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Original Article

Patients with Panton-Valentine leukocidin positive *Staphylococcus aureus* infections run an increased risk of longer hospitalisation

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Abstract: *Staphylococcus aureus* is a major cause of purulent infections. The spectrum of staphylococcal infections varies from mild superficial to invasive life-threatening diseases due to *S. aureus* ability to produce a wide range of virulence factors, including toxins. A prospective observational study was conducted in the Children Clinical University Hospital in Riga, Latvia. During a period of sixteen months from November 2006 to March 2008 224 *S. aureus* isolates were collected. Our study revealed that Panton-Valentine leukocidine (PVL) genes are carried by a high number (75%) of *S. aureus* isolates recovered from children hospitalised in the Children Clinical University hospital. Most of these isolates were associated with abscesess and other skin and soft tissue infections. Patients with PVL positive invasive infections stayed significantly longer in hospital than patients with PVL negative invasive infections. Clonal distribution of PVL positive *S. aureus* isolates were closely related, which provides evidence for the wide spread of PVL producing *spa* type *t435* and ST121 staphylococci in community.

Keywords: *Staphylococcus aureus*, Panton-Valentine leukocidin, methicillin resistance, *S. aureus spa* typing, MLST, BURP, ST121, *t435*

Introduction

Staphylococcus aureus is a major cause of purulent infections. The spectrum of staphylococcal infections varies from mild superficial to invasive life-threatening diseases due to *S. aureus* ability to produce a wide range of virulence factors, including toxins [1]. Panton – Valentine leukocidin (PVL) is an extracellular pore forming *S. aureus* gamma toxin, which consists of two subunits F and S that together are leucocidal and dermonecrotic [2]. This toxin targets the outer membrane of polymorphnuclear cells, monocytes and macrophages. Both of the PVL subunits induce opening of calcium channels, leading to calcium influx and massive release of inflammatory mediators and apoptosis or necrosis of the cell [3]. Panton – Valentine leukocidin injected intradermaly to rabbits, causes severe inflammatory lesions with capillary dilatation, chemotaxis, polymorphnuclear infiltration and skin necrosis [4]. In humans PVL is associated with skin abscesses and necrotizing pneumonia. Toxin is encoded by *lukS/lukF-PV* genes and carried on a bacteriophage [5,6]. *S. aureus* strains which are positive for PVL are usually associated with community-acquired infections which generally affect previously healthy children and young adults. Although Panton-Valentine leucocidin has been strongly associated with community acquired methicillin – resistant *S. aureus* (CA – MRSA), *lukS/lukF-PV* genes can be carried also by methicillin susceptible *S. aureus* (MSSA) isolates [7]. Recent investigations suggest that "PVL positive" *S. aureus* exhibits enhance virulence and are responsible for severe infections such as bone and joint infections and necrotising pneumonia [6,8- 10]. Due to "PVL positive" *S. aureus*, community acquired necrotizing pneumonia is an emerging infection [11] . Pneumonia often arises from the blood born spread of organisms from infected tissues and can follow viral respiratory infec-

tions, especially influenza [12]. Necrotizing pneumonia mainly affects children and young adults and up to 75% of cases are lethal [2]. In Europe most cases of necrotizing pneumonia are due to MSSA strains [10].

The aim of this study was to determinate PVL genes among *S. aureus* isolates recovered in the microbiological laboratory of the Children Clinical University Hospital, as well as to definite the molecular features of the collected isolates.

Methods

Participants and clinical methods

A prospective observational study was conducted in the Children Clinical University Hospital in Riga, Latvia. The hospital is the only tertiary level children hospital in Latvia which serves a population of approximately 420,000 children and shares 600 beds. During a period of sixteen months from November 2006 to March 2008 224 *S. aureus* isolates were collected.

The study inclusion criterion was positive *S. aureus* culture taken from pus, blood or other material from organism sterile sites excluding bronchial lavage and sputum. Patients older than 18 were excluded. Detalised analysis of the patients' medical cards was performed using standardized forms.

The study protocol was approved by the Central Medical Ethics Committee of Latvia.

Case definitions

Severe invasive infections were defined by one or more of the following conditions: bacteremia, endocarditis, pneumonia, septic arthritis, osteomyelitis, or other illnesses in which *S. aureus* was isolated from normally sterile body fluids. Infections involving the skin or soft tissue structures were regarded as mild superficial infections. This section includes – furuncules, carbuncules, hidradenitis, mastitis, impetigo, folliculitis, paronychia.

Superficial abscesses were defined as the abscesses of skin or skin derivates that arise in epidermis or dermis.

Community-associated infection was defined as a positive *S. aureus* culture taken in the first 48 hours after admission to hospital with an illness. For individuals with multiple hospital admissions for *S. aureus* infection during a single year, data were obtained from the first hospitalization.

Laboratory methods

The hospital-based diagnostic microbiology laboratory processed all samples using routine procedures. Antibacterial susceptibility was determined by disk diffusion method according to CLSI standards (M2-A9, M100-S14) [13]. Susceptibilities reported to hospital physicians and investigators were oxacillin, erythromycin, fusidic acid, vancomycin kanamycin, cefoxitin, clindamycin, ciprofloxacin, rifampicin, gentamicin, nitrofurantoin, novobiocin. A total of 224 *S. aureus* isolates (first positive for each patient) were obtained and available for further investigations.

Isolates were identified as *S.aureus* using BD BBL Crystal Identification Systems; Gram – positive ID kit (Becton, Dickinson and Company 7 Loveton Circle Sparks,Maryland 21152U.S.A. 800-638-8663,) and methicillin-resistant Staphylococcus aureus (MRSA) were verified by the detection of *mecA* gene by PCR [14]. *lukS/-lukF-PV* genes were detected by PCR [6, 15].

Spa typing of *S. aureus* (n=219) was performed as described [16]. Chromatograms of spa sequences were analyzed by Ridom StaphType software (Ridom GmbH). The *spa* types were clustered with the BURP algorithm (Ridom GmbH).

Seven PVL-positive *S. aureus* strains with closely related *spa* types belonging to the CC435 and two CA-MRSA isolates with *spa* type t012 were analysed by multi locus sequence typing (MLST) as described [17]. The multiplex PCR method for SCCmec typing was applied [18].

Statistical analysis

The data was analyzed using SPSS version 18.0 for Windows. The results are presented as numbers (n), frequencies (%), medians with their interquartile ranges (IQR). Differences in variables between different groups of infections were performed using the Mann - Whitney

Table 1. Clinical and epidemiological characterization of analyzed patients

		***S. aureus* PVL(+) n=168**			***S. aureus* PVL(-) n=56**		
		Severe invasive infections n=42	Mild superficial infections n=126	p	Severe invasive infections n=25	Mild superficial infections n=31	p
Demographics							
Median age (months) (min.-max.)	114(1-214)	112 (1-214)	112(1-214)	0,947	142 (1- 210)	87(2-213)	0,959
Sex (female)	92 (41,1%)	16(39,0%)	51(40,0%)	0,897	11(47,8)	14(42,4%)	0,689
Co - morbidities	42 (18,8%)	20(48,8%)	11(8,7%)	<0,001	9(39,1%)	2(6,1%)	0,004
Source of infection							
Community-associated	176(78,6%)	24(48,8%)	124(91,3%)	<0,001	13(56,5%)	27(81,8%)	0,039
Hospital-associated	48 (21,4%)	17(51,0%)	3(8,7%)	<0,001	10(43,5%)	6(18,2%)	<0,001
Drug resistance							
MRSA	6 (2,7%)	0	3	1,000	0	3	0,261
Interventions							
Antibiotic therapy	214(95,5%)	39(95,1%)	121(95,3%)	1,000	23(100%)	31(93,9%)	0,507
Procedure for infectious source control	172(75,7%)	29(72,5%)	95(74,8%)	0,771	19(82,6%)	25(78,1%)	0,745

p value for comparison between severe invasive infections and mild superficial infections

test as the continuous variables did not follow a normal distribution, Pearson chi-square and Fisher's Exact test. A p-value of less than 0.05 (two-tailed) was considered statistically significant for all tests.

Results

Clinical and molecular characterization of the recovered S. aureus isolates

Investigation of 224 *S. aureus* cultures (blood isolates (n=8) , isolates from pus obtained by aspiration or operative procedures (n=206), other source (n=10, where abdominal fluid n=1, pleural fluid n=1, exudates n=2, intubation tube n=2, peripheral intravenous catheter n=1, urine n=2, granulation tissue n=1) from patients, who were admitted to the Children Clinical University Hospital in Riga from November 2006 up to March 2008 was cunduced. All patients were divided into two groups – patients with severe invasive infections (n=67) and patients with mild superficial infections (n=157). PCR investigations of all 224 *S. aureus* isolates showed that 168 (75,0%) carried genes for PVL synthesis. According to PVL presence patients were divided into two categories – patients with PVL positive infections and PVL negative infections (each group had patients with severe invasive infections and mild superficial infections). The characteristic features of the patients were median age, gender, co-morbidities, source of infection and surgical interventions. P value was used to compare patients with severe invasive infections and patients with mild superficial infections (**Table 1**).

There were no significant differences in median age, surgical interventions between patients with severe invasive infections and patients with mild superficial infections. Severe invasive infections were found more often in patients with hospital-associated (p<0.001). community-associated infections were found more often in patients with mild superficial infections (p<0.001). Differences were statistically significant in both groups PVL positive and PVL negative (**Table 1**).

One hundred and seventy-six isolates (78,6%) of the all isolates were community -associated and 136 (60,7%) of them were PVL positive *S.*

Table 2. Association of PVL-positive isolates with types of staphylococcal infection

Clinical presentations	No. of *S. aureus* strains	No. of PVL + strains n (%)	No of PVL – strains n (%)	Odds ratio* (95% CI)	P value
Superficial abscesesses	38	33(86,8%)	5 (13,2%)	2.49(0.92,6.74)	0.072
Skin and soft tissue infections	119	93(78,2%)	26 (21,8%)	1.43(0.78,2.62)	0.247
Bone and joint infections**	33	23(69,7%)	10 (30,3%)	0.72(0.32,1.65)	0.447
Other infections***	34	19(55,8%)	15 (44,2%)	0.34(0.16,0.75)	0.007

*Odds ratio is the ratio of the risk of the presence of a particular type of infection if *s. aureus* isolate is PVL positive. CI, confidence interval. **Bone and Joint infections – osteomylitis n=26, bursitis n=7. ***Other infections – sepsis n = 10, pneumonia n=2, deep abscesses n=3, ventriculoperitoneal shunt infection n=1, polytrauma n=1, paraproctitis n=2, pyelonephritis n=2, necrotizing enterocolitis n=1, purulent conjunctivitis n=4, intrauterine infection n=1, encephalopathy n=1, pilonidal abscess n=1, lymfangioma n=1, purulent atheroma n=1, bone cyst n=1, phlebitis n=1, infected haemathoma n=1.

Table 3. Molecular characterization of PVL positive S. aureus strains of CC435

ID	Diagnosis	Material	Spa type	MLST
9.	streptodermia	pus	t308	MSSA ST121
20.	flegmona	pus	t435	MSSA ST121
42.	osteomyelitis	pus	t284	MSSA ST121
43.	bursitis	pus	t159	MSSA ST121
53.	osteomyelitis	pus	t435	MSSA ST121
211.	furunculus	pus	t435	MRSA ST121
253.	lymphadenitis	pus	t435	MSSA ST121

aureus. Six (2,6%) out of 224 were methicillin resistant. Three of the obtained MRSA isolates were community associated and PVL positive. Patients with community- associated MRSA were hospitalised from home except one child who was hospitalised from a child care centre. All the patients with community acquired MRSA were hospitalised with superficial skin and soft tissue infections (furunculosis n=2, lymphadenitis n=1).

Antibiotic therapy was prescribed to 216 of 224 patients. All patients, except two, who did not receive antibacterial therapy, received surgical operative procedures like incision and drainage alone. Information on two remaining patients was not available.

Surgical interventions were performed in 172 (75,5%) patients.

To calculate the association of PVL-positive isolates with types of staphylococcal infection all *S. aureus* isolates were categorized in four groups according to clinical details provided – superficial abscesses, superficial skin and soft tissues infections, bone and joint infections and other infections (including pneumonia and bacteremia) (**Table 2**). Panton-Valentine leukocidin positive isolates were more likely to cause all types of infections (p=0,014) than isolates that were PVL negative. The obtained results of odds risk calculations revealed that if isolated *S. aureus* is PVL positive, the risk of superficial abscesses development increases 2, 49 times. The risk of the development of bone and joint infections, and other infections remains equal in both groups – PVL positive/PVL negative.

Spa typing of *S. aureus* isolates revealed 69 different *spa* types. The majority of the typed S. *aureus* strains (n=90) belonged to the *spa* type *t435* (n=52), or closely related types (*t159, t308, t284*) and were assigned to CC435 by BURP clustering. 86% (n=78) of CC435 isolates were PVL positive and 70 % (n=63) were isolated from patients with mild superficial skin infections. *S. aureus* isolates belonging to the CC435 were isolated from patients with shorter lenght of hospitalisation (mean 8,9 days (SD

10,0); median 5,00 days), comparing with remaining *S. aureus* isolates (mean 11,7 days (SD 17,7); median 7,00 days), differences were statisticaly significant ((z= -2,235 (Mann-Witney test); p=0,025).

MLST results for PVL positive *S. aureus* isolates with *spa* type *t435* and closely related spa types revealed that all of them were ST 121 (**Table 3**).

Hospitalization length among patients with PVL-positive and PVL-negative S. aureus infections

The length of hospital stay was analyzed according to PVL presence in patients with severe invasive infections and patients with mild superficial infections. There were no significant differences in duration of hospitalization between all PVL positive and PVL negative infection cases (median duration 6 days, p = 0, 088), but among patients with severe infections as osteomyelitis, deep abscesses, pneumonia, bacteremia duration of hospitalization was significantly longer in PVL positive group - median duration 19 days, the length of hospitalization in PVL negative group was 12 days (p=0,033). None of the hospitalized patients died.

Hospitalization was significantly longer in PVL positive patient group with underlying diseases – median duration 15 days in comparison with 10 days in PVL negative group (p< 0,001). There was no relation between the duration of hospitalization with the patientts' age and presence of PVL.

Discussion

This study highlights important issues. Firstly the results of this study showed that PVL-positive *S. aureus* were large proportion of all the obtained isolates (75,0%) and the majority were (60,7%) methicillin-sensitive. Investigations of the PVL positive MRSA and MSSA isolates obtained from pus specimens (by aspiration or during operative procedures), blood and pleural fluid in Thailand showed that PVL gene positive *S. aureus* isolates were 49%, and all of them were MSSA [19]. In Europe the prevalence of PVL positive *S. aureus* isolates is lower. It has been estimated that from 2002-2003 isolates <2% of *S. aureus* in the UK were PVL positive, majority were methicillin sensitive, with 65% of them associated with skin and soft tissue infections, 17% with pneumonia [20]. Higher rates of PVL positive *S. aureus* were reported from Greece, where the frequency of PVL positive isolates was 27%, but PVL production among skin and soft tissue infections associated MSSA isolates was 12% [21]. A high PVL positive methicillin susceptible *S. aureus* prevalence (70%) was reported from France from surgically drained abscesses [22]. The discrepancy between the above-mentioned study and reports describing PVL as a very infrequent toxin (`2%) in *S. aureus* is probably due to the differences in *S. aureus* cultures selection and geographic area [23] .

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 [2]. In the present study isolates from patients with invasive infections such as bone and joint infections (including osteomyelitis) harboured genes for PVL production which contradicts the findings of Lina et al [6].

Patients with PVL positive invasive infections stayed significantly longer (12/19 days, p=0.022) in hospital than patients with PVL negative invasive infections. Longer hospitalization was observed in patients with underlying diseases (7 days, p<0,001). The role of PVL in a longer hospital stay is controversial.There are few reports of PVL positive *S. aureus* infection association with the lenght of hospital stay and limitation of available studys is its small

sample size. It was reported that the duration of hospital stay was similar in pediatric patients with and without PVL positive community acquired invasive and non invasive *S. aureus* infections, while another authors reported that pediatric patients with PVL positive bone and joint infections had 3 time longer median hospitalisation time versus control group with PVL negative *S. aureus* bone and joint infections [9, 26].

Most of the patients were hospitalized in surgical profile units as most of them had purulent skin and soft tissue infections. In 76% of the cases chirurgical procedures were performed while 96% received antibacterial therapy. According to some local guidelines incision and drainage is an optimal management of superficial abscesses and minor skin and soft tissue infections such as furunculosis do not need systematic antibiotic therapy [28]. Antimicrobial therapy may be maintained for patients with larger abscesses (> 5cm) and for patients with systemic signs of infection like fever and tachycardia or patients with poor response to surgery [29].

The *spa* sequence analysis revealed that most of the *S. aureus* isolates belong to the *spa* type *t435* or are closely related. Panton- Valentine leukocidin positive *S. aureus* isolates with *spa* type *t435* are mostly methicillin susceptible and is common in Latvia with sporadic cases in Poland, Austria, Romania and Hungary [30]. MLST results showed that PVL positive MSSA with spa type t435 belongs to ST 121. Recent studies of *S. aureus* isolates obtained from children showed that most isolates with such ST were MRSA [19]. Methicillin-susceptible *S. aureus* (MSSA) ST 121 from skin isolates in the South Africa, Russia, India, United states [31]. Recent reports of involvment of MSSA-ST121 PVL positive isolates as well in furunculosis outbreake as in therapy refractory sepsis reveal significance of this clone [32, 33]. MSSA –ST 121 (t435) are uncommon and firstly described in Latvia.

Conclusions

Our study revealed that PVL genes are carried by a high number of *S. aureus* isolates obtained from children hospitalised in the Children Clinical University hospital. Most of these isolates were associated with abscesess and other skin and soft tissue infections. Patients with PVL positive invasive infections stayed significantly longer in hospital than patients with PVL negative invasive infections. Clonal distribution of obtained PVL positive S. aureus isolates were homogenous, the obtained isolates were closely related, which provides evidence for the wide spread of PVL producing *spa* type t435 and ST121 staphylococci in community. Close surrveilance of PVL positive strains is essential to monitor their spread, antimicrobial resistance, and association with clinical features.

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