Clinical Epidemiology and Global Health 15 (2022) 101006

Contents lists available at ScienceDirect

Clinical Epidemiology and Global Health

journal homepage: www.elsevier.com/locate/cegh

Epidemiology of Panton Valentine Leukocidin in clinical *Staphylococcus aureus* isolates - A prospective study at a tertiary care centre in North India

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ARTICLE INFO

Keywords:
PVL
Staphylococcus aureus

ABSTRACT

Background: Staphylococcus aureus is a cause of wide range of infections. The pathogenicity of *Staphylococcus aureus* is related to a number of virulence factors. Panton-Valentine Leukocidin (PVL), a cytolysin that has the ability of leukocyte destruction and tissue necrosis is one such virulence factor determining the pathogenicity of *Staphylococcus aureus* infections. This study aims to know the distribution of *pvl* gene in various clinical specimens and associate *pvl* gene with antimicrobial resistance in *Staphylococcus aureus*, particularly MRSA.

Methods: Consecutive clinical samples including pus, blood, respiratory samples and body fluids received in laboratory, during the study period, which gave growth of *Staphylococcus aureus* were included in the study. Further antibiotic sensitivity testing was done using CLSI guidelines. Detection of *pvl gene* was done by extraction of DNA using boiling method followed by conventional PCR. The amplified DNA was analysed by 264 nm wavelength UV transillumination via Gel Electrophoresis.

Results: Out of 258 *Staphylococcus aureus pvl* gene was detected in 24.03%(62/258, $p < 0.05$). The distribution of PVL in *Staphylococcus aureus* isolates among various clinical samples was 29.9% in pus samples, 12% blood culture samples, 26.4% in respiratory samples. Only 1 of 5 body fluid *Staphylococcus aureus* isolate detected PVL. Out of 62 *pvl* gene positive isolates 79% of were MRSA and 20.9% were Methicillin sensitive.

Conclusion: Association with *pvl* gene was significantly higher in skin and soft tissue infection but it also detected in blood stream infection and pneumonia, which indicate association of *pvl* gene with invasive infection.

1. Introduction

Staphylococcus aureus is recognized as a cause of a wide range of infections. It is also a healthy colonizer of human skin and mucosa.[1] bone infections to devastating septicemia and endocarditis. *Staphylococcus aureus* is the most common cause of skin and soft tissue infections (SSTIs), ranging from the benign (eg; impetigo and uncomplicated cellulitis) to acute life-threatening necrotizing fasciitis and myositis. It is the most common pathogen isolated from surgical site infection, cutaneous abscess and purulent cellulitis.[2] Apart from superficial infections it is also associated with multiple invasive infections like bacteraemia, pneumonia, osteomyelitis etc.

The pathogenicity of *Staphylococcus aureus* is related to a number of virulence factors including various bacterial surface components, extracellular proteins and cytotoxins. However, the precise role of single virulence determinants in relation to particular infection is very difficult to establish. Panton-Valentine leukocidin (PVL), a cytolysin that has the ability of leukocyte destruction and tissue necrosis is one such virulence factor determining the pathogenicity of *Staphylococcus aureus* infections. It is frequently detected in *Staphylococcus aureus* isolates from patients with deep skin and soft tissue infections, particularly furunculosis, cutaneous abscesses, and severe necrotizing pneumonia.[2–4] PVL carrying *S. aureus* strains are more virulent and highly transmissible strains than PVL negative *S. aureus*. PVL positive *Staphylococcus aureus* strains are more toxic and have a high association with mortality and morbidity.[4]

Panton Valentine leukocidin was named after Sir Philip Noel Panton and Francis Valentine who associated it with soft tissue infections in 1932. Panton–Valentine leucocidin (PVL) cytotoxin, is a bi-component, non-haemolytic toxin that causes cytotoxic and cytolytic changes in polymorphonuclear cells, monocytes and macrophages. It encodes *pvl* gene that comprises two exoprotein subunits, encoded by LukS-PV and

Abbreviations: SSTI, Skin and Soft Tiisue Infections; MRSA, Methicillin Resistant Staphylococcus aureus.

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https://doi.org/10.1016/j.cegh.2022.101006
Received 26 February 2021; Received in revised form 8 November 2021; Accepted 9 March 2022
Available online 18 March 2022

LukF-PV.[3,4] These two co-transcribed genes act together as a subunit to form a pore by assembling in the cell membranes of host immune cells particularly the white blood cells, monocytes, and macrophages.[5]

Studies from worldwide associate *PVL* harbouring strains of *Staphylococcus aureus* to skin and soft tissue infections. Prevalence of PVL in *Staphylococcus aureus* isolates have been reported from different countries, i.e., 12.8% in China, 30% in Germany, 45.3% in Japan, and most remarkably 97% in USA.[5–7] Studies from various parts India have reported prevalence of PVL gene in *S. aureus* between 16% and 64%.[8–11]

PVL-positive *Staphylococcus aureus* infections have been associated less commonly with uncomplicated suppurative skin infections such as furunculosis, abscesses and boils and More commonly with complicated necrotic SSTIs include necrotizing fasciitis and purpura fulminans.[4] These infection need immediate intervention and patient management. There is a conflict on the role of *PVL* in the pathogenicity of blood stream infections. Studies report association of *PVL* with disease severity, suggesting *PVL* to be an important factor as an epidemiological marker of bacteraemia. *PVL* carrying *S. aureus* strains are more virulent and highly transmissible strains than *PVL* negative *S. aureus*. These strains are mainly seen in young and healthy individuals who have less health care exposures, defining *PVL* to be associated with Community acquired *Staphylococcus aureus* infections.[9]

PVL positive CA-MRSA infections are common in parts of Europe and USA. The first *PVL* positive MRSA was noticed in the late 1990s and these strains got scattered worldwide in recent years. Data suggests these *PVL* producing MRSA strains have high virulence.[6,12] Limited data is available on the frequency of PVL positive MRSA isolates from various other clinical infections.

Comprehensive literature search on data in Northern India, suggests lack of data on the role of *PVL* in pathogenicity of *Staphylococcus aureus* infections. Moreover, there is undue scarcity of data regarding presence of *pvl* gene *Staphylococcus aureus* bacteraemia cases and its association with methicillin resistance. Therefore, this study aimed to find (i) distribution of PVL gene in various clinical specimens and (ii) associate *pvl* gene with antimicrobial resistance in *Staphylococcus aureus*, particularly MRSA.

2. Materials and methods

2.1. Study setting

The study was conducted at the Bacteriology laboratory, Department of Microbiology, King George's Medical University, Lucknow. The tertiary care hospital attached to medical university caters a wide variety of patient population from northern India.

2.2. Sample collection

Consecutive clinical samples including pus, blood, respiratory samples and body fluids received in laboratory, during the study period, which gave growth of *Staphylococcus aureus* were included in the study. The study was approved by Institutional ethical committee (ECMIIB-Thesis/P12) who follows the Indian Council of Medical Research (ICMR) ethical guidelines for biomedical research on human subjects.

2.3. Microbiological methods

Growth of *Staphylococcus aureus* was identified based on colony characteristics, grams staining (Gram-positive cocci arranged in clusters), catalase test (Positive) and coagulase test (Positive). These isolates were reconfirmed by MALDI-ToF (VITEK MS).

All the *Staphylococcus aureus* isolates were subjected to Kirby Bauers disk diffusion method using antibiotic disks as per CLSI 2017 guidelines.[13] For detection of methicillin resistance, cefoxitin disc 30 mg was used. A zone size of <22 was considered as resistant and the strain was reported Methicillin resistant *Staphylococcus aureus*.

2.4. Molecular methods

All *Staphylococcus aureus* isolates were stocked in 50% glycerol broth at 80degC. These stocks were revived for further molecular detection through conventional PCR of *pvl* gene and *mec*A gene. Detection of *mec* A was done only on that strains that were resistant to cefoxitin.

DNA Extraction: Genomic DNA was extracted from cultures grown on blood agar by suspending a 2–3 colonies in 200 µl nuclease free water and heating at 95 °C for 15 min, and then centrifuging at 2500 rpm for 5 min. The supernatant (200 µL) was used for PCR analysis.

PCR for detection of *PVL* gene: Pre-published primer sequences for the *pvl* genes detection, Luk-PV-1 (5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′) and Luk-PV-2 (5′-GCATCAAGTGTATTGGATAGCAAAAGC-3′) which amplify a 433 base pair fragment specific for *lukS/F –PV* genes, encoding the *PVL* S/F bicomponent proteins were used as described by McClure et al.[13] The DNA thermocycler was programmed for initial denaturation at 95 °C for 5 min; 35 cycles of amplification (denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min); and a final extension at 72 °C for 10 min.

Post PCR gel electrophoresis: To visualize, 5 µl of the PCR amplicon was loaded with dye in 1.5% agarose gel containing 0.5 µl/ml of ethidium bromide (0.5 mg/ml, Medox biotech India Pvt Ltd) along with molecular weight marker (100bp DNA ladder; Bangalore Ganei, India) followed by electrophoresis at 80 V for 2 h and multiple amplified DNA was analysed by 264 nm wavelength UV transillumination and gel was documented. Fragments of DNA 433 bp corresponded amplification of a fragment to the *pvl* genes. Fig. 1

2.4.1. Data management

The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 21.0 statistical Analysis Software. The values were represented in Number (%) and Mean ± SD.

3. Results

Out of 258 *Staphylococcus aureus* strains isolated from various clinical samples during the period of study, 144 (55.81%) were from pus samples, 75(29.07%) were blood samples, 34(13.18%) respiratory samples and 5(1.9%) samples were from various body fluids.

pvl gene was detected in 24.03%(62/258, $p < 0.05$) of all the *Staphylococcus aureus* isolates. The distribution of PVL in *Staphylococcus aureus* isolates among various clinical samples was 29.9% in pus samples, 12% blood culture samples, 26.4% in respiratory samples. Only 1 of 5 body fluid Staphylococcus isolate detected PVL (Table 1).

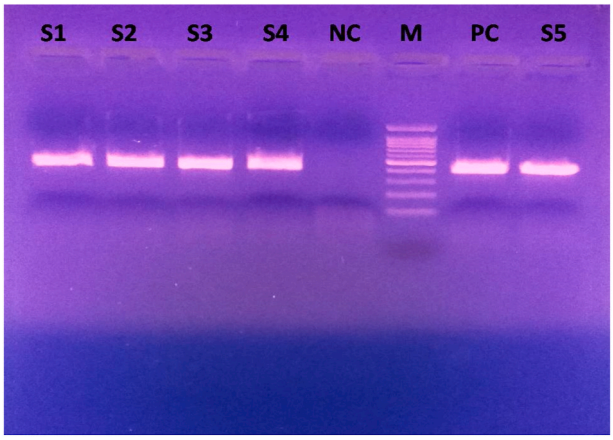

Fig. 1. Agarose gel electrophoresis result showing PVL positive samples (433 bp). M is 100 bp marker, NC is negative control, PC is Positive control and S1–S5 are PVL positive samples.

Table 1
Distribution of *pvl* gene in *S. aureus* among different clinical specimens.

Clinical Specimens	PVL +ve (%)	PVL –ve (%)	p value
Pus (n = 144)	43 (29.9)	101(70.1)	**0.002**
Blood (n = 75)	9(12)	66(88)	**0.020**
Respiratory (n = 34)	9(26.4)	25(73.5)	0.339
Body fluid(n = 5)	1(20)	4(80)	0.576

*p value < 0.05 is significant.

Antimicrobial susceptibility pattern of all *Staphylococcus aureus* isolates was analysed (see Fig. 2). Resistance to Methicillin, Amikacin, gentamycin and levofloxacin was higher in PVL gene positive isolates which was statistically significant. No association was found between resistance to other antibiotics and *pvl* gene (Fig. 2).

Out of 62 *pvl* gene positive isolates 79% of were MRSA and 20.9% were Methicillin sensitive (Table 2). Association of MRSA and PVL in various clinical isolates was analysed. 93% of pvl gene positive pus samples were MRSA (Table 3).

4. Discussion

This study confirms the presence of *pvl* gene in *Staphylococcus aureus* isolates from clinical infections in our setting. Evidence from various studies suggest that PVL positive strains carry a high predilection to invasive infections.[2,14]

In our study, 24.03% of the total *Staphylococcus aureus* isolates had *pvl* gene detected. Comparing to various studies done in the past, our results are similar to Hu Q et al., who reported a prevalence of 28.6% of PVL in various clinical isolates.[12] In the past 10 yrs, studies from various parts of India have reported higher prevalence of PVL associated infections.[9,10,15] A lower prevalence of PVL has been reported from France (5%), UK (4.9%), Saudi Arabia(8.1%), and Bangladesh(14.3%) reflecting the significant variation in prevalence of PVL among geographical areas.[2,14–16]

The distribution of *pvl* gene in various clinical samples varied in our study. Our study shows PVL positive *Staphylococcus aureus* is strongly associated with skin and soft tissue infection. Studies world-wide have demonstrated an overall prevalence of PVL associated skin and soft tissue infections ranging from 8 to 60%.[2,12,15] 30% pus samples were PVL positive which was statistically significant. This is in correlation to various studies that PVL is indicator of pathogenesis of skin and soft tissue infections. In our study PVL was strongly associated with skin and soft tissue infections as compared to rest of bacteraemia and respiratory tract infections.

Few studies have reported PVL in association with pathogenesis of bacteraemia. Our study reported 12% *Staphylococcus aureus* bacteraemia strains to be PVL positive. On the other hand, PVL is infrequently associated with *Staphylococcus aureus* bacteraemia and other infections. The cases which were PVL positive may have some underlying skin and soft tissue infection which led to generalised sepsis. Ellington MJ et al. detected only 1.6% PVL positivity in 244 blood samples.[16] Various other studies have also reported very less prevalence of PVL in *Staphylococcus aureus* bacteraemia. This suggests that PVL has no particular significance in *Staphylococcus aureus* bacteraemia in our setting. These finding are consistent with the systemic review published in met-analysis by Shallcross et al.[2]

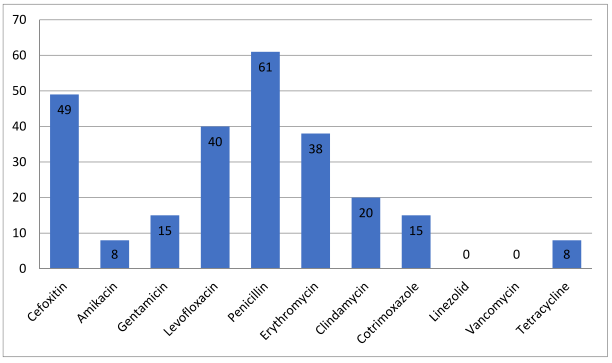

Fig. 2. Resistance to various antibiotics in *pvl* gene positive *S. aureus* isolates.

Table 2
Association of *pvl* gene with MRSA.

pvl gene	MRSA (n = 162)	MSSA (n = 96) (%)
Present (n = 62) (%)	49(79.01)	13 (20.96)
Absent (n = 196) (%)	113(57.65)	83(42.35)

$\chi^2 = 9.214$(df = 1); p = 0.002 (Sig).

Table 3
Association of PVL and methicillin sensitivity among different clinical specimens.

Clinical Specimens	PVL positive		PVL negative	
	MRSA (%)	MSSA (%)	MRSA	MSSA
Pus (144)	40(93.02)	3(6.9)	33	68
Blood (75)	6(66.66)	3(33.33)	52	14
Respiratory(34)	3(33.33)	6(66.66)	25	0
Body fluid (5)	0(0)	1(100)	3	1

Reports from countries world-wide show increasing prevalence of *pvl* gene positivity among MRSA isolates[17–19] The first PVL positive MRSA was noticed in the late 1990s and these strains got scattered worldwide in recent years. The role of PVL in increasing virulence of *S. aureus* leading to MRSA and increasing pathogenicity is being deliberated. On comparing the association of *pvl* gene among MRSA and MSSA isolates from different clinical samples, though association of *pvl* gene was higher among MRSA as compared to MSSA for all the clinical samples but differences were found to be statistically significant only for pus samples (39.8% vs. 16.4%; p = 0.002). Subarna Roy et al. from India, have reported overall 62.85% of PVL prevalence and association among MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%).[20] Similar study by D'Souza et al. from Mumbai, India, reported prevalence of 64% PVL positive isolates among MRSA.[8] Clinically, presence of *pvl* gene cluster along with methicillin resistance is a major concern in treating *Staphylococcus aureus* infections as it can lead to recurrent skin and soft tissue infections and even life-threatening conditions like necrotising fasciitis, necrotising pneumonia and even osteomyelitis. PVL positive MRSA cases have reported to have a prolonged hospital stay, though we could not correlate it in our study. Case series from USA,[2] France[3] have reported an enormous high mortality, prolonged hospital stay and even life-threatening conditions in PVL positive *Staphylococcus aureus* infections.[2]

This study analysed the sensitivity patterns of other antibiotics on *pvl* gene positive isolates. The percentage of MDR *Staphylococcus aureus* among PVL positive isolates was higher than PVL negative isolates of *Staphylococcus aureus*. None of the PVL positive isolate was resistant to Linezolid and Vancomycin. The antibiotic resistance was higher for Penicillin, levofloxacin, erythromycin, clindamycin and trimethoprim-sulfamethoxazole drugs, suggesting frequent use of these antibiotics in the community.

This is the first study done in this area, and has confirmed the prevalence of PVL in this setting and association of MRSA to PVL positive infections. Thus, detection of PVL in various *Staphylococcus aureus* infections is necessary to know the pathogenesis of the disease and to understand the impact of disease for the treating physician.

However, there are several limitations of our study. Firstly, as it was time bound study and specimen from SSTIs was greater in number than blood, it required further studies with larger sample size for better association PVL gene with blood stream infection. It needs further studies

for better understanding of clonal diversity and its association with PVL gene or antimicrobial susceptibility pattern. Wider application of molecular typing (detection of PVL gene, *mec*A, SCC*mec* typing) is of utmost importance to understand the pathogenesis, disease severity and final outcome of different types of *Staphylococcus aureus* infections.

5. Conclusion

This study demonstrates the presence of PVL gene cluster in clinical *Staphylococcus aureus* infections ranging from skin and soft tissue to bacteraemia in our setting, in Northern India. As PVL is a marker of pathogenicity and PVL positive strains are more virulent, these cases need to be monitored and adequately treated. Antibiotic therapies need to guide for these cases as these strains are potentially Methicillin resistant. Appropriate clinical and public health measures such as screening and decolonisation should be undertaken for PVL positive *Staphylococcus aureus* infections.

Funding

None.

Competing interest

None to declare.

Ethical approval

The work plan of the present study was approved by the Ethics Committee of King George's Medical University, Lucknow (UP).

Authorship

Category 1 Conception and design of study: Dr. Vimala Venkatesh, Dr. Rani Jaiswal, acquisition of data: Dr Rani Jaiswal, Dr. Aditi Garg, Dr. Piyush Tripathi analysis and/or interpretation of data: Dr. Vimala Venkatesh, Dr. Rani Jaiswal, Dr. Aditi Garg, Dr. Piyush Tripathi.

Category 2 Drafting the manuscript: Dr. Aditi Garg, Dr Rani Jaiswal, revising the manuscript critically for important intellectual content: Dr. Vimala Venkatesh, Dr. Aditi Garg Dr. Piyush Tripathi.

Category 3 Approval of the version of the manuscript to be published. Dr. Rani Jaiswal Dr. Aditi Garg Dr. Piyush Tripathi, Dr. Vimala Venkatesh,

Acknowledgement

Authors thank to contribution of staff of bacteriology Laboratory, Department of Microbiology for their immense help and support

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