CLINICAL ARTICLES

Involvement of Panton-Valentine Leukocidin–Producing *Staphylococcus aureus* in Primary Skin Infections and Pneumonia

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Panton-Valentine leukocidin (PVL) is a cytotoxin that causes leukocyte destruction and tissue necrosis. It is produced by fewer than 5% of *Staphylococcus aureus* strains. A collection of 172 *S. aureus* strains were screened for PVL genes by polymerase chain reaction amplification. PVL genes were detected in 93% of strains associated with furunculosis and in 85% of those associated with severe necrotic hemorrhagic pneumonia (all community-acquired). They were detected in 55% of cellulitis strains, 50% of cutaneous abscess strains, 23% of osteomyelitis strains, and 13% of finger-pulp–infection strains. PVL genes were not detected in strains responsible for other infections, such as infective endocarditis, mediastinitis, hospital-acquired pneumonia, urinary tract infection, and enterocolitis, or in those associated with toxic-shock syndrome. It thus appears that PVL is mainly associated with necrotic lesions involving the skin or mucosa.

The pathogenicity of *Staphylococcus aureus* infections is related to various bacterial surface components (e.g., capsular polysaccharide and protein A), including those recognizing adhesive matrix molecules (e.g., clumping factor and fibronectin binding protein), and to extracellular proteins (e.g., coagulase, hemolysins, enterotoxins, toxic-shock syndrome [TSS] toxin, exfoliatins, and Panton-Valentine leukocidin [PVL]) [1]. In general, the precise roles of individual staphylococcal factors in invasive infections are difficult to assess, but PVL production has been preferentially linked to furuncles, cutaneous abscesses, and severe necrotic skin infections [2–4].

PVL, together with γ-hemolysin and other leukocidins (e.g., bovine leukocidin R), belongs to the recently described family of synergohymenotropic toxins [5]. These toxins damage membranes of host defense cells and erythrocytes by the synergistic action of 2 nonassociated classes of secretory proteins, designated S and F [6]. γ-hemolysin is produced by >99% of *S. aureus* clinical strains; its activity results from three proteins (HlgA and HlgC, belonging to class S, and HlgB, belonging to class F) forming 2 (S + F) pairs: HlgA + HlgB and HlgC + HlgB. PVL was detected in <5% of *S. aureus* isolates from a general hospital [4].

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All PVL-producing isolates produce the class S and class F proteins specific for PVL (LukS-PV and LukF-PV, respectively), as well as the three proteins forming γ-hemolysin. Hence these strains produce 3 class S and 2 class F proteins, resulting in 6 (S + F) biologically active pairs: HlgA + HlgB, HlgC + HlgB, LukS-PV + HlgB, HlgA + LukF-PV, HlgC + LukF-PV, and LukS-PV + LukF-PV. The 2 pairs composing γ-hemolysin have leukotoxic properties and are also able to lyse rabbit, sheep, and human erythrocytes, whereas purified PVL is only leukotoxic (by pore induction) for rabbit and human polymorphonuclear cells (PMNs) and macrophages [6, 7]. Purified PVL induces severe inflammatory lesions when injected intradermally in rabbits, leading to capillary dilation, chemotaxis, PMN infiltration, PMN karyorrhexis, and skin necrosis [4, 8]. By contrast, γ-hemolysin is inflammatory but not necrotic in the rabbit skin model [9, 10].

The importance of PVL as a potential virulence factor led us to investigate the frequency of PVL-producing *S. aureus* in diverse clinical syndromes, including not only spontaneous primary infections and secondary infections after skin injury but also various deep-seated infections such as pneumonia, infective endocarditis, osteomyelitis, and enterocolitis. For this purpose, a simple and rapid PCR method was developed and used to detect the PVL and γ-hemolysin genes.

Materials and Methods

Characteristics of clinical cases and strains. The *S. aureus* strains were sent to the Centre National de Référence des Toxémies à Staphylocoques (Lyon, France) between 1 January 1985 and 30

September 1998 for various purposes, such as: (1) detection of toxin production in the context of TSS, scalded skin syndrome, bullous impetigo, furunculosis, and severe infections (infective endocarditis, pneumonia, cellulitis, etc.); (2) molecular typing in the context of nosocomial outbreaks; and (3) molecular identification for strains difficult to identify by conventional methods. From this collection, a subset of 172 *S. aureus* isolates was chosen for the present study, which represented only a fraction of staphylococcal infections occurring in France between those dates.

These strains were collected from hospitals throughout France and were associated with the following syndromes. Seventy-four cases of pyogenic skin infections were recognized after a gram stain of pus showed many polymorphonuclear leukocytes with intracellular and/or extracellular gram-positive cocci and a culture yielded pure growth of *S. aureus* on blood agar plates. The types of infections in these 74 cases were defined according to the methods of Roberts and Rook [11] and included 30 cases of furuncles, characterized by necrotic infection of the hair follicles and involment of subcutaneous tissue; 10 cases of superficial folliculitis, characterized by superficial infection (pustules) of the hair follicles, confined to the ostium, with no involvement of subcutaneous tissue; 15 felons, characterized by infection in the tightly enclosed fibrous compartments of the finger pulp and usually acquired by pin- or thorn-prick injury [12]; 4 cases of nonbullous impetigo, defined as very thin vesicles (created by splitting of the epidermis just below the stratum granulosum) that became covered with a crusting exudate; 9 cases of cellulitis, defined as a skin infection spreading to subcutaneous tissue; and 6 abscesses, defined as subcutaneous infection with no involvement of sebaceous glands or hair shafts.

Forty cases of pneumonia were defined according to published recommendations [13], by acute onset of respiratory symptoms, by physical findings such as crackles and rhonchi, by radiographic signs (often with pneumatoceles in community-acquired cases), and by bacterial diagnosis, on the basis of at least one of the following biological criteria: (1) identification of *S. aureus* from puncture of a pleural abscess or lung abscess; (2) positive *S. aureus* culture (by semiquantitative methods) of a bronchoalveolar lavage specimen ($\geqslant 10^4$ cfu/mL), Wimberley brushing sample ($\geqslant 10^3$ cfu/mL), or protected tracheal aspirate ($\geqslant 10^3$ cfu/mL); (3) positive *S. aureus* culture of pleural liquid, with similar bacteria isolated from tracheal secretions; and/or (4) positive blood culture, with a similar *S. aureus* strain isolated from tracheal secretions. Hence, 27 of the 40 cases of pneumonia were considered community-acquired, as they occurred within 24 hours before admission, and 13 cases were considered hospital-acquired, as they involved patients in intensive care units.

Urinary tract infections in 5 patients with abnormal bladder function who had undergone instrumentation or catheterization were defined by a concentration of $\geqslant 50$ leukocytes/mm^3 and a pure culture of *S. aureus*, yielding $>10^5$ cfu/mL. In addition, 5 cases of enterocolitis were identified, which were characterized by fever, abdominal distension, collapse, profuse diarrhea, and heavy growth of *S. aureus* in stool cultures.

Other types of infections were mediastinitis, which occurred in 5 patients who had undergone heart surgery and was defined by a purulent discharge from the sternal area that was culture-positive; 13 cases of osteomyelitis, i.e., infective bone lesions with positive blood cultures; 21 definite cases of infective endocarditis, selected on the basis of the Duke criteria [14]; and 9 cases of TSS fulfilling the criteria for definite cases [15]. Cases were assessed on the basis of a proforma sent to the clinicians and/or the microbiologists in charge of the patients.

Strains were characterized as *S. aureus* by their ability to coagulate citrated rabbit plasma (bioMérieux, Marcy-l'Etoile, France) and to produce a clumping factor (Staphyslide Test, bioMérieux). Strains from patients with deep-seated or severe staphylococcal infections such as pneumonia, infective endocarditis, osteomyelitis, and mediastinitis were usually isolated from a number of different sites, e.g., blood, throat, tracheal aspirate, bronchoalveolar lavage fluid, cardiac valve, wound, skin, and/or soft tissue. When multiple *S. aureus* strains were isolated from a single patient, only one isolate was included in this study (preferentially that from blood culture or from the infected site, e.g., bronchoalveolar lavage in cases of pneumonia).

S. aureus ATCC 49775 served as the reference strain for PVL and γ-hemolysin production; it was originally isolated from a patient with chronic furunculosis [16] and initially used to characterize the genes coding for PVL [5]. *S. aureus* Newman was used as the *hlg*-positive control strain (negative for *lukS-PV* and *lukF-PV*), and its *hlg*-deficient derivative strain (N65), constructed by allelic replacement of the whole *hlg* locus (*hlgA, hlgC,* and *hlgB*), was used as a negative control for both the PVL and γ-hemolysin genes [17].

Characterization of PVL and γ-hemolysin production by S. aureus *isolates.* Genomic DNA was extracted from cultures grown on agar plates and was then used as a template for amplification [18]. Oligonucleotide primers were designed according to the published sequences of the PVL genes (GenBank accession numbers X72700 and AB006796) and the γ-hemolysin genes (GenBank accession numbers X81586 and L01055), the former to obtain coamplification of *lukS-PV* and *lukF-PV* [19, 20], and the latter to obtain coamplification of *hlgC* and *hlgB*. The primer sequences for the PVL genes were as follows: for *luk-PV-1*, 5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′; for *luk-PV-2*, 5′-GCATCAASTGTATTGGATAGCAAAAGC-3′. The primer sequence for the γ-hemolysin genes were as follows: for *hlg-1*, 5′-GCC AATCCGTTATTAGAAAATGC-3′; for *hlg-2*, 5′-CCATAGACGTAGCAACGGAT-3′.

The primers do not amplify other class S or F genes, i.e., *lukE-lukD* of *S. aureus* (GenBank accession number Y13225) and *lukS-I* and *lukF-I* of *Staphylococcus intermedius* (GenBank accession number X79188). DNA amplification of *gyrA* [18] was used as a control for each DNA extract to confirm the quality of the extraction and the absence of PCR inhibitors. All these oligonucleotides were synthesized by Eurogentec (Seraing, Belgium).

After amplification for 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C), the PCR products were resolved by electrophoresis through 1.5% agarose gels (Sigma, Saint Quentin Fallavier, France). This was followed by ethidium bromide staining and analysis. To assess the specificity of *lukS-PV* and *lukF-PV* amplification, PCR products were subjected to DNA sequencing (Genome Express, Grenoble, France). *S. aureus* ATCC 49775 and Newman were used as positive amplification controls for all PCR runs, whereas N65 was used as a negative amplification control.

Statistical analysis. The categorical variables were compared with the χ^2 test or with Fisher's exact test when appropriate.

Results

The *gyrA* gene was detected in all 172 *S. aureus* strains tested. The *S. aureus* reference strain ATCC 49775 gave amplification products of the expected sizes (433 bp and 937 bp) with the PVL and γ-hemolysin gene–specific primers, respectively, whereas the Newman strain gave positive amplification with the γ-hemolysin-gene–specific primers only, and no amplification was observed with strain N65 (figure 1). Confirming the specificity of the PCR conditions, DNA sequencing of amplicons from two clinical isolates (cultured from furuncles) and from the reference strain ATCC 49775 revealed PVL- and γ-hemolysin–gene sequences that were 99% identical to the published sequences (not shown).

Among the 172 clinical isolates, 64 yielded positive PVL amplification (table 1), whereas the γ-hemolysin genes were detected in all isolates. There was a definite association between detection of the PVL genes and furunculosis (93% of cases) or community-acquired pneumonia (85% of cases). *S. aureus* strains associated with other syndromes harbored the PVL genes with a frequency ranging from 13% (felons) to 50% (abscesses) and 55% (cellulitis). Conversely, the strains responsible for hospital-acquired pneumonia, impetigo, folliculitis, infective endocarditis, urinary tract infections, mediastinitis, TSS, and enterocolitis were not shown to harbor the PVL genes.

Discussion

The PCR system used in this study was shown to be specific for the PVL and γ-hemolysin genes by sequencing 3 different strains, including the ATCC reference strain, and comparing them with the published sequences. Since previous methods for PVL detection are somewhat cumbersome, requiring detection of the toxin by immunodiffusion with rabbit antibodies [3, 21] or DNA hybridization with oligonucleotide probes [4], this simple method should prove useful for routine testing.

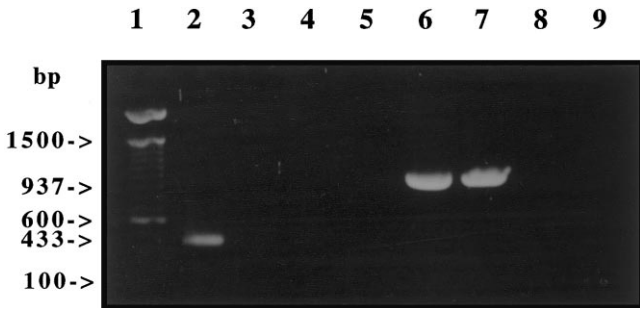

Figure 1. Detection of the *luk-PV* genes (lanes 2–5) and *hlg* (lanes 6–9) by polymerase chain reaction and analysis by 1.5% agarose gel electrophoresis. Lane 1, molecular weight marker; lanes 2 and 6, strain ATCC 49775 (*lukS-PV*$^+$, *lukF-PV*$^+$, *hlgA*$^+$, *hlgC*$^+$, *hlgB*$^+$); lanes 3 and 7, strain Newman (*lukS-PV*$^-$, *lukF-PV*$^-$, *hlgA*$^+$, *hlgC*$^+$, *hlgB*$^+$); lanes 4 and 8, strain N65 (*lukS-PV*$^-$, *lukF-PV*$^-$, *hlgA*$^-$, *hlgC*$^-$, *hlgB*$^-$); lanes 5 and 9, H_20.

Table 1. Production of Panton-Valentine leukocidin by 171 *Staphylococcus aureus* strains associated with various clinical syndromes.

Type of infection	No. of strains tested	No. (%) of PVL-positive strains	*P* value
Pneumonia			
Hospital-acquired	13	0 (0)	—[a]
Community-acquired	27	23 (85)	<.001
Skin infection			
Superficial folliculitis	10	0 (0)	—[b]
Impetigo	4	0 (0)	NS
Finger pulp (felon)	15	2 (13)	NS
Cutaneous abscess	6	3 (50)	.03
Cellulitis	9	5 (55)	.01
Furunculosis	30	28 (93)	<.001
Other infection			
Infective endocarditis	21	0 (0)	—[c]
Osteomyelitis	13	3 (23)	NS
Urinary tract infection	5	0 (0)	NS
Enterocolitis	5	0 (0)	NS
Mediastinitis	5	0 (0)	NS
Toxic-shock syndrome	9	0 (0)	NS

[a] Reference group for statistical analysis for pneumonia.
[b] Reference group for statistical analysis for skin infections.
[c] Reference group for statistical analysis for infective endocarditis.

PVL has been detected only occasionally in strains of *S. aureus* from western Europe (<5%) [4]. An interesting finding in our study was that the PVL genes were much more frequent in strains causing disease by direct invasion and tissue destruction (primary skin infections and primary community-acquired pneumonia) than in strains causing secondary infections after skin injury, hospital-acquired pneumonia, infective endocarditis, mediastinitis, urinary tract infection, TSS, or enterocolitis.

Strains isolated from primary necrotic infections involving the dermis, such as furuncles, harbored the PVL genes in 93% of cases, confirming the results of Couppié et al. [2], who detected the toxin in 86% of the *S. aureus* strains responsible for furuncles. Furuncles result in a central area of subcutaneous necrosis that can be due to direct activation/lysis of PMNs by PVL. In contrast, superficial, nonnecrotic processes such as impetigo and folliculitis were not associated with PVL-harboring *S. aureus,* again confirming the observations of Couppié et al. [2]. Other subcutaneous infections were associated with a moderate percentage of PVL-positive strains (50% of abscesses and 55% of cases of cellulitis) or a very low percentage (13% of felons, which were defined as infections of the finger pulp).

It cannot be excluded that some of the abscesses (those caused by PVL gene–positive strains) had primarily been true furuncles in which the hair sheath was destroyed by the necrotic process and so presented as primary skin abscesses. Conversely, most of the abscesses associated with PVL gene–negative isolates could have been caused by another pathophysiological mechanism such as skin injury. Similar observations were made for felons, which are mainly considered secondary to skin damage; felons were rarely associated with PVL gene–positive

strains (13%). The high proportion (45%) of cases of cellulitis associated with PVL gene–negative strains might be explained by a secondary role of *S. aureus* in a number of instances, as suggested by Leppard et al. [22].

As soon as we suspected the importance of PVL in this syndrome in the course of this study, we collected as many staphylococcal strains as possible that were associated with primary community-acquired pneumonia. The PVL genes were detected in 23 of the 27 *S. aureus* strains causing primary community-acquired pneumonia, including all the strains associated with the 14 fatalities. Since cases of pneumonia referred to the Centre National de Référence des Toxémies à Staphylocoques are usually those considered severe, the distribution of PVL genes in the corresponding strains may not reflect their actual prevalence in community-acquired staphylococcal pneumonia. These cases mainly involved immunocompetent children and young adults with previously normal lungs. Autopsy usually showed diffuse bilateral necrotic hemorrhagic pneumonia, and histopathologic studies showed necrotic lesions of the tracheal mucosa and alveolar septa, with numerous clusters of gram-positive cocci (data not shown).

Typical patients had a predisposing viral infection, leukopenia, blood cultures positive for *S. aureus*, and a chest radiograph showing pneumatoceles or patchy infiltrates over the lungs, which are features that corresponded to the few severe cases previously reported [23, 24]. By contrast, hospital-acquired staphylococcal pneumonia was rarely associated with a necrotic process. Cases secondary to hematogenous dissemination from another focus such as an indwelling intravenous device were not associated with PVL gene–positive *S. aureus* strains either. There is no explanation for the lack of typical nosocomial pneumonia with necrotic lesions associated with PVL-producing strains, except for the possible lack of diffusion of these PVL-producing *S. aureus* strains in the hospital environment.

The PVL genes were not detected in *S. aureus* strains responsible for deep-seated infections, such as infective endocarditis and mediastinitis, nor in those causing urinary tract infections and enterocolitis. Couppié et al. [2] detected PVL in only 1 of 86 *S. aureus* strains isolated from blood. Three of our 13 cases of osteomyelitis were due to PVL gene–positive strains (table 1). No clear clinical difference was observed between cases due to strains harboring the PVL genes and the other cases. Finally, staphylococcal syndromes associated with the toxin production, such as TSS, were not associated with PVL toxin–positive strains (table 1).

In conclusion, PVL appears to be a possible virulence factor associated with necrotic lesions of the skin and subcutaneous tissues (e.g., furuncles) and also with community-acquired severe necrotic pneumonia. Association of PVL genes with other virulence factors is not known; the PVL genes have been recently localized on a phage particle that contains, in addition to *lukS-PV* and *lukF-PV,* >60 potential open reading frames, some of which could have a participating role in virulence [25]. Experimental comparisons of isogenic PVL-positive and PVL-negative strains in relevant animal or cellular models will help us understand this question. Ultimately, this may lead to specific therapeutic approaches targeting PVL in severe PVL-related staphylococcal syndromes.

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