Detection of Pvl gene for the presence of leukocidin among clinical isolates of staphylococcus aureus

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Running Title: Detection Pvl gene in S.aureus.

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Abstract:

Aim: Detection of Pvl gene for the presence of leukocidin among clinical isolates of staphylococcus aureus

Background: PVL and γ -haemolysin are considered to be members of a toxin family known as synergohymenotropic toxins, as they act on cell membranes by the synergy of two proteins that form a pore. Only 2% of S. aureus isolates produce PVL, while γ -haemolysin is produced by more than 99% of S. aureus isolates. PVL is the most leukocytolytic toxin in the family, however it does not exhibits no haemolytic activity on human erythrocytes. A sum of 20 clinical isolates of S. aureus were subjected to antibiotic sensitivity pattern followed by the detection of pvl gene by PCR. We have observed increased resistance to most of the routinely used antibiotics and 10% of our isolates found to have pvl gene. As this gene is directly associated with skin and soft tissue infections by S. aureus, our two isolates may even cause such infections, although none of these strains were not obtained from cutaneous lesions. Panton–Valentine leukocidin (PVL) is one of many toxins associated with S. aureus infection. Because it can be found in virtually all CA-MRSA strains that cause soft-tissue infections, it was long described as a key virulence factor, allowing the bacteria to target and kill specific white blood cells known as neutrophils. This view was challenged, however, when it was shown that removal of PVL from the two major epidemic CA-MRSA strains resulted in no loss of infectivity or destruction of neutrophils in a mouse model.

Materials and Method: These antibiotics were procured from Himedia, Mumbai. This was performed by Kirby-bauer disc diffusion method as per CLSI guidelines.

Conclusion: The pathogenicity of *Staphylococcus aureus* depends on various bacterial surface components and extracellular proteins. uggests that the Panton-Valentine leukocidin (PVL) is 1 such virulence factor that has a major role in pathogenicity.

Keywords: Staphylococcus aureus, Pvl gene, PCR, Leukocidin ,Haemolysin.

Introduction:

Staphylococcus aureus is an important human pathogen that causes a wide range of diseases from mild superficial skin infection to life-threatening bacteremia and infective endocarditis, as well as toxin-mediated conditions such as toxic shock syndrome.[1] They produce more than 30 different extracellular products.[2]Nearly all strains exhibit a group of enzymes and cytotoxins that includes haemolysins (α, β, γ and δ), nucleases, proteases, lipases, hyaluronidase and collagenase. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA-E, G-I), the exfoliative toxins (ETA and ETB) and Panton–Valentine leukocidin (PVL) [3].

Two major mechanisms of eukaryotic cell death have been identified, namely necrosis and apoptosis. Necrotic cell death has classically been considered as a passive process resulting from physical or chemical injury. In contrast, cells undergoing apoptosis play an active role in their own demise: extracellular stimuli or genetic programming activates a cascade of intracellular events resulting in morphological and biochemical alterations. Apoptosis has already been described in monocytes and PMNs exposed to S. aureus α-toxin and Fusobacterium necrophorum leukotoxin, respectively (15, 16). PVL-sensitive target cells, including PMNs, macrophages, and the promyelocytic cell line HL-60 (8, 17, 18) are thought to undergo osmotic lysis secondary to pore formation in the cell membrane. This process would be consistent with necrosis.

Although PVL has potent membrane-disturbing and cytolytic activities in vitro, the mechanisms by which it exerts its lethal effects on target cells, particularly on the first line of host defense cells such as neutrophils, and the sequence of events in the overall cytotoxicity are not known. Furthermore, in other clinical situations such as fulminant hepatitis (19), the apparent predominance of necrosis has been shown to be actually secondary to apoptosis. Hence, the mechanisms by which PVL causes injury to host tissues and potentially to peripheral blood cells during the course of infection are not clearly established, and the role of necrosis versus apoptosis in the pathogenesis of PVL-associated infections remains to be defined. The focus of the present study was to characterize the biological effects of S. aureus PVL on human peripheral PMNs in relation to the possible involvement of this toxin in necrotizing pneumonia pathogenesis.

Apoptosis was only triggered at low PVL concentrations, whereas higher concentrations induced necrotic alterations. These different actions may be related to the molecular properties of PVL. At low concentrations, PVL, which binds to specific but unidentified cell surface receptors (17), could produce small numbers of octameric pores (9). These pores could favor the entry of other PVL molecules into the cell, allowing the toxin to act on mitochondria and induce apoptosis. However, at high concentrations (200 nM), PVL may nonspecifically adsorb to the lipid bilayer, forming larger pores that are Ca2+ permissive, forming more numerous octameric pores, or leading to Ca2+ channel opening (36), all of which can result in necrosis. Moreover, larger pores would also lead to a loss of ATP, which is required for most apoptotic processes (37, 38). Thus, the modes of cell death in vivo (necrosis versus apoptosis) may critically depend of the PVL concentration. In staphylococcal infection, cells of the host tissue surrounding the bacteria are likely to be exposed to a PVL concentration gradient: necrosis would occur locally, while apoptosis would occur in the periphery and even in circulating blood cells.

In previous study, the population structure of S. aureus, isolated from the nares of healthy persons in the Rotterdam area, the Netherlands, was elucidated (8). Strains were obtained from healthy children (<19 years) and elderly persons (>55 years). Invasive strains (blood culture, skin and soft tissue infections, and impetigo isolates) were included in this study (Table). All carriage and clinical isolates (n = 1,033) were mecA negative. We used the same strain collection to study the PVL prevalence in carriage and invasive isolates of S. aureus from a single geographic region.

Detection of pvl gene in Staphylococcus aureus:

Staphylococcus aureus isolates were detected for the presence of *pvl* gene by PCR analysis. Detection of the gene was carried out using primer as depicted in table 1. Bacterial DNA was extracted by boiling lysis method. 1 µL of DNA extract was used as template for PCR reaction. The reaction mixture contained 2mM of $Mgcl_2$ 0.2mM dNTP mix and 0.8µM of *pvl* gene with IU of Taq polymerase (New England Biolabs) in a 1x PCR buffered reaction. A positive control of S. aureus with *pvl* gene was also included in this study. PCR amplification was carried out using thermal cycler (Eppendorf) with the following cycling condition. Initial denaturation at 96°C for 2 min and 30 cycles for 30s, 52°C for 60s and 73° C for 30s, followed by a final extension of 6 min at 72°C. PCR products were resolved in 1.5% agarose gel. A 100bp ladder was including in all the gel analysis. [8]

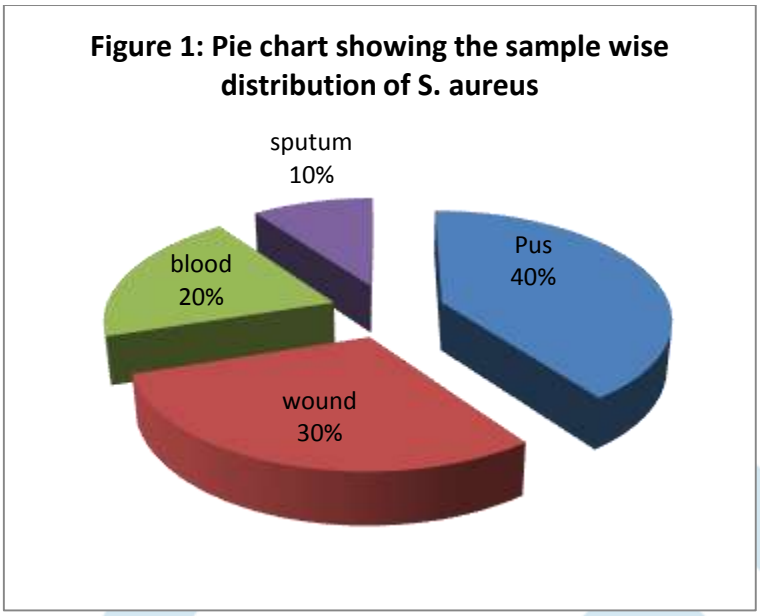
Table 1: Primer detail of *pvl* gene

Primer	Primer sequence	Product size
pvl	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A GCA TCA AST GTA TTG GAT AGC AAA AGC	433 bp

Results:

Sample wise distribution of clinical isolates of S. aureus

Of 20 clinical isolates of S. aureus, 8/20 (40%) were obtained from pus, 6/20 (30%) were from wound, 4/20 (20%) and 2/20 (10%) were from blood and sputum respectively (Figure 1).



Antibiotic susceptibility pattern

We have observed a varied pattern of sensitivity among one *S.aureus* isolates. There was complete resistance observed for penicillin(100%), 9/20(45%)isolates were shown to the resistant to erythromycin,6/20(30%) were to cotrimoxazole,4/20(20%)were to linezolid followed by 3/20(15%) were resistant to ciprofloxacin and clindamycin respectively (Table 1).

Table 2: Results of antibiotic susceptibility pattern of S.aureus

Antibiotics	**Sensitive(%)**	**Intermediate(%)**	**Resistant(%)**
Penicillin	0	0	20(100)
Erythromycin	14(70)	4(20)	2(10)
Clindamycin	15(75)	2(10)	3(15)
Ciprofloxacin	9(45)	8(40)	3(15)
Tetracyclin	14(70)	4(20)	2(10)
Cotrimoxazole	10(50)	4(20)	6(30)
Linezolid	10(50)	6(30)	4(20)

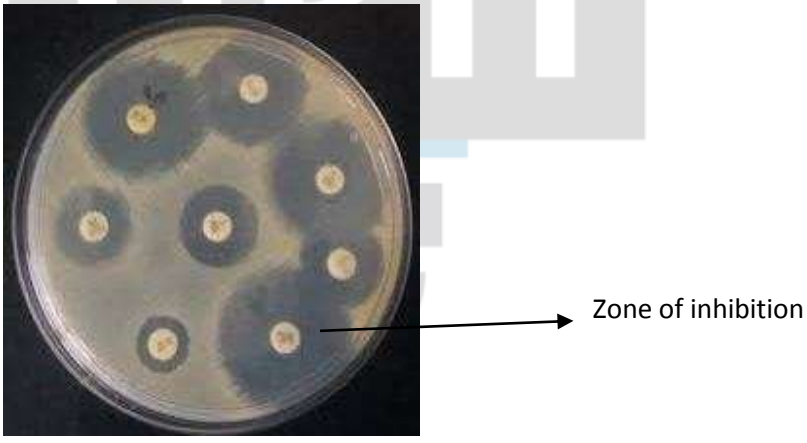
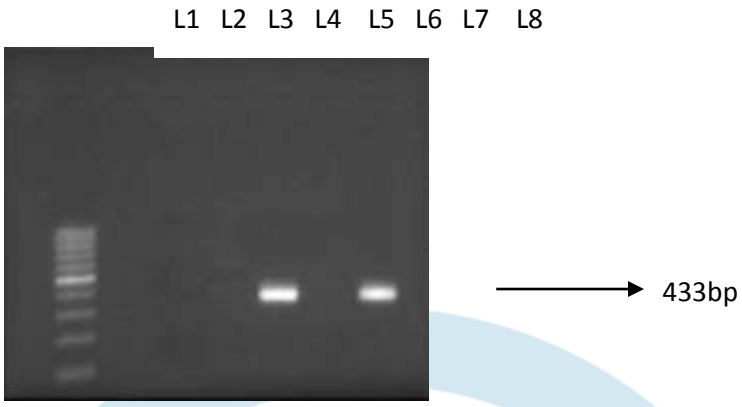


Figure 2: Representative picture showing antibiotic sensitivity pattern of *S. aureus*

Result of *pvl* gene in Staphylococcus aureus:

2/20 (10%) clinical isolate of S. aureus were found to possess *pvl* gene.

Figure 3: Representative gel picture showing pvl gene



-100bp; L6,L8-pvl gene positive

Discussion:

S. aureus is one of the most common Gram-positive bacterial pathogens in humans. It is an opportunistic pathogen that colonizes the skin of approximately 20% of the population without causing clinical symptoms (22, 23). However, breached mucocutaneous membranes or impaired host immunity facilitate tissue invasion and bloodstream dissemination of S. aureus (24). This bacterium can cause serious infections often associated with abscess formation such as osteomyelitis, endocarditis, and pneumonia that often require a prolonged and aggressive antibiotic treatment. Among the most serious complications of S. aureus infections are manifestations of septic and toxic shock syndromes that may lead to multiple organ failure (25). We recently described a novel clinical entity, S. aureus necrotizing pneumonia, which is associated with a massive cell lysis of host pulmonary tissue and is highly lethal in young immunocompetent patients (1). We also found that these cases were strongly associated with S. aureus strains producing the otherwise very infrequent exotoxin PVL (1). In the present report, we demonstrated that PVL is a remarkable bacterial virulence factor because this toxin is localized in the pulmonary lesions of patients who died from necrotizing pneumonia and induces PMN apoptosis by directly targeting mitochondria.

Staphylococcal infections are typically associated with tissue death. Induction of apoptosis by S. aureus may cause tissue damage, compromise the antimicrobial immune response, and thereby facilitate bacterial spread. S. aureus can induce apoptosis of epithelial cells (26–28), endothelial cells (29–31), keratinocytes (32), osteoblasts (33), lymphocytes, and macrophages (34). In epithelial cells, keratinocytes, or osteoblasts, S. aureus–induced apoptosis may require internalization of the bacteria (26, 27, 30, 32, 33). Conversely, Bantel et al. (16) showed that S. aureus α-toxin does not require bacterial internalization to induce apoptosis. Similarly, our data indicate that bacterial invasion of PMNs is not required for PVL to induce apoptosis. Hence, soluble PVL can be regarded as a mediator of S. aureus–induced neutrophil apoptosis, depleting neutrophils available for phagocytosis. Neutrophils transit through the human circulation en route to tissues, where they form the first line of cellular defense against invading bacterial pathogens. Phagocytosis of complement-opsonized targets is a primary function of neutrophils at sites of inflammation, and clearance, by apoptosis, of PMNs that have phagocytosed microbes is important for the resolution of inflammation (35). By inhibiting neutrophil phagocytosis, PVL could enhance the pathogenicity of S. aureus. Neutropenia is significantly more frequent in patients with necrotizing pneumonia caused by PVL-positive S. aureus than in patients with pneumonia caused by PVL-negative S. aureus (1) and could be due to the presence of PVL in pulmonary endothelial cells. Circulating PMNs could encounter PVL during their passage into the pulmonary vessels and therefore enter the cascade that leads to their apoptosis. As the cellular systems used in this study may not accurately reflect host-pathogen interactions in vivo, we cannot exclude the possibility that other staphylococcal exoproteins might contribute to human cell death.

In the present study, we have detected pvl gene in 2/20 (10%) of our S. aureus isolates. Similar kind of study conducted by Johnsson and colleagues in 2004 reported PVL gene was detected in one out of 65 S. aureus isolates collected prospectively from septicaemic patients.[9] This finding was in agreement with our study and other previous studies showing prevalences of 0–2% in positive blood cultures.[10] In addition, none of the S. aureus isolates causing infective endocarditis was PVL-positive.[11]Thus, PVL does not seem to represent an important virulence factor in invasive bloodstream infections. Our isolates were not collected from skin and soft tissue related infection, even though, two strains were showed positive for this gene. In cutaneous infections, PVL has been associated more frequently with direct invasion and tissue destruction (e.g., necrotising primary skin infections such as furunculosis) than with secondary infections after skin injury. he Staphylococcus aureus Panton-Valentine leukocidin (PVL) is a pore-forming toxin secreted by strains epidemiologically associated with the current outbreak of community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) and with the often-lethal necrotizing pneumonia. To investigate the role of PVL in pulmonary disease, we tested the pathogenicity of clinical isolates, isogenic PVL-negative and PVL-positive S. aureus strains, as well as purified PVL, in a mouse acute pneumonia model.

Isolates categorized by type of staphylococcal infection revealed that PVL positive isolates were strongly associated with superficial abscesses and other skin and soft tissue infections, whereas the association of the PVL positive isolates with bone and joint infections was low. These results confirm reports from previous studies where it was detected that 93% of PVL positive S. aureus isolates were associated with furunculosis and other skin and soft tissue infections [6]. The current study and recent reports from Europe demonstrate that PVL positive methicilin susceptible S. aureus has emerged as a significant cause of skin and soft tissue infections and invasive infections such as necrotising pneumonia, soft tissues necrosis [24-26]. Alhough PVL positive S. aureus are often associated with fatal necrotising pneumonia cases in the present study there were only two PVL positive MSSA caused pneumonia cases with positve outcome.

PVLpositive S. aureus is isolated rarely from cases of folliculitis or impetigo. PVL and γ -haemolysin are considered to be members of a toxin family known as synergohymenotropic toxins, as they act on cell membranes by the synergy of two proteins that form a pore. Only 2% of S. aureus isolates produce PVL, while γ -haemolysin is produced by more than 99% of S. aureus isolates. [4]Pvl is the most leukocytolytic toxin in the family, however it does not exhibits no haemolytic activity on human erythrocytes.[5] It also dermo-necrotic, as observed after .intradermal injection of rabbit skin. [6] At sub-lytic concentrations, PVL has been demonstrated to induce granule secretion and release of leukotriene B4 and interleukin-8 from human polymorphonuclear leukocytes.[6] Based on this background, we have undertaken this study to detect the presence of pvl gene among S. aureus by PCR. In 1932, Panton and Valentine described PVL as a virulence factor belonging to the family of synergohymenotropic toxins (4). These toxins form pores in the membrane of host defense cells by synergistic action of 2 secretory proteins, designated LukS-PV and LukF-PV, which are encoded by 2 cotranscribed genes of a prophage integrated in the S. aureus chromosome (5). PVL is mostly associated with community-acquired methicillin-resistant S. aureus (MRSA) infections and distinguishable from nosocomial MRSA by nonmultidrug resistance and carriage of the type IV staphylococcal chromosome cassette element .

Conclusion:

In our study, we have seen only 10% of them were found to harbor pvl gene. As this gene is directly associated with skin and soft tissue infections by S. aureus, our two isolates may even cause such infections, although none of these strains were not obtained from cutaneous lesions. In order to prove its virulence, it is necessary to include more samples especially from cutaneous lesions.

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