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Detection of Panton–Valentine leukocidin gene in *Staphylococcus aureus* by LightCycler PCR: clinical and epidemiological aspects

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ABSTRACT

The prevalence of the Panton–Valentine leukocidin (PVL) gene in *Staphylococcus aureus* was investigated with a simple, reproducible and rapid real-time LightCycler SYBR Green I PCR assay. The PVL gene was detected in one isolate from 65 patients with *S. aureus* bacteraemia, in four isolates from 55 patients with respiratory tract infections, and in two isolates from 91 patients with cutaneous infections. In contrast, 15 of 25 cutaneous isolates of methicillin-resistant *S. aureus* (MRSA) were positive. All PVL-positive cutaneous MRSA isolates were community-acquired and comprised three different clones as determined by pulsed-field gel electrophoresis. The PVL gene was detected in isolates from patients with recurrent primary skin infections and *S. aureus* bacteraemia, but PVL did not seem to be an important virulence factor in the pathogenesis of staphylococcal bacteraemia.

Keywords LightCycler, MRSA, Panton–Valentine leukocidin (PVL) gene, PCR, pneumonia, *Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is an important human pathogen that causes a diverse range of diseases, from mild superficial skin infection to life-threatening bacteraemia and infective endocarditis, as well as toxin-mediated conditions such as toxic shock syndrome [1]. *S. aureus* produces more than 30 different extracellular products [2]. Nearly all strains secrete 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].

PVL and γ-haemolysin are considered to be members of a toxin family known as synergohymenotropic toxins, since 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 >99% of *S. aureus* isolates [4]. PVL is the most leukocytolytic toxin in the family, but exhibits no haemolytic activity on human erythrocytes [5]. It is 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 B_4 and interleukin-8 from human polymorphonuclear leukocytes [4].

There are at least five different proteins in the family, divided into two compatible classes, termed S (slow-eluted) and F (fast-eluted) on the basis of their separation by column chromatography, namely HlgA, HlgC and LukS-PV (class S) and HlgB and LukF-PV (class F) [4]. The LukS-PV and LukF-PV components of PVL have been purified from a V8 strain (ATCC 49775) and were determined to be 32 and 38 kDa in size, respectively [7]. The PVL genetic determinant contains two open reading frames, *lukS-PV* and *lukF-PV*, which are 939 and 978 nucleotides in size, respectively, and are separated by a single thymine nucleotide and transcribed as a single mRNA molecule [4]. This gene has been found in

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different prophages on the genomes of different *S. aureus* strains [8,9].

Most *S. aureus* strains responsible for primary skin infections and necrotising pneumonia harbour the PVL determinant [10,11]. The PVL gene has been detected in 93% of isolates associated with furunculosis, 55% of isolates associated with cellulitis, 50% of isolates associated with cutaneous abscess, and 13% of isolates associated with finger-pulp infection, but was absent in isolates associated with superficial folliculitis and impetigo [11]. Highly lethal necrotising pneumonia caused by PVL-positive *S. aureus* has been described in previously healthy young patients [10]. In a study of 593 *S. aureus* isolates [12], 83 were PVL-positive, and 14 of these were from patients with community-acquired methicillin-resistant *S. aureus* (CA-MRSA). Two patients with necrotising pneumonia have been identified in Sweden [13], and one previously healthy patient presented at Örebro University Hospital with a pulmonary abscess caused by PVL-positive *S. aureus*. This may represent an emerging problem with the spread of PVL-positive *S. aureus* in the community. Thus, a rapid and reliable method for detecting PVL-positive isolates is required, as the optimal therapy for this disease might be antimicrobial agents in combination with intravenous immunoglobulin. The present study describes the development of a rapid real-time LightCycler PCR assay for detection of the PVL gene, and its use for determining the frequency of PVL-positive *S. aureus* isolates in a Swedish setting.

MATERIALS AND METHODS

Bacterial isolates and patients

The bacteria investigated comprised *S. aureus* isolates identified and stored at the Department of Clinical Microbiology, Örebro University Hospital, Sweden from 1999 to 2002 (Table 1). Some were from previous consecutive prospective studies, while others were collected from the routine diagnostic laboratory. Isolates from patients with *S. aureus* bacteraemia, skin infections or respiratory tract infections were investigated. *S. aureus* strains were identified by conventional methods, including DNase and coagulase tests. Colonies were suspended in preservation medium (trypicase soy broth containing yeast extract 0.3% w/v and horse serum 30% v/v) and stored at −70°C. The study was approved by the Örebro ethical committee.

Bacteraemia isolates ($n = 65$) were collected from 65 patients diagnosed with *S. aureus* bacteraemia [14]. The median age was 66 years (range: 10–91 years) and 59% were men.

Table 1. Detection of the Panton–Valentine leukocidin gene in *Staphylococcus aureus* isolates from patients with bacteraemia, various cutaneous infections and pulmonary infections

Type of infection	No. of isolates	No. of MRSA isolates	No. of PVL-positive isolates
Bacteraemia	65	0	1
Complicated	45		0
Infective endocarditis	15		0
Cutaneous infection	116		
Prospective study	43	0	1
Impetigo/SSSS	3		
Chronic wound infection	21		
Post-operative wound infection	4		
Erysipelas/cellulitis	2		
Skin abscesses	4		1
Ear secretion	3		
Paronychia	1		
Miscellaneous skin infections	5		
Retrospective study	73	25	16
Impetigo/SSSS	15		
Erysipelas/cellulitis	1		
Ear secretion	3	1	1
Skin abscesses	5	1	1
Furunculosis	5	3	4
Miscellaneous skin infections	44	20	10
Pulmonary infection	55		
Prospective study	23	0	2
Retrospective study	25	0	2
Pneumonia and bacteraemia	7	0	0

SSSS, staphylococcal scaled skin syndrome.

Consecutive *S. aureus* isolates ($n = 43$) from 39 patients with cutaneous infections were collected for 2 days in September 2002. The isolates were from inpatients ($n = 8$) and outpatients ($n = 31$). The median age of the patients was 66 years (range: 0.4–93 years) and 36% were men. A further 73 *S. aureus* isolates obtained since 1999 from 68 patients with cutaneous infections were also analysed. These isolates represented more severe infections or unusual clinical findings, and included 25 MRSA isolates from 24 patients, which were hospital-acquired (HA-MRSA) in six cases and CA-MRSA in 18 cases. The median patient age was 33 years (range: 3 days to 88 years), although 40% were aged <10 years, and 41% were men.

Pulmonary infection isolates were obtained from the following sources:

1. Twenty-one patients included in a prospective study of pneumonia aetiology conducted during 1999–2002 at the Department of Infectious Diseases, Örebro University Hospital. The median age of these patients was 71 years (range: 52–93 years). Eighteen isolates were obtained from the nasopharynx, by swab and/or aspiration. Five other isolates were obtained from representative sputum samples, i.e., >5 neutrophils/epithelial cell. In addition, eight nasopharyngeal control isolates were obtained from adult patients ($n = 114$) hospitalised for orthopaedic surgery or urinary tract, soft tissue or bone infections without respiratory symptoms.
2. Twenty-five *S. aureus* isolates, including four MRSA, from respiratory tract samples of 23 patients since 1999, were analysed. Six isolates were from nasopharyngeal swabs, two were from nasopharyngeal aspirates, three were from sputum or bronchial secretions, and 14 were from pleural effusions or lung abscesses. The median age of these patients was 52 years (range: 0.1–89 years).

3. A third group of isolates was obtained by searching a database of all patients treated at the Department of Infectious Diseases during the periods 1997–1998 and 2000–2002 for pulmonary infections and concurrent bacteraemia. Thirty-nine patients were identified, but *S. aureus* was isolated in only seven cases from blood culture. Two of these isolates had already been included in the previous group.

Detection of PVL gene

Genomic DNA was extracted from cultures grown on blood agar by suspending a few colonies in sterile water and heating at 98°C for 15 min, and then centrifuging at 13 000 *g* for 30 s. The supernatant (2 μL) was used for LightCycler (Roche Diagnostics, Mannheim, Germany) PCR analysis.

Oligonucleotide primers (forward: 5′-GTAAAATGTCTG-GACATGATCCA-3′; reverse: 5′-CAA(C/G)TGTATTGGA-TAGCAAAAGC-3′ (Scandinavian Gene Synthesis AB Köping, Sweden)) were designed from sequences used in a previous study [11] to amplify a region consisting of the last section of *lukS-PV* and the beginning of *lukF-PV* [4,15]. The specificity of the primers was assessed by performing a similarity search of the National Centre for Biotechnology Information (NCBI) sequence databases with the BLAST program (blastn) [15].

The PCR mastermix (20 μL) contained LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) with 4 mM $MgCl_2$, 0.7 μM forward primer and 0.5 μM reverse primer. The LightCycler program consisted of 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 52°C for 5 s and 72°C for 18 s, with single fluorescence acquisition at the end of each extension step. Finally, a melting curve analysis was conducted by continuous fluorescence acquisition during temperature elevation from 65°C to 95°C at 0.1°C/s. This enabled an assessment of the specificity of the amplification reaction, since the melting point is determined by the length and GC content of a specific amplicon.

A PVL-positive *S. aureus* isolate (16575) [13] was used as a positive extraction control in every amplification run. In addition, a DNA extract (4 μg/mL) prepared with the Dynabeads DNA DIRECT system I (Dynal, Oslo, Norway) was used as a positive amplification control (8 ng DNA/reaction) in every run. *S. aureus* strain CCUG 35601 (RS85) and molecular-grade water were used as negative controls.

PCR specificity

The LightCycler amplicon from control isolate 16575 was purified with the High Pure PCR Product Purification Kit (Boehringer Mannheim, Indianapolis, IN, USA) and sequenced using the same PCR primers with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK). The reactions were purified with a DyeEx Spin Kit (QIAGEN, Hilden, Germany) before analysis on an ABI PRISM 310 (PE Applied Biosystems). Sequence data were compiled and analysed with ABI PRISM AutoAssembler DNA Sequence Assembly Software (PE Applied Biosystems), and compared with sequences in the NCBI databases with the BLAST program (blastn) [15].

Pulsed-field gel electrophoresis

Preparation and *Sma*I digestion of bacterial DNA was performed with the GenePath Group 1 Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA), used according to the manufacturer's instructions, except that the agarose plugs were washed six times. Analysis was on agarose 1% w/v gels in 0.5× TBE buffer (89 mM Tris-borate, 1 mM EDTA) at 6 V/cm with ramped pulse times of 5.3–34.9 s over 20 h using a contour-clamped homogeneous electric field (GenePath System; Bio-Rad). Restriction patterns were analysed with GelCompar software v. 4.0 (Applied Maths, Kortrijk, Belgium), but were finally documented by visual examination and interpreted as recommended by Tenover *et al.* [16].

RESULTS

Amplification of the PVL gene

The *S. aureus* positive control isolate (16575) yielded a 421-bp amplification product with the developed LightCycler PCR, whereas no amplification was observed with strain RS85 (Fig. 1). The sequence of this amplicon was >99% identical to three PVL sequences published previously [8,9,17] and 98.6% identical to a fourth sequence [4]. The positive amplification control was amplified in each run with a crossing point of 23 cycles and a melting point (T_m) of 81.6 ± 0.4°C (range: 80.9–82.2°C). The positive extraction control amplified in every run showed a T_m of 81.5 ± 0.4°C (range: 80.9–82.1°C).

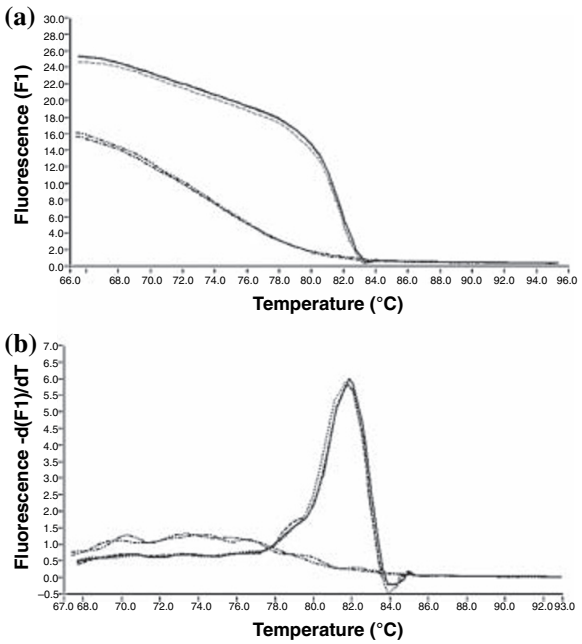

Fig. 1. Melting curve (a) and melting peaks (b) for real-time PCR using the LightCycler System with SYBR Green I for detecting the PVL gene of *Staphylococcus aureus*. Lower lines represent profiles obtained with negative controls.

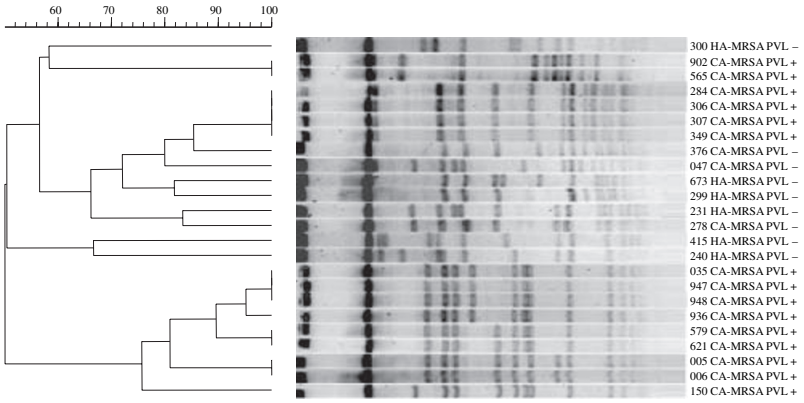

Fig. 2. Pulsed-field gel electrophoresis patterns of 24 methicillin-resistant *Staphylococcus aureus* isolates from cutaneous infections.

Detection of the PVL gene in bacteraemia isolates

Of 65 *S. aureus* bacteraemia isolates, only one was PVL-positive by LightCycler PCR (Table 1). The patient was male, aged 62 years, with an infected haematoma in the groin following percutaneous transluminal coronary angioplasty. There was no evidence of further complications. A blood cell count showed no leukopenia on admission, but did show slight thrombocytopenia. The strain did not produce staphylococcal enterotoxins A–D or TSST-1. Three *S. aureus* isolates from patients with concomitant pneumonia were PVL-negative.

Detection of the PVL gene in cutaneous infection isolates

Among the 43 clinical isolates collected consecutively during 2 days of routine activity, only one was PVL-positive. This was from a male infant aged 5 months who was being treated as an inpatient following two episodes of extensive subcutaneous abscesses.

Of the 73 isolates stored since 1999, 16 were PVL-positive by PCR. One was a methicillin-sensitive isolate from a female aged 34 years with relapsing folliculitis and a furuncle, while the other 15 were MRSA isolates from patients (median age 17 years; range 4–52 years; 53% male) with furunculosis (n = 3), abscess (n = 1), ear secretion (n = 1) or miscellaneous pyogenic skin infections (n = 10). These PVL-positive isolates were all regarded as CA-MRSA and belonged to three different genotypes as determined by pulsed-field gel electrophoresis (Fig. 2). Among the PVL-negative isolates, three were CA-MRSA and seven were HA-MRSA (obtained from six patients).

Detection of the PVL gene in pulmonary infection isolates

Among the 23 isolates from 21 patients in the prospective study of pneumonia aetiology, two were PVL-positive (Table 1), while the eight control isolates were PVL-negative. Of these 21 patients, 16 had X-ray verified pneumonia, of whom nine had no microbiological cause identified apart from *S. aureus*. *S. aureus* was isolated from the sputum of three of the nine patients (sputum samples were not obtained from three patients). One of the PVL-positive isolates was from the sputum of a female, aged 52 years, who was also serologically positive for influenza B. The other was isolated from the nasopharynx of a male aged 85 years with bronchitis. *Haemophilus influenzae* was also isolated from sputum and the nasopharynx. Both patients remained alive after 6 months.

Among the 25 respiratory tract isolates from 1999–2002, two were PVL-positive. One was isolated from the pus of a pulmonary abscess in a male aged 46 years, while the other was isolated from the bronchoalveolar lavage of a female aged 3 years who was positive for human immunodeficiency virus and was hospitalised with diffuse multilobar infiltrates.

None of the seven *S. aureus* isolates from patients with simultaneous pneumonia and bacteraemia were positive for the PVL gene.

DISCUSSION

The specific, simple and reliable PVL LightCycler assay could be performed in <2 h following the identification of *S. aureus* in pure agar culture. The method was less cumbersome than previous

techniques (e.g., immunodiffusion with rabbit antibodies) and combined PCR amplification and detection. It should therefore prove useful for routine testing.

In the present study, the PVL gene was detected once among 65 *S. aureus* isolates collected prospectively from septicaemic patients. This finding was in agreement with previous studies showing prevalences of 0–2% in positive blood cultures [6,7,18,19]. 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.

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 [6,11,18,19]. PVL-positive *S. aureus* is isolated rarely from cases of folliculitis or impetigo [6,11,18,19]. In the present study, the isolates collected consecutively from cutaneous cultures mostly represented secondary skin infections. However, the single PVL-positive isolate was from a patient with two episodes of extensive subcutaneous abscesses. Thus, this finding is not in conflict with previous knowledge.

Among the 73 *S. aureus* isolates from cutaneous cultures which had been stored for various reasons, 16 were PVL-positive. According to information received in the case referrals, most isolates were associated with primary skin infections. Fifteen of these isolates was CA-MRSA, and none of the six HA-MRSA isolates was PVL-positive. Dufour *et al.* [12] reported similar findings with French isolates. Fourteen cases of community-acquired infection were reported, caused by *mecA*- and PVL-positive *S. aureus* isolates belonging to a single clone, but the 15 PVL-positive CA-MRSA isolates in the present study belonged to three different pulsed-field gel electrophoresis clones.

The presence of the PVL gene in *S. aureus* has been associated previously with community-acquired pneumonia [11]. Gillet *et al.* [10] reported that PVL-producing *S. aureus* strains caused rapidly progressive, haemorrhagic, necrotising pneumonia with a high mortality rate, mainly in immunocompetent children and young adults. Most cases had a preceding influenza-like syndrome (fever and cough with coryza or pharyngitis), but no laboratory diagnostic procedures were undertaken to verify this. In the present investigation, one isolate was PVL-positive from nine adult patients with verified pneumonia, respiratory cultures yielding *S. aureus*, and no laboratory evidence of other aetiologies. If the diagnostic criteria for staphylococcal pneumonia were restricted to include only those patients with a positive sputum culture, one of three patients yielded PVL-positive *S. aureus*. This patient showed a significant rise in titre against influenza B (1/160–1/640). The second patient had a high single titre against influenza A (1/320), and the third showed low titres in the acute-phase serum against influenza A and B (1/20 and 1/40, respectively). Thus, an influenza infection may have preceded the development of these staphylococcal pneumonias. No further data were available on the two patients with PVL-negative *S. aureus*, as they died within 11 days of admission. The patient with PVL-positive *S. aureus* survived.

None of the eight control patients had PVL-positive *S. aureus* isolated from the nasopharynx. This is consistent with previous reports on asymptomatic nasal carriers [18,19]. However, in the present study, PVL-positive *S. aureus* was isolated from the nasopharynx of one patient with *H. influenzae* bronchitis; this may represent colonisation of the upper respiratory tract.

In the group of 25 stored isolates from 23 patients, two were PVL-positive. The first patient presented with a pulmonary abscess as well as infiltrates (X-ray and CT scan) in the left lower lobe, and had prodromal illness with flu-like symptoms. The patient's condition improved following intravenous antibiotic treatment and percutaneous drainage of pus from the lung abscess. Microscopic examination of pus from the abscess revealed disrupted leukocytes. This patient had a long history of ulcers in the anterior nares, and a culture from this location revealed a PVL-positive *S. aureus* isolate 10 days after termination of antibiotic treatment. This may indicate that this patient is a persistent carrier of a PVL-positive strain. The second patient was immunocompromised and showed miliary infiltrates of the lungs. Bronchoscopy revealed that the respiratory tract was unaffected, with no haemorrhagic or necrotic alterations. Culture from bronchoalveolar lavage showed heavy growth of *S. aureus* as well as α-haemolytic streptococci and yeast. No *Pneumocystis carinii* or

Mycobacterium tuberculosis strains were isolated. The infiltrates disappeared following treatment with trimethoprim–sulphamethoxazole.

In contrast, autopsies of patients with necrotising pneumonia associated with PVL-positive *S. aureus* have revealed extensive ulceration of the entire respiratory tract, but lack of infiltration by inflammatory cells, which could be caused by the cytolytic properties of PVL. This indicates that PVL is an important virulence factor in the development of severe necrotising pneumonia, particularly that affecting young patients, which may represent a specific clinical entity [10]. However, in the present study, the median age of the patients was relatively high and no young adults were included, which may explain, in part, the absence of necrotising pneumonia.

In conclusion, the LightCycler PCR assay was found to be a simple, reproducible and rapid method for detection of the PVL gene in *S. aureus*. PVL-positive isolates were obtained from patients with recurrent primary skin infections and *S. aureus* pneumonia, but PVL did not seem to be an important virulence factor in the pathogenesis of staphylococcal bacteraemia. Notably, all the PVL-positive cutaneous MRSA isolates were community-acquired and belonged to three different clones.

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