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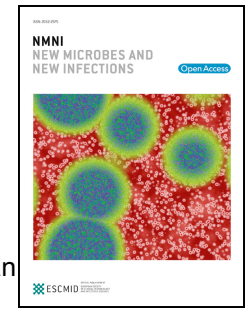

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Molecular characterisation of Panton Valentine leukocidin (PVL) toxin encoding phages from South India

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Summary:

A total of 19 MRSA isolates was investigated for Panton Valentine (PVL) toxin, PVL gene sequence variation and PVL-encoding phages. Whole genome sequencing was performed for all the isolates. Analysis of MRSA isolates (n=19), confirmed that majority of MRSA (n=11) were positive PVL gene and multi-drug resistant. ST772-MRSA-V was the predominant PVL positive MRSA clone and all of them were found to carry ϕ IND772PVL phage in the genome. This study provides an insight into the evolution of new lineage of PVL-MRSA and highlights the potential risk of the emergence of multi-drug resistant community acquired (CA) MRSA with high virulence.

Key words: MRSA, PVL, ST772, ϕ IND772PVL

Introduction:

Panton-Valentine leukocidin (PVL) toxin is the specific virulent entity, often associated with recurrent *Staphylococcus aureus* skin and soft tissue infection (SSTIs) and necrotising pneumonia. Mortality due to PVL positive necrotising 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 i) inflammasome activation, ii) induces a huge IL-1β release within minutes, iii) cell death (apoptosis) [2]. Despite clear epidemiological data, the function of PVL in causing pathogenesis is controversy. Some animal models and clinical studies in bone and joint infections (BJI) and necrotizing pneumonia have recognized PVL as an indicator of disease severity independent of methicillin resistance [3]. However, several other clinical trials show 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 co-transcribed 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 epidemiology linked with community-acquired methicillin resistant *S. aureus* (CA-MRSA), but some CA- MRSA strains do not carry PVL genes [6]. PVL positive HA-MRSA strains have also been reported [7].

In India, CA-MRSA clones are genetically diverse and apparently three-fourth is PVL positive [8]. ST772 and ST22 were the major clones reported from India [9,10]. ST772-MRSA-V called as “Bengal Bay clone” is a multi- drug resistant, PVL positive CA-MRSA clone initially isolated and reported from India [8]. Four studies from India, have reported 16% - 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* has not been adequately reported from India.

This study was planned to investigate i) the distribution of PVL genes in CA and HA-MRSA, ii) to compare antimicrobial resistant pattern of PVL positive and negative MRSA isolates, iii) analyse of mutation in PVL genes iv) typing of PVL encoding phages and iv) epidemiology and molecular characteristics of PVL positive and negative *S. aureus* isolated from bloodstream infection.

Methods:

A total of 19 non-duplicate MRSA isolates collected during 2015-2016 from patients with sepsis were included in this study. Antibiotic susceptibility testing was performed by using disk diffusion method for the following antibiotics; cefoxitin (30 µg), gentamicin (10µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), netilmicin (30µg), rifampicin (5µg), erythromycin (15µg), clindamycin (2µg), tetracycline (30µg) and linezolid (30µg). Inducible clindamycin resistance was detected by using D-zone test. Minimum inhibitory concentration (MIC) of vancomycin was determined by microbroth dilution method according to CLSI guidelines [13]. SCC *mec* typing was performed as previously described [14].

DNA isolation from pure cultures was performed using QiAamp DNA mini Kit (Qiagen, Germany) .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.5.0.4. Genome sequence was annotated using PATRIC, the bacterial bioinformatics database and analysis resource (http://www.patricbrc.org) [15], and the 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 genes profile was analysed using ResFinder 2.1 from the CGE server (https://cge.cbs.dtu.dk//services/ResFinder/). The sequence type (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 *S. aureus* database maintained at the MLST website (http://saureus.mlst.net/). PHASTER (PHAge Search Tool Enhanced Release) has been used for annotation and identification of prophage sequences in bacterial genome.

Results:

Of the analysed genome (n=19), 11 MRSA isolates were found to be PVL positive and multi-drug resistant, except two isolates. Among PVL positive isolates, 5 were CA-MRSA and 6 were HA-MRSA. In half of the PVL positive cases, bloodstream infection occurred secondary to skin and soft tissue infection (SSTIs)/pneumonia. All PVL positive isolates were ciprofloxacin resistant and are often susceptible to clindamycin (D-zone negative).

The genome was analysed for SNPs 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 non-synonymous (2 in *luk*S-PVL and 1 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; alanine (A) to valine (V) substitution at amino acid residue 47 (nucleotide, 140) in *luk*F-PVL. Remarkably, all PVL positive MRSA isolates was 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 were seems to be similar in all the isolates.

A specific association between PVL phages and ST was observed. "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 USA 400 clone (ST1-MRSA-IV) and Hungarian clone (ST239-MRSA-III). It is noteworthy, that majority of PVL positive MRSA carried ϕ IND772PVL phage in the genome, identified with the clonal lineage of ST722-MRSA-V and belongs to CC1 (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 was identified in HA-MRSA. The phage ϕ IND772PVL was found to carry PVL as well staphylococcal enterotoxin (*sea*) gene, while ϕPVL was only identified with PVL gene. *spa* typing showed high genetic diversity, as indicated by the presence of 11 different *spa* types (n=19) among PVL positive and PVL negative isolates.

Discussion:

PVL toxin is considered as an important marker for differentiation of HA-MRSA and CA-MRSA. The present study provides an insight into micro-epidemiology of PVL positive MRSA isolates from bloodstream infection. We observed that majority of the PVL positive HA-MRSA are associated with skin and soft tissue infection (SSTIs), but less likely with pneumonia or sepsis. Multidrug resistant (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, the present showed that all PVL positive MRSA are ciprofloxacin resistant.

PVL gene variation identified in this study was similar to the previously described non-synonymous SNPs [7,18-20]. All the PVL positive isolate was identified as "H" variant

and are capable of causing invasive disease was observed in this study. Besseyre *et al*., demonstrated that histidine to arginine amino acid substitution doesn't impaired leucotoxicity of PVL toxin [21]. Despite of PVL gene variation, H or R variant has demonstrated with significant leucotoxicity. However, possible association between other non-synonymous mutation and leucotoxicity remains unclear. This could be an important cause for evasion of host immune response to invasive disease [22]. Acquisition of PVL genes by HA-MRSA strain could raise the morbidity and mortality.

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 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 ϕ IND772PVL phage identified in this study was found to carry PVL as well *sea* toxin on the propahage 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*., has reported that PVL distribution was significantly associated with ST22-MRSA-IV (66%) as compared to ST772-MRSA-V (27%) in clinical isolates [9]. D'souza *et al*., has reported that 65% of ST22-MRSA-IV and ST772-MRSA-V were positive for PVL and 27% of them were multi-drug resistant from mixed CA-MRSA and HA-MRSA infection [10]. In contrast, the present study showed, ST772-MRSA-V (63%) was the predominant PVL positive clone and was multi-drug resistant.

Conclusion:

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

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Conflict of interest: The authors declare no conflicts of interest.

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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.

						***luk*S-PVL**			***luk*F-PVL**							
						Non-synonymous mutation		**Synonymous mutation**	**Non-synonymous mutation**	**Synonymous mutation**						
Isolate ID	**CA/HA MRSA**	**Source of MRSA sepsis**	**Antimicrobial resistant profile**	**Accession no**	**PVL gene**	**470 (T)**	**527 (G)**	**663 (T)**	**140 (C)**	**456 (A)**	**789 (G)**	**PVL encoding phage**	**SCC *mec* types**	***spa* type**	**Sequence type (ST)**	**Clonal complex (CC)**
VB9939	CA	Skin and soft tissue infection	Gen, SXT, ery, cip	MLQK00000000	+	T	**A**	**G**	C	**G**	**A**	ϕ IND772PVL	V	t657	ST722	CC1
VB16578	CA	Epidural MRSA abscess	Gen, SXT, ery, cip	MLQD00000000	+	T	**A**	**G**	C	A	**A**	ϕ IND772PVL	V	t657	ST722	CC1
VBA46389	CA	Skin and soft tissue infection	Gen, SXT, ery, cip	MLQG00000000	+	T	**A**	**G**	**T**	A	**A**	ϕ IND772PVL	V	t548	ST772	CC1
VB9352	CA	Postatic abscess	Gen, SXT, ery, cip	LXWR00000000	+	T	**A**	**G**	C	A	**A**	ϕ IND772PVL	V	t657	ST772	CC1
VB23686	HA	Epidural MRSA abscess	Gen, SXT, ery, cip	MANS00000000	+	T	**A**	**G**	C	A	**A**	ϕ IND772PVL	V	t657	ST772	CC1
VB26276	HA	Necrotising soft tissue infection	Gen, SXT, ery, cip	LWMF00000000	+	T	**A**	**G**	C	A	**A**	ϕ IND772PVL	V	t657	ST772	CC1
VBA4283	CA	MRSA sepsis	Gen, SXT, ery, cip	Yet to receive	+	**A**	**A**	**G**	C	A	**A**	ϕ IND772PVL	V	t021	ST772	CC1
VB31683	HA	Necrotising soft tissue infection	Ery, clin, cip	MANT00000000	+	T	**A**	**G**	C	A	**A**	ΦPVL	IVc	t657	ST22 (EMRSA-15)	CC22
VBA44094	HA	Necrotising soft tissue infection	Ery, clin, cip	MLQH00000000	+	T	**A**	**G**	C	**G**	**A**	ΦPVL	IVc	t474	ST22 (EMRSA-15)	CC22
VB9982	HA	Necrotising 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	Skin and soft tissue infection	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	Yet to receive	_	NA	NA	NA	NA	NA	NA	NA	IVd	t304	ST6	Singleton
VB44746	CA	Necrotising fasciitis	Susceptible to all tested antibiotics except cefoxitin	MLQF00000000	_	NA	NA	NA	NA	NA	NA	NA	IVh	t131	ST1290	Singleton

SCC – Staphylococcal cassette chromosome, *spa* – staphylococcal protein A