



RĪGAS STRADIŅA
UNIVERSITĀTE

Liene Cupane

MOLECULAR EPIDEMIOLOGY AND
CLINICAL COURSE OF PANTON-
VALENTINE LEUKOCIDIN POSITIVE *S.*
AUREUS INFECTION IN CHILDREN OF
LATVIA

Summary

PhD Thesis in Pediatric infectious diseases

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the Department of Pediatrics of the Riga Stradins University in Riga,

the Children's Clinical University Hospital,

Molecular Biology and Genetics Department of the United Laboratory of P. Stradins Clinical University Hospital,

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ABBREVIATIONS

BD – *Becton Dickinson*

BURP – based upon repeat pattern.

CA-MRSA – Community-associated methicillin-resistant *Staphylococcus aureus*

CC – Clindamycin

CCC – Children care centre

CCUH – Children's Clinical University Hospital

CIP – Ciprofloxacin

CLSI – *Clinical and Laboratory Standards Institute*

ECDC – European Center for Disease Prevention and Control

EMRSA – epidemic methicillin-resistant *S. aureus*

ERY – Erythromycin

ETA – exfoliative toxin A

ETB – exfoliative toxin B

EU – European Union

GEN – Gentamicin

HA - MRSA – hospital - associated methicillin-resistant *S. aureus*
luk-PV - PVL – toxin gene

mecA – methicillin-resistance gene

MLST – multilocus sequence typing

MRSA – methicillin-resistant *Staphylococcus aureus*

MSSA – methicillin-susceptible *Staphylococcus aureus*

OX – Oxacillin

PCR – polymerase chain reaction

PFGE – pulsed field gel electrophoresis

PSCUH – Pauls Stradins Clinical University Hospital

PVL – Panton-Valentine leukocidin

RIF – Rifampicin

S. aureus – *Staphylococcus aureus*

SCC *mec* – staphylococcal chromosomal cassette

spa – staphylococcal protein A gene

ST – sequence type

STX – Trimethoprim-Sulfamethoxazole

TET – Tetracycline

USA – United States of America

UV transiluminator – ultraviolet light transiluminator

VAN – Vancomycin

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 – furuncles, carbuncles, hidradenitis, mastitis, impetigo, folliculitis, paronychia.

Superficial abscesses were defined as the abscesses of skin or skin derivatives 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.

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. 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. 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. In humans PVL is associated with skin abscesses and necrotizing pneumonia. Toxin is encoded by *lukS/lukF-PV* genes and carried on a bacteriophage. *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. 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. Due to PVL positive *S. aureus*, community acquired necrotizing pneumonia is an emerging infection. Pneumonia often arises from the blood born spread of organisms from infected tissues and can follow viral respiratory infections, especially influenza. Necrotizing pneumonia mainly affects children and young adults and up to 75% of cases are lethal. In Europe most cases of necrotizing pneumonia are due to MSSA strains.

Aim of the research paper was to determine PVL genes among *S. aureus* isolates recovered in the microbiological laboratory of the Children's Clinical University Hospital, as well as to define the molecular features of the collected isolates and detect the impact of PVL on the clinical course of *S. aureus* in children.

Objectives of the work was:

1. Determination of the prevalence of PVL positive *S. aureus* among *S. aureus* isolates collected from children with *S. aureus* infections.
2. Determination of the effect of PVL on the clinical course of *S. aureus* infections in hospitalized children.
3. Determination of the effect of PVL on the length of hospitalization in children with *S. aureus* infections.
4. Identification of dominating spa types among hospitalized children with *S. aureus* infection.
5. Determination of the effect of clonal spa type on the clinical course in hospitalized children.

The study questions

1. Is spread of PVL positive *S. aureus* among hospitalized children observed in Latvia?
2. Which is the most prevalent clinical manifestation of the PVL positive *S. aureus* infections in Latvia?
3. Does the presence of PVL affect the course and outcome of patients' disease?
4. Is the PVL positive *S. aureus* genotypic characterization by spa gene similar to the spa types found in Europe?

Scientific novelty of the study-

1. Statistically significant effects of PVL on the length of hospitalization for children with severe invasive diseases have been demonstrated.
2. Common PVL positive *S. aureus* isolates in Latvia forming CC435 cluster have been described.

Structure and extent of the work - the PhD thesis is written in the Latvian language and consists of 14 chapters: summary in the Latvian and English languages, introduction, statement of the importance of the problem, aim and objectives of the research work, scientific novelty of the work, literary description, materials and methods, results, discussion, conclusions, practical recommendations, references and four attachments. The thesis consists of 128 pages, including 19 tables and 17 figures. The reading list comprises 187 items.

During research process molecular, genetical investigations of 224 *S. aureus* isolates were done as well as retrospective analysis using questionnaires of all 224 patients' medical cards information

There are 22 publications regarding the present PhD Thesis, 1 of which published in internationally quoted medical research edition (registered in PubMed database), 4 in Latvian scientific editions, 1 monograph, 6 thesis of the study are published in international scientific congresses, 4 oral presentations in the domestic scientific congresses and 10 theses in domestic scientific congresses and conferences.

The approbation of the Doctoral Thesis - Molecular epidemiology and clinical course of Panton-Valentine leukocidin positive *S. aureus* infection in children of Latvia - took place on March 13, 2012 at the session of the Department of Pediatrics, RSU with participation of representatives from the Department of Biology and Microbiology.

Results of the study on PVL positive *S. aureus* molecular epidemiology and clinical forms in children were used for creation of practical

recomendations.

1. MATERIALS AND METHODS

1.1. Structure of the study

A retrospective study was conducted in the Children Clinical university hospital, molecular biology and genetics department of United laboratory of P. Stadins Clinical university hospital and Hereditary Cancer institute of the Riga Stradins university, and department of Pediatrics of the Riga Stradins university in Riga (Figure 1.1.)

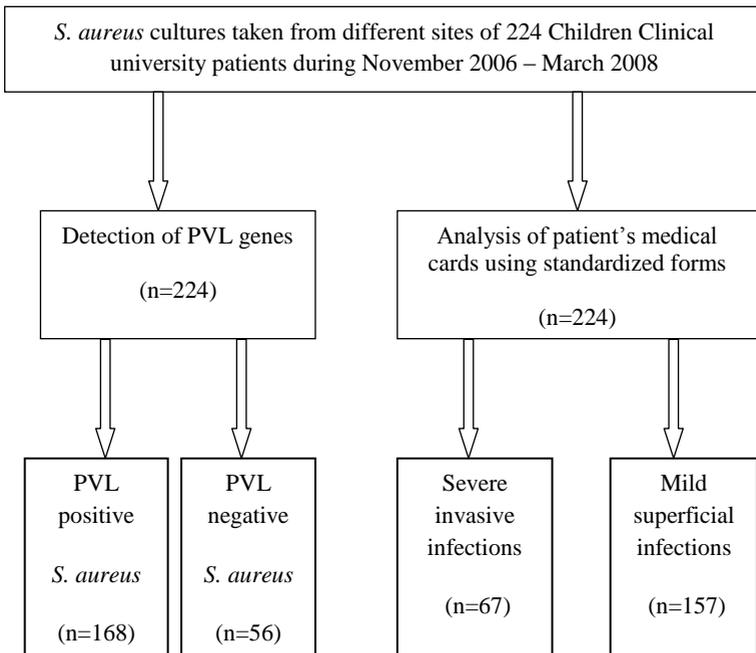


Figure 1.1. Structure of the study

224 *S. aureus* cultures taken from Children Clinical University Hospital patients' blood, cerebrospinal fluid, pus obtained by aspiration or during operative procedures, intravenous catheters were included. Microbiological investigation of collected isolates was performed in the Department of Microbiology of the Children' s Clinical University Hospital, further investigations were performed in the Molecular Biology and Genetics Department of United Laboratory of P. Stadins Clinical University Hospital and Hereditary Cancer Institute of the Riga Stradins University. A detailed analysis of 224 patients' medical cards was performed using standardized forms. All patients were divided into two groups – patients with severe invasive infections and patients with mild superficial infections. 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. Example of the form added in the Annex.

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. **Excluded** were patients older than 18.

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

1.2. 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 (actual version). Susceptibilities reported to hospital physicians and investigators were oxacillin, erythromycin, fusidic acid, vancomycin kanamycin, ceftioxin, 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).

Methicillin-resistant Staphylococcus aureus (MRSA) were verified by the detection of *mecA* gene by PCR. *lukS/-lukF-PV* genes were detected by PCR. *Spa* typing of *S. aureus* was performed as described previously. 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. The multiplex PCR method for SCCmec typing was applied.

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 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.

2. RESULTS

2.1. Analysis of hospitalized patients with *S. aureus* positive cultures

2.1.1. Characteristics of patients

Patients who were hospitalized in Children Clinical University Hospital from November 2006 till March 2008 and had positive *S. aureus* cultures identified by hospital's microbiological laboratory and were suitable for inclusion criteria were included in demographic analysis and disease clinical course investigation.

S. aureus isolates more often were collected from boys (59,9%, n=134), than from girls (41.1%, n=92). Mean age of patients with positive *S. aureus* cultures was 8,9 years or 107, 4 months (SD 73,4) from one month to 17, 8 years (214 months) (Fig. 2. 1.)

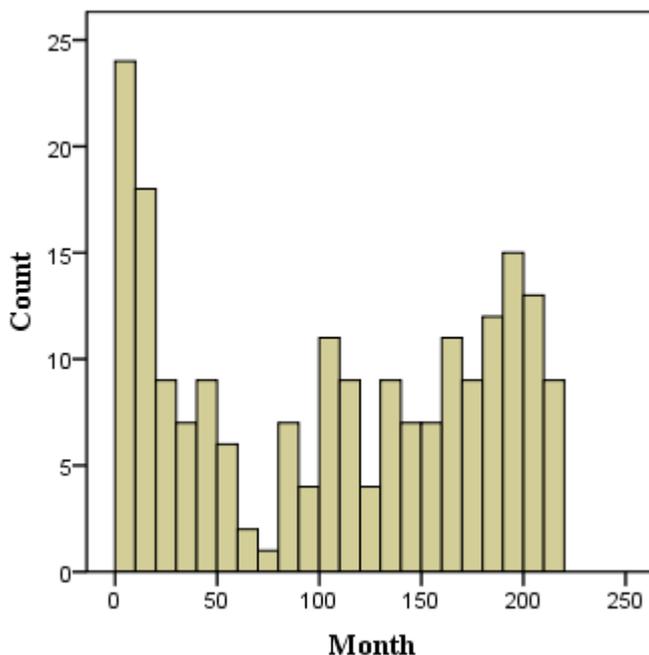


Figure 2.1. Histogram of patients age with positive *S. aureus* cultures identified in hospital's microbiology laboratory. Median age 9,5 years (114 months) (IQR 29;114).

Children were hospitalized from home in most cases - 83,9% (n=188), from other hospital - 13,8 % (n=31) cases, from children care centre in 2,2% cases (n=5).

Of all analysed patients 18,8% (n=42) had side diseases - dermatological (atopic dermatitis, ichthyosis, scabies) (n=7), CNS pathologies (n=6), bone system diseases (juvenile osteochondrosis, chronic osteomyelitis) (n=4), premature (n=3), anemia (n=3), heart diseases (congenital heart diseases, heart rhythm disorders) (n=3), pyloric stenosis (n=2), immunodeficiency (n=2), dermatomyositis (n=2), food allergy (n=2), other diseases (renal anomalies,

tracheomalacia, narcomania, obesity, congenital eye pathology, glucose tolerance disorders, chronic appendicitis). Main hospitalization reasons were purulent skin and soft tissue infections (furunculosis, paronychia, lymphadenitis, mastitis) – 53,1% (n=119), abscesses – 17% (n=38). Osteomyelitis diagnosed in 11, 6% (n=26) cases, sepsis developed in 4, 4% (n=10) cases. Other patients had different diseases (Figure 2. 2.).

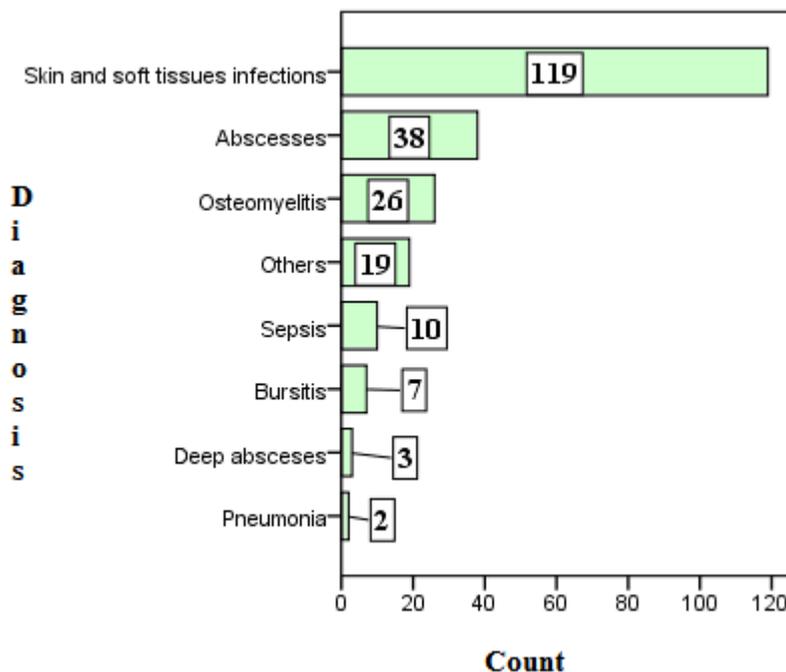


Figure 2.2. Hospitalization diagnosis of analysed patients.

Most of all *S. aureus* infections were acquired in outpatient settings - 78,6% (n=176). Nosocomial *S. aureus* infection was detected in 21,4% (n=48) cases.

More often *S. aureus* cultures were collected in the first 48 hours after admission to hospital – 76,7% (n=171) (Figure 2.3.).

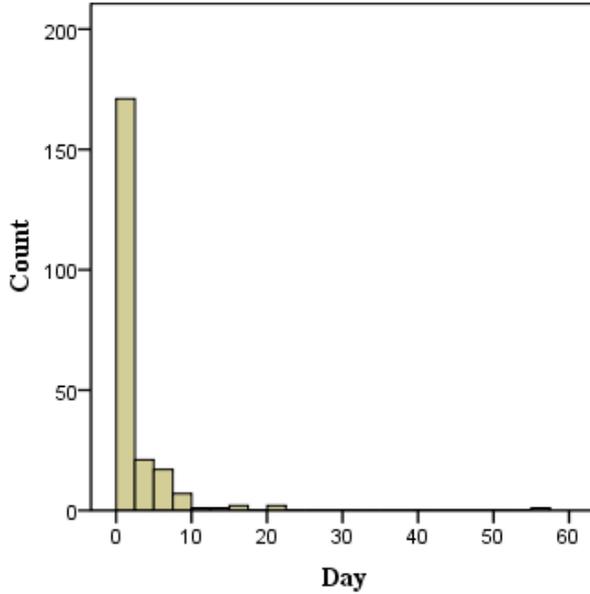


Figure 2.3. Histogram of *S. aureus* culture collecting time.

Frequently *S. aureus* isolates were collected from pus obtained during operation or by aspiration, others were collected from blood or other sites (Figure 2.4.).

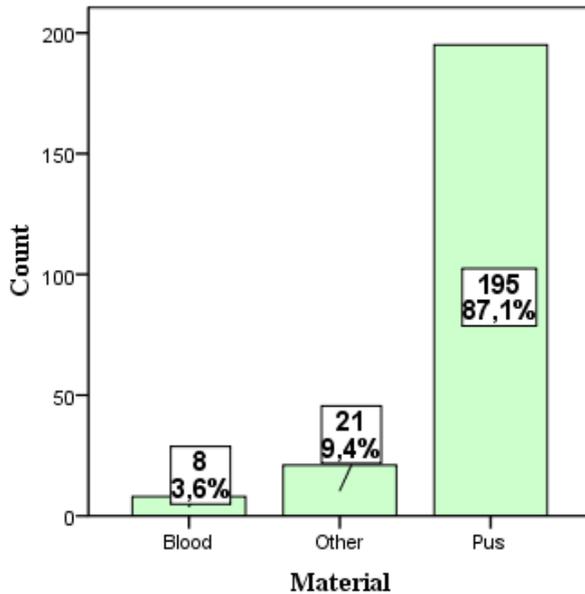


Figure 2.4. Material for cultivation of *S. aureus* culture

Other – from intra-abdominal fluid n=1, pleural fluid n=1, exudate n=2, intubation tube n=2, peripheral intravenous catheter n=1, urine n=2, granulation tissue n=1.

2.1.2. Analysis of therapy of hospitalized patients with *S. aureus* infection

In most cases patients were hospitalized in surgical profile departments (abdominal surgery, neurosurgery, thoracic surgery, traumatology), other patients hospitalized in neonatal units, pediatric departments and intensive care unit. (Figure 2.5.).

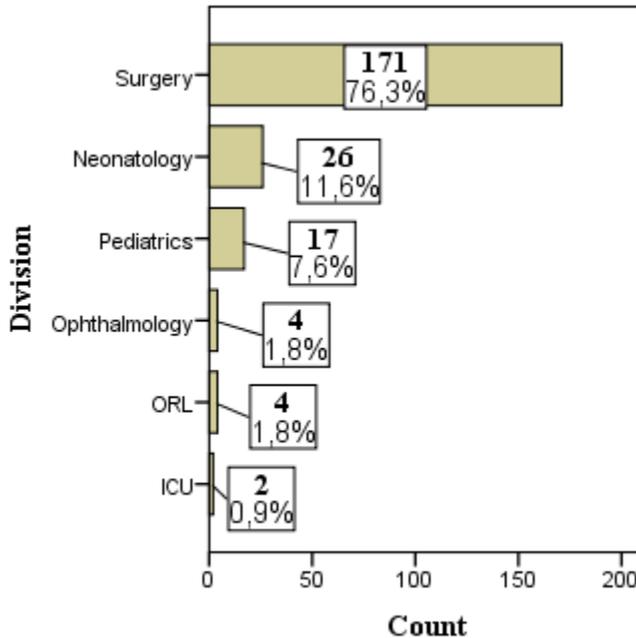


Figure 2.5. Analysed of patient’s distribution in units.

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. The most often performed procedures were incision and drainage-79,1% (n=136). Osteoperforation was done in 15 patients, remaining patients received paraneural cavity drainage (n=1), ventriculoperitoneal shunt removal and replacement (n=1), toracotomy and pleural cavity drainage (n=2), needle aspiration (n=1), frontoetmoidotomy (n=1), laparotomy (n=2), laparoscopy and

apendectomy (n=1), sekvestrektomy (n=1), excision of exostosis (n=1), excision of lymfangioma (n=1), ophtalmological operation (n=1).

Median length of hospital stay for all patients was six days (IQR 4,0;12,0), from one to 174 days (Figure 2.6.). None of the children included in the study died during hospitalization.

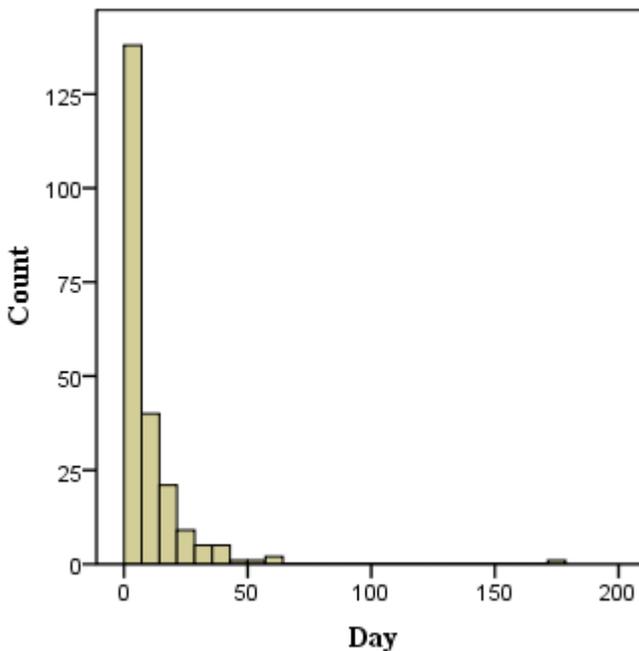


Figure 2.6. Histogram of patient's hospitalization length.

2.1.3. Prevalence of PVL positive

S. aureus

Results are based on the analyses of questionnaire data which was processed by Microsoft Excel 2003, Windows SPSS 18.0 version. See the Questionnaire form in the Annex. Two hundred twenty four hospitalized patients with laboratory confirmed *S. aureus* infections during November 2006 till March 2008 was conducted.

PVL positive *S. aureus* prevalence in children with *S. aureus* infection hospitalized in the Children's Clinical University Hospital was demonstrated in 75,0 % (n=168) patients (95% CI 68,9 –80,2%) (Figure 2.7.).

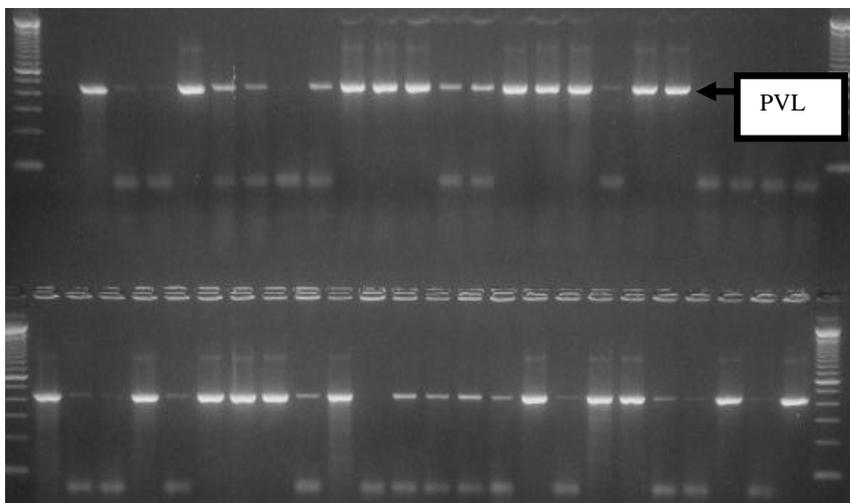


Figure 2.7. Distribution of amplification products in 2% agarose gel of *S. aureus* isolates collected by microbiology laboratory of Children Clinical university hospital.

Characterization of patients according to PVL presence shown in tables

2.1., 2.2.

Table 2.1.

Characterization of patients according to PVL presence and clinical course of disease

Indicators	All patients	<i>S. aureus</i> PVL(+) n=168		
		Severe invasive infections n=42	Mild superficial infections n=126	p value ¹
Median age, months, (min.-max.)	114 (1-214)	112 (1-214)	112 (1-214)	0,947
Gender (girls)	92(41,1%)	16(39,0%)	51(40,0%)	0,897
Underlying diseases	42(18,8%)	20(48,8%)	11(8,7%)	<0,001
Community associated	176(78,6%)	24(48,8%)	124(91,3%)	<0,001
Nozocomial	48(21,4%)	17(51,0%)	3(8,7%)	<0,001
Antibacterial therapy	214(95,5%)	39(95,1%)	121(95,3%)	1,000
Surgical interventions	172(75,7%)	29(72,5%)	95(74,8%)	0,771

¹ p value for comparison between severe invasive infections and mild superficial infections.

Table 2.2.

Characterization of patients according to PVL absence and clinical course of disease

Indicators	All patients	<i>S. aureus</i> PVL(-) n=56		
		Severe invasive infections n=25	Mild superficial infections n=31	p ² value
Median age, months, (min.-max.)	114 (1-214)	142 (1- 210)	87 (2-213)	0,959
Gender (girls)	92(41,1%)	11(47,8)	14(42,4%)	0,689
Underlying diseases	42(18,8%)	9(39,1%)	2(6,1%)	0,004
Community associated	176(78,6%)	13(56,5%)	27(81,8%)	0,039
Nozocomial	48(21,4%)	10(43,5%)	6(18,2%)	<0,001
Antibacterial therapy	214(95,5%)	23(100%)	31(93,9%)	0,507
Surgical interventions	172(75,7%)	19(82,6%)	25(78,1%)	0,745

² p value for comparison between severe invasive infections and mild superficial infections.

There were no significant differences in median age, antibiotic therapy subscriptions, surgical interventions between patients with severe invasive infections and patients with mild superficial infections in both groups – PVL positive and PVL negative.

Severe invasive infections were found more often in patients with hospital-associated infections ($p < 0.001$). Community-associated infections were found more often in patients with mild superficial infections ($p < 0.001$). Patients with co – morbidities more often had severe invasive infections ($p < 0.001$). Differences were statistically significant in both groups PVL positive and PVL negative (Table 2. 1., 2.2.).

Distribution of diagnosis in patients with and without PVL positive *S. aureus* infections is shown in Table 2.3. In patients with isolated PVL positive *S. aureus* mild superficial infections were diagnosed more often 75% (n=126), but in patients with isolated PVL negative *S. aureus* severe invasive infections were found more frequent than in cases when PVL positive *S. aureus* was isolated, 45% (n=25)/25% (n=42) respectively.

Table 2.3.

Clinical features of PVL positive *S. aureus* cases compared with PVL negative *S. aureus* cases (p=0,014)

Clinical features		PVL positive <i>S. aureus</i> (n=168)	PVL negative <i>S. aureus</i> (n=56)
Mild superficial infections (n=157)	Superficial abscesses (n=38)	126 (75%)	31 (55%)
	Other skin and soft tissue infections (n=119)		
Severe invasive infections (n=67)	Bone and joints infections (n=33)	42 (25%)	25 (45%)
	Other infections (n=34)		

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. This risk increases with 10% statistical confidence (p<0.1 or p=0.07). The risk of the development of bone and joint infections, and other infections remains equal in both groups – PVL positive/PVL negative.

Most of the analyzed patients with PVL positive *S. aureus* infections were hospitalized in surgery profile units. (Figure.2.8.).

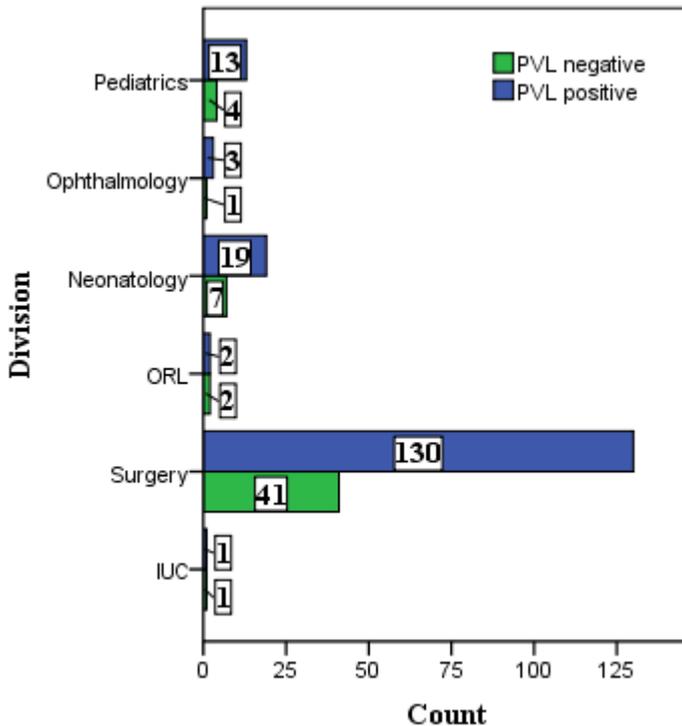


Figure 2.8. Distribution of patients by hospital units according to PVL presence

2.1.4. Impact of PVL on the length of hospitalization

There were no significant differences ($p = 0,088$) in duration of median hospitalization length between all PVL positive (median duration 6 days, (IQR 4,0;10,8)) and PVL negative infection cases (median duration 8 days, (IQR 4,0; 14,0)). Among patients with severe infections such as osteomyelitis, deep abscesses, pneumonia, bacteremia duration of hospitalization was

significantly ($p=0,033$) longer in PVL positive group - median duration 19 days (IQR 12,5;19,0), the length of hospitalization in PVL negative group was 12 days(IQR 6,0; 24,0) while among patients with mild superficial PVL positive or PVL negative infections the length of hospitalization was similar, 5 (IQR 4,0;7,0) days and 6,5 (IQR 4,0; 10,0) days respectively (Table 2.4.).

Table 2.4.

Median length of hospitalization in patients with *S. aureus* infection according to PVL presence in *S. aureus* isolates.

Indicators	PVL(+) Median length of hospitalization (days)	PVL(-) Median length of hospitalization (days)	p value
All patients (n=224)	6,0 (IQR 4;10,5)	8,0 (IQR 4;14)	0,088
Svere invasive infections (n=67)	19,0 (IQR 12,5;28)	12 ,0 (IQR 6;24)	0,033
Superficial skin and soft tissue infections (n=157)	5,0 (IQR 4;7)	6,5 (4;10)	0,034

Hospitalization was longer in patients with underlying diseases compared with patients without underlying conditions, 11/5 days respectively ($p<0,001$). 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 ($p< 0,001$). Hospitalization length among patients without underlying diseases was similar in both groups, PVL positive and PVL negative - 5/7, 5 days (Table 2.5.).

Table 2.5.

Median length of hospitalization among patients according to underlying diseases and PVL presence

Median length of hospitalization (days)	Underlying diseases (n=42)	No information about underlying diseases (n=182)
All patients p<0,001	11 (IQR 6;25)	5 (IQR 4;10)
PVL + p<0,001	15 (IQR 6;27)	5 (IQR 4;8)
PVL – p=0,164	10 (IQR 6;17)	7,5 (IQR 4;13,5)

2.1.5. Molecular characterization of recovered *S. aureus* isolates

Spa typing was performed to 219 *S. aureus* isolates and 98 different *spa* types recovered. The majority of the typed *S. aureus* strains (41% (n=90)) belonged to the *spa* type *t435* (n=52), or closely related types (*t159* (n=5), *t308* (n=10) , *t284*(n=10)) and were assigned to CC435 by BURP clustering (distance between two *spa* types was two or less genetic steps). Other most frequently identified *spa* types were *t012* (8,6% (n=19)), *t015* (6,8% (n=15)), *t318* (2,7% (n=6)) un *t1397* (1,8% (n=4)) (Figure 2.9).

Table 2.6.

Typing of collected *S. aureus* isolates by PVL genes and *spa* type

<i>Spa</i> type (n=219)	PVL +			PVL -		
	Mild superficial infections n(%)	Severe invasive infections n (%)	P value	Mild superficial infections n (%)	Severe invasive infections n (%)	P value
CC 435* (n=90)	62 (68,8%)	16 (17,7%)	0,135	9 (10,0%)	3 (3,3%)	0,040
Other <i>spa</i> types n=129)	63 (48,8%)	26 (20,2%)		19 (14,7%)	21(16,3%)	

*CC 435 (t435, t159, t308, t284, txAB)

S. aureus isolates belonging to the CC435 were isolated from patients with a shorter length of hospitalization (mean 8,9 days (SD 10,0); median 5,00 days (IQR 4,0;10,0)), comparing with remaining *S. aureus* isolates (mean 11,7 days (SD 17,7); median 7,00 days (IQR 4,0;12,8)), the differences were statistically significant ((z= -2,235 (*Mann-Witney* test); p=0,025). Analyses of CC435 isolates alone revealed no statistically significant differences (*Mann-Witney* z=0,331; p=0,741) in hospitalization length between PVL positive *S. aureus* (mean 8,6 days(SD10,0); median 5,0 days) and PVL negative *S. aureus* isolates (mean 10,8 days(SD10,3); median 7,0 days) respectively.

MLST was performed for seven isolates from CC435. All of them belonged to ST121 (Table 2.7.).

Table 2.7.

Typing of PVL positive *S. aureus* isolates by MLST

Nr.	Clinical features	Material	<i>Spa</i> type	MLST
1.	streptodermia	pus	t308	MSSA ST121
2.	phlegmona	pus	t435	MSSA ST121
3.	osteomyelitis	pus	t284	MSSA ST121
4.	bursitis	pus	t159	MSSA ST121
5.	osteomyelitis	pus	t435	MSSA ST121
6.	furunculus	pus	t435	MRSA ST121
7.	lymphadenitis	pus	t435	MSSA ST121

2.1.6. The clinical case of PVL positive *S. aureus* in Children's Clinical University hospital

(The clinical case - PVL positive *S. aureus* pneumonia in a child with influenza A H1N1 infection)

A 15 – year old boy was admitted to the Children' s Clinical University Hospital on November 30, 2009 from Daugavpils Regional Hospital with a 4-day history of flu- like illness and approved total right sided pneumonia.

On arrival (fifth day of illness) at CCUH, Riga, the patient had difficulty breathing and had signs of severe respiratory failure; he was sitting in an enforced position, had tachypnea (35 – 40 times per min.) with loud, groaning breathing and intercostal retractions. Auscultation of the lungs showed

unilateral dullness on the right side. Laboratory findings showed significant changes in blood gases. Changes in biochemical and blood count analyses indicate severe bacterial infection (Table 2.8., 2.9., 2.10.).

Table 2.8.

Investigations of blood gases

Indicator	30.11.09. 15.25	30.11.09. 20.18	01.12.09. 4.04	02.12.09.
pO ₂ mmHg (N 71-104)	70,6	64,7	55,4	132,2
pCO ₂ mmHg (N 32-46)	78,6	75,1	59,5	42,9
Be (B) mmol/l (-5-5)	6,0	10,3	11,2	6,7
pH (N 7,37- 7,45)	7,28	7,30	7,40	7,40
HCO ₃ std mmol/l (N 21-26)	29,9	30,8	32,9	30,0

Table 2.9.

Complete blood count

Indicator	30.11.09.	02.12.09.	03.12.09.	22.01.10.
WBC x10 ³ /μl (4,5-13)	6,85	6,54	9,87	9,66
RBC x10 ⁶ /μl(4,5-5,3)	5,37	4,31	4,35	5,10
PLT x10 ³ /μl (181-521)	143	166	197	413
HGB (g/dl)	14,3	11,5	11,5	12,6
Mo%	10	7	-	-
Ly%	12	15	-	-
Neutrophils "segs" %	52	61	-	-
Neutrophils "bands" %	25	15	-	-
Mylocytes%	1	-	-	-

Table 2.10.

Investigations of blood chemistry

Indicator	30.11. 2009.	02.12. 2009.	03.12. 2009.	07.12. 2009.	16.12. 2009.	27.01. 2010.
CRP mg/l	261,0	163,0	179,0	96,0	50,7	5,1
Protein total g/l	71,0	50,4	-	-	68,3	-
Lactate	2,26	1,30	-	-	-	-
Crea µl/l	63	69	-	45	51	-
Urea mmol/l	7,3	8,9	-	6,4	4,2	-
AST – aspartate aminotransferase U/l	14,2	91,4	-	134,7	97,6	-
ALT – alanine aminotranferase U/l	7,7	20,3	-	150,7	134,7	-
Bilirubin µl/l	6,1	-	-	11,1	5,2	-
LBP	-	44,4	108,0	-	-	-
Il6 pg/ml	-	172	135	-	-	-

The chest X-ray showed multiple focal shadows on both sides of the lungs and unilateral intensive infiltration in the middle part of the right lung that suggested severe bilateral pneumonia (Figure 2.10., 2.11.).

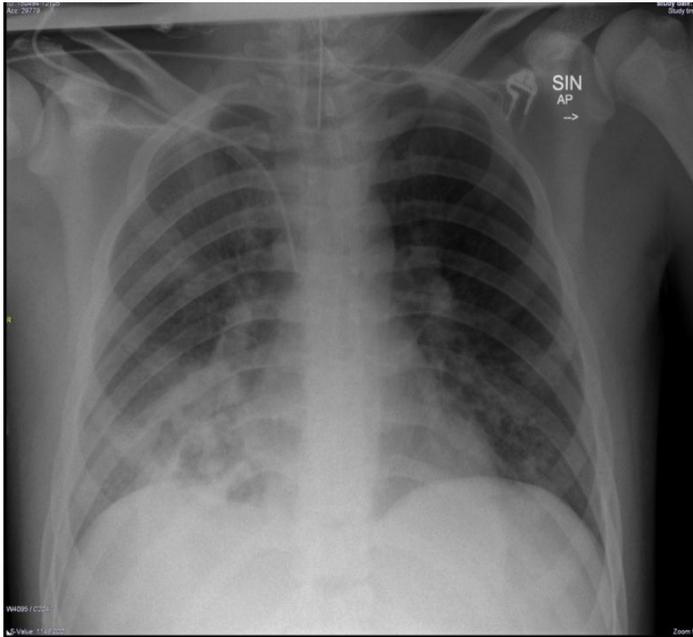


Figure 2.10. Chest X-ray 01.12.2009. 7.35

Multiple focal shadows on both sides of the lungs and unilateral intensive infiltration in the middle part of the right lung

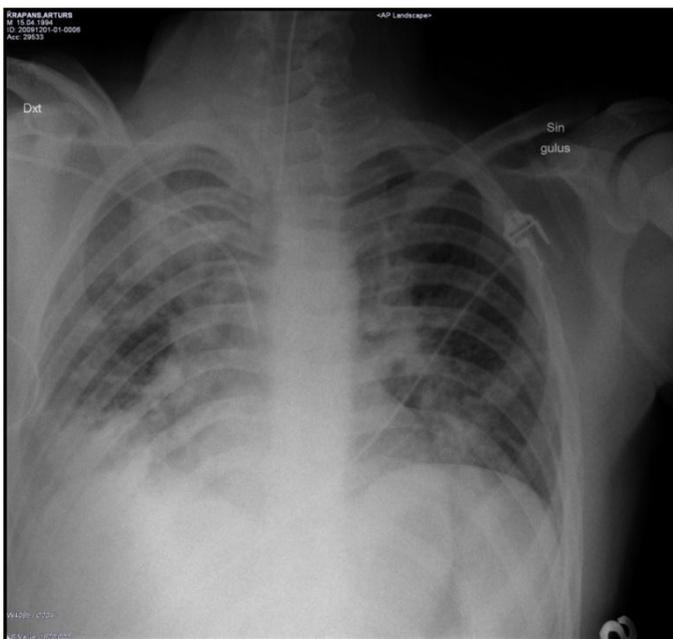


Figure 2.11. Chest X-ray 01.12.2009. 11.36

Negative dynamics, rapidly progressing bothsided multiple focal shadows, unilateral intensive infiltration in the middle part of the right lung.

Eleven hours after admission due to increasing respiratory insufficiency mechanical pulmonal ventilation was started and continued for 15 days. The general condition of the patient remained severe for more than five days.

Novel influenza A H1N1 infection was confirmed by PCR and *S. aureus* isolated from blood and pleural fluid on the day of admission were methicillin susceptible, Panton – Valentine leukocidin producing and were *spa* type *t435*. Antibacterial susceptibility was determined according to CLSI standards (actual version). The *lukSF-PV* genes were detected by PCR. Chromatograms of the *spa* sequences were analysed by Ridom StaphType software (Ridom GmbH).

According to flu-like symptoms empiric oral antiviral therapy with

oseltamivir phosphate (five days) and intravenous antibacterial therapy with ceftriaxone (14 days) and oxacillin (14 days) in addition to symptomatic therapy were commenced. One day later clindamycin was added (21 days).

On the 16th day the boy underwent operative therapy with a right side thoracotomy and resection of S4, S5 of the right lung because of the severe condition due to pneumothorax and empyema. Further investigations of postoperative material revealed necrosis and inflammation of lung tissues. After the operation his general condition improved and it was decided to continue conservative therapy with antibiotics, but due to a post-operative fistula of the right lung, the surgery was repeated after 3 weeks and the fistula was closed. With this treatment blood cultures became negative on the 14th day of hospitalization. His general condition improved and after 58 days in hospital the patient was discharged.

The described case exposes that PVL – positive *S. aureus* with *spa* type *t435* can complicate influenza in otherwise healthy children, with rapid progression to severe pneumonia that needs complicated and long management of the illness.

2.2. Community acquired MRSA carriage among children in an outpatient child care centre

An 8-month-old girl with purulent inflammation of neck lymphatic nodes was admitted to the Children' s University Hospital from a child care centre in August 2007.

On the day of admission a *S. aureus* isolate was obtained from pus after a lymphatic node incision and was identified as CA-MRSA. *S. aureus* was

susceptible to all antibiotics, except cefoxitin, carried the *mecA* gene and genes for PVL production, and *type IV SCCmec*.

After incision and anti-bacterial therapy with gentamicin and oxacillin, the baby was discharged from the hospital in good condition after 2 weeks.

The girl was hospitalized from a children's home for children with special needs with a different degree of mental disabilities. Approximately 62 children up to the age of 4 years, divided into 6 separate groups, live in the children's home. Each group has 2 caretakers who take care of the children 24 hours a day.

There was a high risk of CA-MRSA spread in a close community directly after the nasal swabs were obtained from the personnel and children from the child care centre which was the presumptive source of the infection.

Close contact persons were tested in September 2007. Seven *S. aureus* cultures were obtained. All seven cultures were susceptible to cefoxitin (FOX). A molecular investigation revealed that four of the obtained *S. aureus* strains displayed the characteristics of CA-MRSA; the strains were *mecA* gene-positive and carried the type IV SCCmec and genes necessary for PVL production.

The *spa* typing of *S. aureus* strains from the child care centre, showed that four of the strains were closely related, but three of the strains were identical and belonged to the same *spa* type – t1298. (Table 2.11.)

Decolonisation of CA-MRSA carriers from the child care centre was not done. After 1 year, the screening was repeated. Thirteen isolates of *S. aureus* were identified, but no MRSA was found.

Table 2.11.

Characterization of *S. aureus* isolates

Isolate	Site of isolate acquisition	Time and place	Personne	Antibakteriālā rezistence*											luk-PV	mec-A	SCC mec type	spa repeats	spa type
				P	F O X	G M	E	C IP	C C	R A	T	S T X	V A						
14.	Lyphadenitis purulenta acuta	27.08. 2007. CCUH	patient	R	R	S	S	S	S	S	S	S	S	S	poz.	poz.	IV	15-12-16-16-02-16-02-25-17	t433
13.	nose	07.09. 2007. CCC	child	R	S	S	S	S	S	S	S	S	S	S	poz.	poz.	IV	23-21-22-16-34-33-13	jauns
10.	nose	07.09. 2007. CCC	personell	R	S	S	S	S	S	S	S	S	S	S	poz.	poz.	IV	15-16-02-16-02-25-17-24	t1298
9.	nose	07.09.2007. CCC	child	R	S	S	S	S	S	S	S	S	S	S	poz.	poz.	IV	15-16-02-16-02-25-17-24	t1298
7.	nose	07.09. 2007. CCC	child	R	S	S	S	S	S	S	S	S	S	S	poz.	poz.	IV	15-16-02-16-02-25-17-24	t1298
5.	nose	07.09.07. CCC	personell	R	S	S	S	S	S	S	S	S	S	S	poz.	neg.	-	14-44-13-17-17-17-23-18--17	t435
4.	nose	07.09.07. CCC	child	R	S	S	S	S	S	S	S	S	S	S	neg.	neg.	-	-	
1.	nose	07.09.07 CCC	child	R	S	S	S	S	S	S	S	S	S	S	neg.	neg.	-	-	-

*Ppenicillin, FOX- cefoxitin, GM- gentamicin, E – erythromycin, CIP – ciprofloxacin, C- chlorampfenicol, CC – clindamycin, RA – rifampin, T – tetracycline, STX – trimethoprim/sulfamethoxazole, VA – vancomycin.

3. DISCUSSION

S. aureus is a successful nosocomial and community acquired pathogen. The pathogen can cause a wide range of infections from mild superficial to severe invasive infections like deep seated abscesses, pneumonia, endocarditis, sepsis. Pathogenicity is related to a number of virulence factors like the ability of rapid adaption to most frequently used antibacterial substances, factors that affect the immune system, promote *S. aureus* adhesion to the host tissues and degrade them and contribute to the penetration of bacteria into host tissues. One of these virulence factors is Pantone-valentine leucocidin. (Holmes A *et al*, 2004). More than fifteen years ago PVL genes were identified only in 2% of *S. aureus* isolates. (Prevost G *et al*, 1995). Further reports indicate an increase of PVL *S. aureus* isolates. (Issatrel B *et al*, 2005, Chini V *et al*, 2005, Del Giudice P *et al*, 2009, Nickerson E, *et al*, 2009, Masiuk H *et al*, 2010).

Although Pantone-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 collected from blood due to severe invasive infections as well as from pus in cases of mild superficial infections.

The discrepancy between the reports describing PVL prevalence is probably due to the differences in *S. aureus* cultures selection and geographic area, in general PVL positive *S. aureus* spread in the world has increased. Another concern is the large PVL positive *S. aureus* prevalence and the fact that this toxin is often identified in *S. aureus* collected from the samples of not only purulent skin infections, but also severe invasive diseases in children and young people (Gillet Y *et al*, 2002, Gillet Y *et al*, 2007).

3.1. Prevalence of PVL positive *S. aureus* in hospitalized patients with *S. aureus* infection

In Latvia the prevalence of PVL positive *S. aureus* has not been studied. A study was done in the Children's Clinical University Hospital in Riga, Latvia which is the only tertiary level children's hospital in Latvia which serves a population of approximately 420,000 children and shares 600 beds, therefore the study can characterize the situation of children in Latvia. In order to make an accurate accounting of PVL positive *S. aureus* cases, centralisation of data from pediatric departments of all hospitals in Latvia is required.

Data obtained during the study indicates a high prevalence of PVL positive *S. aureus* in children with *S. aureus* infection. The high prevalence of PVL positive *S. aureus* infection is determined by two factors. Firstly, no study of PVL positive *S. aureus* spread among children in Latvia has previously been done, so we cannot claim that PVL genes were not widely spread in Latvia before the study. The studied population (children with purulent skin and soft tissue infections) could be another factor which influenced the result of prevalence studies. Data from literature show a connection of PVL positive *S. aureus* with purulent skin and soft tissue infections in children, too. (Table 3.1.).

Table 3.1.

Data from literature about prevalence of PVL in *S. aureus* isolates

Place, date	Studied population	<i>S. aureus</i> cultures	Material	PVL prevalence	References
New Caledonia, France, February –April, 2002	Hospitalized adults	65 MSSA	Pus from abscesses	89%	Issatrel B et al, 2005
France, 2003-2006	Not specified	57	Pus from abscesses	70%	del Giudice P et al, 2009
Thailand, 2006 - 2007	All ages	248	Pus, blood, pleural liquide, cerebrospinal fluid .	49%	Nickerson E et al, 2009
USA, 2005.- 2006	Children	214 MSSA	Abscesses, wounds	14,5%	Orscheln RC et al, 2009
Latvia, 2006-2008	Hospitalized children	224 MSSA	Pus, blood, operation material	75%	Results from investigation in CCUH
New Zealand, February –April, 2008	All ages	335 MSSA	Patients with skin infections	37%	Muttaiyah S et al, 2010

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. These results confirm reports from previous studies where it was detected that the high prevalence of PVL positive *S. aureus* isolates was associated with furunculosis and other skin and soft tissue infections (Lina G *et al*, 1999, del Guidice P *et al*, 2009, Baba-Mousa L *et al*, 2011, del Guidice P, 2011). 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 (Masiuk H *et al*, 2010, Melles DC *et al*, 2006). The high prevalence of skin and soft tissue infections caused by PVL positive *S. aureus* among children suggests that local guidelines are needed. The clinical case included in the study indicates the significance of close everyday contacts for spread of PVL positive *S. aureus*, especially in a close community. Outbreaks with PVL positive *S. aureus* infection have occurred among football team members, students in a school, inhabitants of a villige in Germany and within the same family (Fontanilla JM *et al*, 2010, Cheril LM *et al*, 2005, Boubaker K *et al*, 2004, Wiese-Posselt M *et al*, 2007, Tinelli M *et al*, 2009).

S. aureus is one of the most frequent causes of osteomyelitis in children (Darvile T *et al*, 2004, Kao HC *et al*, 2003). Data of osteomyelitis incidence in Lithuania show an increase from 11.5 cases per 100 000 children in 1982 to 14.3 cases in 2003 (Malcius D *et al*, 2005). In the current study one of the most frequent reasons of hospitalization after skin and soft tissue infections was bone and joint infections. More than half of all bone and joint infections were caused by PVL positive *S. aureus*. There is little information in the literature about PVL positive *S. aureus* caused osteomyelitis. The available information suggests that PVL positive *S. aureus* osteomyelitis has been found among children (Kaplan SL *et al*, 2005). In addition, osteomyelitis is often caused not

only by PVL positive MRSA but also by PVL positive MSSA (Sdgoukus D *et al*, 2007). The large amount of patients with PVL positive methicillin susceptible *S. aureus* osteomyelitis indicates the necessity of molecular investigations for identification of PVL genes in *S. aureus* isolates in patients with severe course of illness to carry out toxine mediated therapy.

PVL positive *S. aureus* is a frequent cause of necrotizing pneumonia (O Gorman J *et al*, 2004, Rouzic N *et al*, 2010). The high occurrence of PVL positive *S. aureus* pneumonia among children and young adults is worrying. Median age of such patients is fourteen years and fatal outcomes are observed in more than half of all clinical cases (Gillet *et al*, 2002, Gillet *et al*, 2007). Bacterial infections with PVL positive *S. aureus* is a common cause of severe illnesses often occurring after, and complicating, viral respiratory infections, especially influenza (Roberts JC *et al* 2008, Gillet Y *et al*, 2002). In the present study there were only two PVL positive MSSA caused pneumonia cases. Both children were under three years of age.. There was no information that during hospitalization patients had flu or other respiratory viruses. Information was acquired retrospectively from patients' medical cards. More detailed information was acquired about the patient with PVL positive *S. aureus* pneumonia after influenza infection. The patient's age at the onset of illness and subsequent course coincided with the literature data. In all cases the disease progressed favorably, the children recovered. Although few cases of PVL positive pneumonia are described in the current study, however, this shows the significance of PVL in causing severe infections.

3.2. Impact of PVL on the clinical course of the *S. aureus* infection in hospitalized children

The impact of PVL on the clinical course of *S. aureus* infection is well described in literature. There are reports confirming adverse effect of this toxin by causing severe invasive infections and increasing mortality (Gillet Y *et al*, 2002, Dophin B *et al*, 2007). Experiments with animal models revealed evidences of PVL degrading effect on organism (Labandeira-Rey M *et al*, 2007, Diep BA *et al*, 2010). However, there are studies that doubt the influence of PVL on the clinical course of *S. aureus* infection (Voyich JM *et al* 2006, Bubeck Wardenburg J *et al* 2008).The influence of PVL on the clinical course of *S. aureus* infection in the performed study was detected using information obtained from retrospectively analysed medical cards of patients which can be a drawback of the study. There can be discrepancy between the real situation and medical records.

Information from the performed investigation shows that the presence of PVL genes indicate a more severe course of illness in patients with underlying diseases and in patients with hospital acquired infections. PVL positive *S. aureus* isolates were more prone to cause skin abscesses. There were no suggestion that PVL positive *S. aureus* increased the risk of such severe infection development as sepsis, pneumonia and osteomyelitis. However, a report of a study performed in a hospital which included patients of all ages shows that PVL positive *S. aureus* isolates are connected with a 1.94 times higher risk of sepsis development (Tong SY *et al* 2010).

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 (HPA 2008). 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 (Daum RS, 2007).

3.3. Impact of PVL on the length of hospitalization

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 on the connection between PVL positive *S. aureus* infection and the length of hospital stay and the restricted number of samples included in the studies make them implausible. 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 other authors reported that pediatric patients with PVL positive bone and joint infections had 3 times longer median hospitalisation time versus control group with PVL negative *S. aureus* bone and joint infections (Dophin B *et al*, 2009, Papenburg J *et al*, 2009).

3.4. Phylogenetical characterization of *S.aureus* isolates

In the study of the epidemiology of *S. aureus* infection different typing methods are used. The most popular ones are PFGE (pulsed-field gel electrophoresis), MLST (multi-locus-sequence typing) and *spa* typing (Said-Salim B *et al*, 2003, Enright MC *et al*, 2000.). In the current study collected isolates were typed by *spa* gene. This method is rapid, obtained sequence data are of unambiguous nature and easily electronically portable, which provides exchange of information between laboratories for database development (Aires-de-Sousa M *et al*, 2006). *Spa* typing is suitable for investigation of infection outbreaks in hospitals as well in outpatient settings (Harmsen D *et al*, 2003). Besides using sequence data and BURP algorithm it is possible to trace clonal relationship of isolates as well as with MLST typing which is a more expensive and time consuming method (Mellmann A *et al*, 2007).

The *spa* sequence analysis revealed that most of the *S. aureus* isolates belong to the *spa* type *t435* or are closely related. Pantone- Valentine leukocidin positive *S. aureus* isolates with *spa* type *t435* are mostly methicillin susceptible and are common in Latvia with sporadic cases in Poland, Austria, Romania and Hungary (Grundman H *et al*, 2010) (Figure 3.1.).

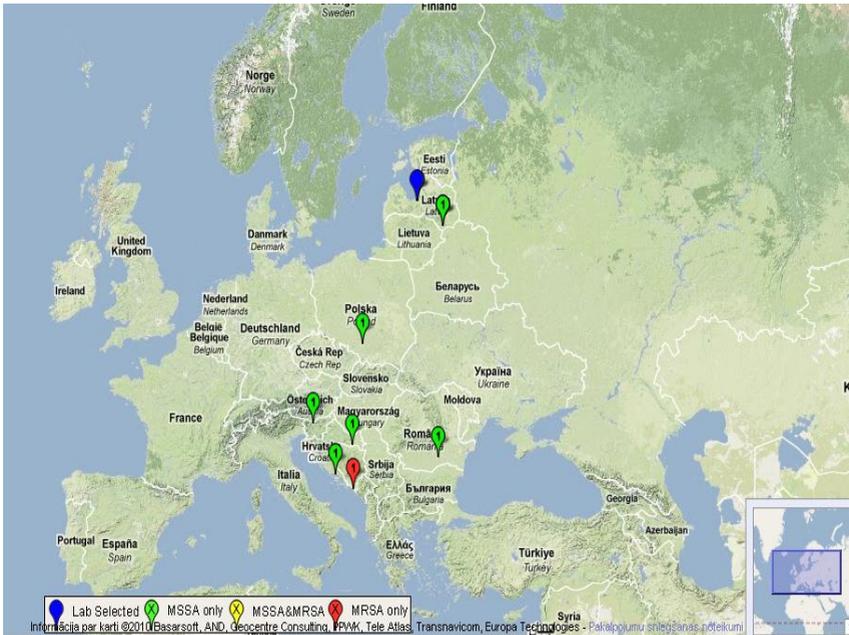


Figure 3.1. Spread of *S. aureus* with *spa* type *t435* in Europe (Grundman H *et al*, 2010)³

In Latvia *S. aureus* isolates with *spa* type *t435* have been described for the first time. Interestingly, *spa* type *t435* is prevalent in children population and is different from *S. aureus spa* types collected from adult population where *t433* is the most prevalent. In the report on *spa* and MLST typing, Strommenger B *et al* identify methicillin susceptible *S. aureus* isolates belonging to CC435 which includes the *t435*, *t159*, *t308*, *t284*, *t269*. The described isolates are PVL negative (Strommenger B *et al*, 2006). However, in the current study most of

³ Methicillin-susceptible isolates in green, methicillin-resistant isolates in red belonging to the *spa* type *t435*.

the isolates belonging to CC435 are PVL positive. There are reports in the literature that PVL genes are carried on bacteriophages that provide their circulation among *S. aureus* isolates and ability to infect PVL negative isolates (Narita S *et al*, 2001, Kaneko J *et al*, 1997). A report from Poland shows that in patients with *S. aureus* furunculosis most frequent detected *spa* types were *t435* and clonally related *spa* type (Masiuk H *et al*, 2010). This finding corresponds with the observations of the current study where isolates with *spa* type *t435* and closely related *spa* types were collected from patients with superficial skin and soft tissue infections.

Spa typing was useful for identification of CA-MRSA carriage in an outpatient children care centre. A patient from this centre was admitted to the Children's Clinical University Hospital with soft tissue infection. A further molecular investigation of *S. aureus* cultures obtained from the persons in contact with the patient from the child care centre revealed an outbreak of CA-MRSA. To detect the relationship between isolates, *spa* typing was used. Disappearance of separate repeats of analysed *S. aureus* isolates, as well as detection of identical strains can designate one source, which determines the spread of *S. aureus* in the child care centre. (Table 2.11.).

Protein A is an important virulence factor of *S. aureus*. It binds to a variety of ligands including Fc region of IgG, von Willebrand factor and tumor necrosis factor receptor-1 and impacts immune system processes, platelets aggregation and pathogenesis of pneumonia caused by *S. aureus*. (Foster TJ, 2005, O'Seaghdha M *et al*, 2006, Gomez MI *et al*, 2006). Claro *et al* reports that protein A plays an important role in *S. aureus* caused osteomyelitis by influencing apoptosis and inhibition of proliferation of osteoblasts (Claro T *et al*, 2011). The study revealed that *S. aureus* isolates belonging to CC435 more often were collected from patients with mild superficial infections, while isolates collected from patients with severe invasive infections belonged to

other *spa* types. This finding can indicate the role of *spa* type in the clinical course of *S. aureus* infection, however, in literature there is no information that small mutations of protein A coding gene *spa* can affect virulence of protein A. Spa connection with the length of hospitalization is not described in literature. The study results show that the length of hospitalization is not connected with identified dominating *spa* types in children.

MLST results showed that PVL positive MSSA with *spa* type t435 and closely related *spa* types belong to ST121. Data about *S. aureus* isolates with ST121 are controversial. Recent studies of *S. aureus* isolates obtained from children showed that most isolates with such ST were MRSA (Nickerson EK *et al*, 2009). For instance, a report from Portugal shows that a large proportion of collected *S. aureus* isolates from children with skin and soft tissue infections belonged to ST121 and were methicillin susceptible (Conceicao T *et al*, 2011). Results of investigations of methicillin-susceptible *S. aureus* (MSSA) ST121 from skin isolates were described in South Africa, Russia, India, the United States showing the worldwide distribution of this ST (Goering RV, 2008). Recent reports on the involvement of MSSA-ST121 PVL positive isolates as well in furunculosis outbreak as in therapy refractory sepsis reveal the significance of this clone (Wiese-Posselt M *et al*, 2007, Schefold JC *et al*, 2007). The study which was carried out over a long period of time and covered many countries revealed that spread of PVL positive methicillin susceptible *S. aureus* is pandemic and they are related with CA-MRSA (Rasigade JP *et al*, 2010). Šī saistība iespējams nozīmē, ka PVL-pozitīvie MSSA var kalpot par CA-MRSA rezervuāriem (Monecke S *et al*, 2007). The results of the study underline the necessity of microbiological sampling and epidemiological typing as a rational basis for treatment of *S. aureus* infections and infection control measures to limit the spread of epidemic MSSA clones.

4. CONCLUSIONS

1. Prevalence of PVL positive *S. aureus* in hospitalized children with *S. aureus* infection was 75 %, (95% CI 0,6894 – 0,8022).
2. Development of severe invasive infections is statistically significantly observed in patients with underlying diseases ($p < 0.001$) and patients with hospital acquired *S. aureus* infections ($p < 0.001$).
3. Presence of PVL genes statistically significantly designates a longer time of hospitalization in patients with severe invasive infections ($p = 0.033$) and patients with underlying diseases ($p < 0.001$).
4. Spread of *spa* clonal type CC435 *S. aureus* isolates in Latvia is observed in hospital-treated children.
5. Dominant in Latvia CC453 does not prolong the pediatric patients' hospitalization time in comparison with *S. aureus* infections of other clonal types.

5. PRACTICAL RECOMENDATIONS

1. Information centralization about *S. aureus* induced necrotic pneumonia, osteomyelitis and other serious invasive infections in children to detect PVL positive *S. aureus* infection cases is needed.
2. Hand hygiene is important tool to limit the spread of PVL positive *S. aureus* in areas of children gathering (preschools, social care centers, schools, sports arenas, etc.).

6. PUBLICATIONS ON THE STUDY SUBJECT

1. **L. Čupāne**, Ņ. Pugačova, I. Selga, A. Balode, D. Gardovska, E. Miklaševičs. Bērnu Klīniskās universitātes slimnīcas MRSA izolātu klīniskais un molekulārais raksturojums. 2007. gada RSU Zinātnisko rakstu krājuma infekcijas slimību sadaļa; 219-223.
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