated that histidine to arginine amino acid substitution does not impaired leucotoxicity of PVL toxin. Despite PVL gene variation, the H or R variant has demonstrated significant leucotoxicity. However, a possible association between other nonsynonymous mutation and leucotoxicity remains unclear. This could be an important cause

for evasion of host immunoresponse to invasive disease [22]. Acquisition of PVL genes by an HA-MRSA strain could increase morbidity and mortality.

The PVL-positive isolates investigated in this study were from three different genetic backgrounds (ST772-MRSA-V, ST22-MRSA-IV, ST2371-MRSA-I/V). This finding reveals the existence of vertical transmission of PVL genes within the same clone or horizontal transmission between different clones. The present study highlights the evolution of new lineages (ST22/ST2371) of PVL-positive HA-MRSA carrying ΦPVL phage. Interestingly, all the ΦIND772PVL phages identified in this study were found to carry PVL as well *sea* toxin on the prophage, as previously described [8].

Four studies have reported the prevalence of PVL with clonal lineage from India. This includes two from carriers and two from clinical isolates [23]. Dhawan *et al.* [9] reported that PVL distribution was significantly associated with ST22-MRSA-IV (66%) compared to ST772-MRSA-V (27%) in clinical isolates. D'Souza *et al.* [10] reported that 65% of ST22-MRSA-IV and ST772-MRSA-V were positive for PVL, and 27% of them were MDR from mixed CA-MRSA and HA-MRSA infection. In contrast, the present study showed that ST772-MRSA-V (63%) was the predominant PVL-positive clone and was MDR.

Conclusion

Variants of the PVL gene and PVL-encoding phages are lineage specific. ST772-MRSA-V (Bengal Bay clone) belongs to CC1 and serves as a major reservoir for the dissemination of phage-mediated PVL toxin. This CA-MRSA clone (ST772-MRSA-V) was found with unique features of high virulence and MDR. In addition, co-carriage of PVL and *sea* toxin enhances both superantigenic and cytotoxic response. This combination of toxins on the same prophage has not been reported in other strains of *S. aureus*.

Conflict of Interest

None declared.

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