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Research Article

Detection of the *Panton-Valentine Leukocidin Gene* in Swedish Isolates of Methicillin-Resistant *Staphylococcus aureus* using a Multiplex PCR Assay

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ABSTRACT

Background: Panton-Valentine leukocidin (PVL) is a bicomponent leukotoxin produced by <2% of *Staphylococcus aureus* (*S. aureus*) strains. PVL forms transmembrane pores that cause cell disruption and death. PVL is associated with skin necrotic lesions and severe necrotizing pneumonia. The methicillin-resistant *S. aureus* (MRSA) strains that harbor the *lukS-PV* gene (the gene encoding PVL toxin) are considered highly pathogenic since they can cause infections that are difficult to treat.

Objective: This study aimed to optimize a multiplex PCR assay that can detect both methicillin-resistant *S. aureus* strains and their ability to produce PVL toxin isolated from Swedish patients.

Methods: An optimized multiplex PCR-assay was developed to detect both *nuc* and *lukS-PV* genes and was applied to a collection of 80 MRSA clinical isolates collected between 1999 and 2004.

Results: The results showed that 30/80 (40%) of all MRSA isolates were PVL-positive. The highest PVL prevalence (86%) was among abscess isolates. When PVL was correlated to staphylococcal protein A (spa) typing, the highest PVL positivity was among spa-type 44.

Conclusion: The results of this study showed that the *lukS-PV* gene is common among Swedish community of the MRSA isolates.

Keywords: Leukotoxin; Panton-Valentine leukocidin; Methicillin-resistant *Staphylococcus aureus nuclease gene; Staphylococcal protein A*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive, catalase and coagulase positive coccus bacterium, that grows single, diplo, tetrads and irregular clusters (grape-like) [1]. *S. aureus* is a major cause of nosocomial infections due to its ability to produce many virulence factors and to develop multiple drug resistance [2,3]. Almost all *S. aureus* strains can secrete a group of enzymes and toxins which include nucleases, proteases, lipases, hyaluronidase, and collagenase, which help the bacteria to spread into human tissue and convert host tissues into nutrients necessary for bacterial survival and growth [4]. All *S. aureus* strains also produce an extracellular thermostable nuclease (thermonuclease, TNase) which is an endonuclease (MW=17kDa), which degrades both DNA and RNA [5]. This nuclease is encoded by the *nuc* gene and may be used to distinguish between *S. aureus* from coagulase-negative Staphylococci [6]. The Staphylococcal protein-A (encoded by the spa-gene) is an extracellular protein that binds to the fragment crystallizable (Fc) region of Immunoglobulin G (IgG) inhibiting the host immune response by disrupting cell opsonization and phagocytosis [7].

Several leukotoxins such as Pantone Valentine (PVL) and γ-Haemolysin (HIg) are produced by *S. aureus* [8,9]. PVL is produced by only 2% of *S. aureus* strains, but HIg is produced by almost all the strains [9]. The PVL has been detected in *S. aureus* strains isolated from human patients with abscesses, furuncles, and in community-acquired severe necrotic pneumonia [10,11]. PVL causes leukocytosis and tissue necrosis by means of pore formation on the target cells, and its toxicity involves two

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soluble, non-associated but synergic exoproteins a slow-eluting from the ion exchange column (class S protein) and a fast-eluting from the ion exchange column (class F protein) [12]. The synergistic action of 2 secretory proteins form pores in the membrane of host defense cells and are encoded by 2 cotranscribed genes (*LukS-PV* and *LukF-PV*) on a prophage integrated into the *S. aureus* chromosome [13]. The S class proteins include *HIgA, HIg*C and *LukS-PV*, whereas proteins of the F class include *HIgB*, *LukF-PV*, and *Luk*M. The PVL activity starts with binding of the *LukS-PVL* (MW=32 kDa) component to certain host membrane receptors on polymorphonuclear (PMN) leukocytes and monocytes, which allows binding of the second component *LukF-PVL* [2,14]. Binding of the *Luk-F* (MW=34kD3) leads to the formation of oligomeric transmembrane channels specific for the traffic of monovalent cations; these sequential interactions at the membrane surface induce opening of Ca^{2+} channels and increase the free intracellular Ca^{2+} concentration [15]. Apoptosis was only triggered at low PVL concentrations, whereas higher concentrations induced necrotic alterations [16]. The activation of the PMN leukocytes induces granule secretion in the PMN leukocytes and the release of inflammatory mediators (leucotriene B4, interleukin-8, interleukin-6, and interleukin-12) which are involved in chemotactic and vasodilatation [17].

The β-lactam antibiotics such as Benzylpenicillin (penicillin G) were used successfully to treat *S. aureus* infections, but by the late 1950s, an increasing rate of *S. aureus* strains resistant to benzylpenicillin was a cause for concern [1]. Strains resistant to penicillin G produce a β-lactamase enzyme, which inactivates the β-lactam-ring of the antibiotics. At the beginning of the 1960s methicillin was introduced, a penicillin derivative that was resistant to β-lactam hydrolysis [18]. Unfortunately, as soon as methicillin was introduced clinically, the first isolates of methicillin-resistant *S. aureus* (MRSA) were reported in 1960 [19]. The resistance to methicillin is induced by the presence of penicillin-binding protein (PBP 2a), which is encoded by the *mecA* gene and has a low binding affinity to β-lactam antibiotics, and substitute the native PBPs and thereby allow a continuous cell assembly [20].

Like other *S. aureus* , MRSA can colonize the skin and body of an individual without causing sickness (carriers), and in this way, it can be passed on to other susceptible individuals unknowingly [21]. Problems arise in the treatment of overt infections with MRSA because the antibiotic choice is very limited although treatment with vancomycin so far remains an acceptable treatment option [22].

In Sweden, the Swedish Institute for Infectious Disease Control (SMI) has reported 3375 cases of human MRSA in 2017 [23]. There are increasing reports of MRSA harboring the PVL toxin, which increases strains pathogenicity and their ability to cause infections that are difficult to treat.

Therefore, this study aimed to optimize a simple and rapid multiplex PCR assay for simultaneous detection of *lukS-PV*, and *nuc* genes and investigate the prevalence of PVL positive Swedish MRSA clinical isolates.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction

The MRSA strains culture collection included in this study (80 strains) were collected at the clinical microbiology and immunology laboratory at Lund University Hospital, Sweden. The strains were isolated from the throat (9), wound (20), perineum (2), abscesses (7), respiratory tract (29), and unknown sites (13). Samples were cultivated on two culture media: a half-blood agar plate with aztreonam disc and on a half selective oxacillin plate with oxacillin disc and incubated aerobically overnight at 37°C. The rest of the sample was suspended in a tryptic-soy broth (TSB) containing 72.0 µg/ml colistin and 0.05 µg/ml penicillin V (PcV) and cultivated on the other halves of the two agar plates. The isolates were characterized as *S. aureus* based on colony morphology, coagulase- and agglutination-test with Pastorex Staph Plus (bioMérieux, France). Resistance to oxacillin (MIC>I mg/L) was determined by using disc diffusion tests according to the Swedish Reference Group for Antibiotics (SRGA, www srga.org). The presence of the mecA gene in the suspected MRSA strains was confirmed by PCR assay. The positive-MRSA strains were preserved in calf serum media at-80°C. The MRSA-strain (SMI 04-1) was used as positive control for the *lukS-PV* gene, whereas sterile Millipore water was added to negative controls.

For molecular testing, clinical strains were thawed and re-cultivated on blood agar plates overnight at 37°C. A ten-fold dilution series (102 to 108 CFU/ml) in sterilized water were prepared. The diluted bacteria were heated at 95°C for 15 min in a thermocycler (Applied Biosystems, PCR system 2700) to lyse bacterial cells and free the DNA.

PCR primers and amplification conditions

Detection of the *lukS-PV* gene was done using previously published primers [11], PVL-f and PVL-r, which were ordered from SyberGene AB (Stockholm, Sweden). The PCR mixture (50 µl) containing 1X PCR buffer, 3.0 mM $MgCl_2$, 0.6 µM of each primer (PVL-F and PVL-R), 200 µM of each dATP, dCTP and dGTP, 300 µM dUTP and 1 U of Taq DNA polymerase (HotStar Taq, VWR International, Stockholm, Sweden). Exactly 5 µl of DNA extracted by heating the suspension was added to the reaction mixture. PCR mixtures were incubated at 95°C for 15 min (Hot Start) followed by 40 cycles of denaturation at 93°C for 50 s, annealing at 53°C for 50 s, and extension at 72°C for 50 s followed by final extension at 72°C for 8 min. A 14 µl PCR product was analyzed on 2% agarose gel electrophoresis in 1X Tris-Acetate-EDTA (TAE)-buffer (40 mM Tris-HCl-20 mM EDTA, pH 8.0) and stained with 0.5 µM Ethidium bromide. Estimation of the PCR product size was done by using a fragment standard (Gen Ruler 100 bp DNA ladder, MBI Fermentas, Vilnius, Lithuania). The gels were run in 1 XTAE-buffer at 130V for 30 min. The PCR products were visualized using a GelDoc 2000 UV-camera (BIORAD, Hercules, CA, USA).

Multiplex-PCR assay (*lukS-PV* and *nuc* genes)

A multiplex PCR-based assay was developed for detection of both *lukS-PV* and *nuc* genes by using 0.6 µM of each *lukS-PV* specific primers (PVL-f, and PVL-r), 0.1 µM of each *nuc*-specific primers [6], *nuc*-forward (N1), and *nuc*-reverse (N2) ordered from SyberGene AB (Stockholm, Sweden; Table 1). The PCR mixture (50 µl) contained 1X PCR buffer, 3.0 mM $MgCl_2$, 200 µM of each dATP, dCTP and dGTP, 300 µM dUTP, and 1U of Taq DNA-polymerase. Exactly 5 µl of DNA extracted by heating was added to the reaction mixture. The MRSA strain CCUG 35602 was used as a positive control for the *nuc* gene. DNA amplification conditions were the same as the *lukS-PV* PCR assay mentioned above.

Table 1: Oligonucleotide primers used in the multiplex PCR assay.

Oligonucleotide	nucleotide sequence	PCR product	Tm[1]
A) *lukS-PV* specific primers			
PVL-f (forward)	5'-GTAAAATGTCTGGACATGATCCAY-3'	433 bp	58°C
PVL-r (reverse)	5'-GCATCAASTGTATTGGATAGCA-3'		
B) *nuc*-specific primers			
N1 (forward)	5'-GCGATTGATGGTGATACGGTT-3'	279 bp	58°C
N2 (reverse)	5'-CAAGCCTTGACGAACTAAAGC -3'		

[1]Tm: Melting Temperature

[2]PVL: Panton Valentin Leukocidin

[3]*nuc*: Thermostable Nuclease Gene

RESULTS

Optimizing of *lukS-PV* PCR assay

The effect of changing the annealing temperature and primer concentration on the detection limit of PVL PCR assay was evaluated by testing different annealing temperature (55°C and S3°C) on the ability of Taq DNA polymerase to amplify *lukS-PV* gene. When a high annealing temperature (55°C) was used the minimum concentration of *S. aureus* detected was 10^5 CFU/ml. Reducing the annealing temperature to 53°C lowered the detection limit and allowed detection of 10^4 CFU/ml. When we studied the effect of *lukS-PV* Primer concentration, it was found that the best Primer concentration among those tested was 0.6 µM which allowed detection of 10^4 CFU/ml. By changing the cycle number from 30 to 40 cycles did not affect the PCR detection limit. The PCR product appears as a single band with a size of 433 bp. The repeating of the testing with other *S. aureus* strains gave the same results.

Optimizing of the multiplex-PCR assay (*lukS-PV* and *nuc*-genes)

The developed multiplex PCR-assay for detection of both the *lukS-PV*, and the *nuc* genes based on already published primers [6,11], the *nuc* primers are used for the routine diagnosis of MRSA strains in the Medical Microbiology and Immunology Laboratory, Lund University Hospital, Lund, Sweden. The selection of the *lukS-PV* and *nuc* specific primers was based on their specificity to the target genes. Both primer sets had almost equal annealing temperatures, and they have different size of amplified PCR products (433 bp for *lukS-PV* gene and 270 bp for the *nuc* gene; Figure 1). The PCR conditions used for the multiplex PCR assay were the same as those mentioned above and allowed amplification of both genes with a detection limit of 10^4 CFU/ml.

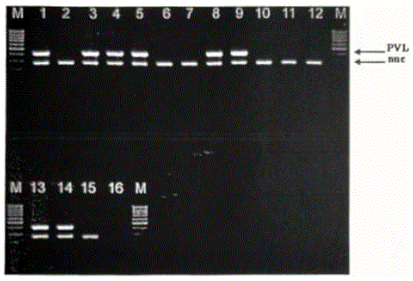

Figure 1: Detection of *lukS-PV* and *nuc* genes in Swedish MRSA clinical strains using a multiplex PCR assay. Lanes: M, DNA ladder (Gen Ruler 100 bp DNA ladder, MBI Fermentas, Vilnius, Lithuania); 1, PVL-gene positive control (SMI 04-1); 16, negative control (sterile water); 2-15, MRSA clinical isolates.

Prevalence of the *lukS-PV* and *nuc* genes among MRSA clinical isolates

All the 80-MRSA isolates collected between 1999 and 2004 were positive for *nuc* gene. When *lukS-PV* PCR assay was applied to these isolates, it was found that 30/80 (40%) were PVL-positive

by amplification of the 433 bp specific PCR product (Table 2). The distribution of PVL positive isolates among the different MRSA isolates were: 11/20 (50%) of wounds, 6/7 (86%) of abscesses, 9/29 (30%) of respiratory tract infections, 1/9 (11%) of throat, and 2/13 (23%) from unknown sites. All the 80 samples were positive for the *nuc* gene PCR assay by amplification of the 279-bp specific PCR product.

Table 2: Prevalence of the *lukS-PV* gene in clinical MRSA strains as established using the PCR-based assay.

Sample type	No.	Positive No. (%)
Throat	9	1 (11)
Wound	20	11 (50)
Abscess	7	6 (86)
Respiratory tract infections	29	9 (31)
Perineal secret	2	0 (0)
Others	13	3 (23)
Total	80	30 (40)

The spa-typing of MRSA strains uses the sequence of a polymorphic variable number tandem repeat (VNTR) in the 3' coding region of the *S. aureus* -specific staphylococcal protein A (spa) [24]. The correlation between the spa-typing of MRSA strains included in this study and PVL-results was investigated (Table 3). It was found that the spa type 44 (UIGBBPB) was the most common type with (95%) frequency. All the isolates with spa types 08 (YHGFMBQBLO) and spa type 131 (UIGBPB) were PVL positive.

Table 3: Correlation between spa-typing and PVL-positive MRSA strains.

Spa-Typing1	Spa-pattern2	No.	Positive No. (%)
1	TIMBMDMGMK	2	0 (0)
2	TJMBMDMGMK	14	1 (7)
8	YHGFMBQBLO	3	3 (100)
10	TMBMDMGMK	1	0 (0)
15	XKAKBEMBKB	3	0 (0)
18	WGKAKAOMQQQ	4	0 (0)
19	XKAKAOMQ	1	1 (100)
30	WGKAQQ	2	0 (0)
32	TJJEJNJ2MNJ2MOMOKR	2	0 (0)
37	WGKAOMQ	2	0 (0)
41	TMBMDMBMDMGMK	3	0 (0)
44	UJGBBPB	19	18 (95)
67	TJMBMDMGM	3	0 (0)
127	UJFKBPE	1	1 (100)
131	UJGBPB	2	2 (100)
148	UJGFGMDMGGM	1	0 (0)
230	XKAKB	1	0 (0)
286	UJEBKBPE	5	0 (0)
296	XKAOB3B3B3	1	0 (0)

Unknown	10	4(40)
Total	80	30 (40)

Spa typing was done as previously described by Shopsin et al. Repeat codes are derived from the organization of individual repeats (random alphabetical code). There are 13 distinct repeat types.

DISCUSSION

DNA amplification by PCR has been applied successfully in the diagnosis of infectious diseases. It offers advantages, such as speed, specificity, and sensitivity [25]. The PVL PCR assay used in this study was found to be specific for the *lukS-PV* gene based on analysis of reference DNA sequences of two *S. aureus* strains, (SMI 04-1, and ATCC 27733), and allowed amplification of 433 bp of *lukS-PV* gene-specific PCR product. The detection limit of the PVL PCR assay was 10^4 CFU/ml of boiled *S. aureus* cells suspension, which was close to the theoretical detection limit of PCR (10^3 CFU/ml). Although lysis of bacterial cells by boiling is a simple, inexpensive, time saving and reduces the risk of contamination between samples, on the other hand, the thick cell wall of *S. aureus* requires boiling cells for a long time, which increases the possibility of DNA degradation and reducing the sensitivity of PCR [12]. Despite the drawbacks of boiling, we decided to use it because we applied the PVL PCR assay on pure cultures that have bacterial cell number concentration above the PCR assay detection limit which was 10^4 CFU/ml. Taq DNA polymerase "Hot start," which was activated at a higher temperature, was used to avoid amplification of unspecific PCR products due to non-specific annealing and primer elongation.

The combination of the production of PVL, which is a potent toxin involved in severe skin infections and necrotizing pneumonia, with resistance to commonly used antibiotics and ability to spread easily in certain communities, possess a potential threat to public health. Outbreaks of PVL-producing strains have recently been reported worldwide [26]. PVL-producing strains have been identified in the US. The PVL producing *S. aureus* stains belong to the USA300 genotype and are associated Skin and soft-tissue infections, necrotizing pneumonia, and sepsis and have been found to colonize healthy children and children with cancer [27]. Similarly, PVL-producing strains have been identified in the gay community in the Netherlands, which was found in 10 % of the MRSA isolates [28]. An outbreak of PVL producing strains among healthy French teenagers was also reported [29].

There is an increasing concern of the outbreaks of *S. aureus* PVL-producing strains, which are associated with rapidly progressive, hemorrhagic, necrotizing, community-acquired pneumonia in young immunocompetent patients with high fatality rate [30]. Familial carriage of *S. aureus* PVL-producing strains has been shown to be a risk factor for subsequent infection with the same strain in neonates [31].

The results of this study showed that the *lukS-PV* gene was prevalent in the Swedish MRSA strain community (40%), which is higher than the 5% PVL reported in Western Europe [9]. The highest prevalence of PVL-positive strains was found among strains had been associated with furuncles which appear as a central area of subcutaneous necrosis due to direct activation and lysis of PMN by the PVL toxin [11]. In this study, the highest prevalence of MRSA PVL positive strains had been isolated from abscesses (86%), skin wounds (50%), and respiratory tract infections (30%), whereas only 11% of isolates collected from throat were PVL-positive. These results are in accordance with results reported by Lina et al. [11] who found that the PVL detected in 50% of *S. aureus* were isolated from the cutaneous abscess, cellulite, or furunculosis. This was also confirmed by Couppie et al. [32,33] who detected PVL in 86% of abscesses. Some of the abscesses could have originated from true furuncles that faced destruction by the necrotic process and appeared as skin abscesses. In contrast, superficial, nonnecrotic processes such as impetigo and folliculitis were not associated with PVL-harboring *S. aureus* .

When we investigated the correlation between the spa-typing (which was done before) and PVL- results, it was found that the spa type-44 was the most common type with (95%) frequency. We had only 3 isolates with spa-type 0 and two spa-type 131, all of them were PVL positive. Unfortunately, we did not have enough samples to compare the prevalence of PVL positive samples with the other spa types. However, it will be interesting to collect more samples and to test the correlation between these two virulence factors.

CONCLUSIONS

PVL appears to be a possible virulence factor associated with specific cases such as necrotic lesions of the skin, and subcutaneous tissues and community-acquired severe necrotic pneumonia. Association of *lukS-PV* gene as a virulence factor and mecA gene as a resistance factor makes *S. aureus* a well-adapted pathogen. Problems arise in the treatment of infections with MRSA because the use of different types of antibiotic over the years has led to the emergence of multiple drug resistant MRSA, which makes the therapeutic choice very limited. Further studies will be performed in order to gain more insight into the microbiological and epidemiological background of these virulent MRSA strains. *lukS-PV* gene could be successfully detected by the PCR assay employed here and allowed simultaneous detection of PVL producing MRSA strains.

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