ORIGINAL RESEARCH

Panton-Valentine leukocidin in pediatric community-acquired *Staphylococcus aureus* infections

Jesse Papenburg MD FRCPC¹

Patricia Fontela MD, MSc²

Lélia Raynal PhD³

Louise Jetté³

Johanne Ismail³

Sadjia Bekal PhD³

Ibrahim Al-Zahrani MD FRCPC¹

Caroline Quach MD, MSc FRCPC¹

¹ Division of Infectious Diseases, Department of Pediatrics, and Department of Microbiology, Montreal Children's Hospital, McGill University Health Centre, Montreal, Québec, Canada

² Department of Epidemiology, Biostatistics and Occupational Health, Faculty of Medicine, McGill University, Montreal, Québec, Canada

³ Laboratoire de Santé Publique du Québec, Institut National de Santé Publique du Québec, Sainte-Anne-de-Bellevue, Québec, Canada

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Abstract

Purpose: Panton-Valentine Leukocidin (PVL) is an exotoxin produced by strains of *Staphylococcus aureus* (SA). Its importance as a virulence factor is controversial. We aim to further characterize the role of PVL in pediatric community-acquired SA infections.

Methods: In a cohort study conducted from July to November 2006, we prospectively collected all strains of SA isolated at the Montreal Children's Hospital causing community-acquired infections in children aged 18 years or younger. The strains were analyzed for the presence of the PVL encoding genes by PCR and were phage typed. Strains resistant to methicillin or *pvl*⁺ were analyzed by pulsed-field gel electrophoresis. A medical chart review blinded to patient *pvl* status was performed to retrieve demographic and clinical data. Data were analyzed by logistic regression.

Results: We identified 74 pediatric community-acquired SA infections. Nineteen strains (25.7%) were positive for the *pvl* genes. Four isolates (5.4%) were resistant to methicillin and three of these were *pvl*⁺. No predominant clone was identified by phage typing or pulsed field gel electro-

phoresis. *Pvl*⁺ and *pvl*⁻ infections were statistically similar for patient age, hospital admission, length of hospital stay, invasive disease, intravenous antibiotics and outcomes. *Pvl*⁺ strains were more likely to cause abscesses (OR 20.79; 95% CI 4.93 – 87.58), less likely to cause superficial skin infections (OR 0.18; 95% CI 0.05 – 0.64) and less likely to be resistant to erythromycin (OR 0.048; 95% CI 0.004 – 0.52).

Conclusions: In a clonally heterogeneous population of pediatric community-acquired SA infections, *pvl*⁺ strains were associated with abscess formation and erythromycin susceptibility, but not invasive disease.

Staphylococcus aureus (SA) causes a wide range of disease, from benign skin infections to life-threatening sepsis. Panton-Valentine Leukocidin (PVL) is a bi-component, pore-forming exotoxin secreted by certain strains of SA, and has been hypothesized to be an important virulence determinant.¹

PVL preferentially targets neutrophils, causing release of inflammatory mediators at low concentrations, and cell lysis at higher concentrations.²⁻⁴ Intradermal injection of the toxin causes localised edema, erythema and dermonecrosis in rabbits.⁵⁻⁷ However, recent reports on the role of PVL as a virulence factor in mouse models have yielded contradictory results.⁸⁻¹⁰

Epidemiological data have associated PVL with a broad spectrum of clinical manifestations, from mild primary skin infections to fulminant invasive disease with high mortality.¹¹ Nevertheless, the role of PVL as a virulence factor is yet to be fully understood. Previous studies on PVL in pediatric community-acquired (CA) SA disease may have been confounded by very high rates of methicillin resistance (MRSA)¹², and the emergence of a PVL-producing epidemic clone, USA300.¹³

Our primary objective is to determine the role of PVL as a virulence factor in pediatric CA-SA infections. We also aim to identify potential risk factors for infection with *pvl*⁺ CA-SA in children, and to estimate its prevalence.

Patients, Materials, and Methods

Study Setting

The greater metropolitan area of Montreal (Québec) Canada has a population of approximately 3.6 million¹⁴, an estimated 19% (684,000) of whom are children aged 18 yr or younger.¹⁵ The Montreal Children's Hospital (MCH) is one of two university-affiliated pediatric tertiary care hospitals serving the area.

Study Population

From July 1 to November 15, 2006, we prospectively screened all new clinical isolates identified as SA at the MCH microbiology laboratory. Isolates eligible for study were from patients aged ≤18 yr, the sole or predominant pathogen identified in the clinical specimen, and community-acquired (CA).

As previously proposed¹⁶, infections were considered CA when (1) symptoms developed in the outpatient setting or within 48 hr of admission to hospital; (2) the patient did not reside in a long-term care facility nor receive dialysis for the year prior to infection; (3) the specimen was not taken from the site of a previous surgery; and (4) the patient had no indwelling medical device. Isolates were excluded if sampling was done for the purpose of screening for colonization.

Antimicrobial susceptibility testing

We performed antimicrobial susceptibility testing in the MCH clinical microbiology laboratory on all study samples by the disk diffusion method, 6 g/L oxacillin agar screen and D-test in accordance with the Clinical and Laboratory Standards Institute guidelines.¹⁷ Isolates resistant to cefoxitin by disk diffusion and growing on 6g/L oxacillin agar were confirmed as MRSA by a real-time polymerase chain reaction (PCR) assay detecting the presence of the *mecA* gene and the SA-specific *sa442* gene.¹⁸

Epidemiological typing of strains and molecular methods

All strains were tested by a conventional PCR assay for the presence (*pvl*⁺) or absence (*pvl*⁻) of the PVL-encoding genes *lukS-PV* and *lukF-PV*.¹⁹ In order to assess for clonal relationships between SA strains, we phage typed all isolates. Strain differences were determined according to international standards.²⁰ Molecular epidemiological typing was performed by pulsed-field gel electrophoresis (PFGE). All MRSA or *pvl*⁺ isolates were typed by PFGE as per the Canadian protocol²¹, and strain relatedness was interpreted as per previously described criteria²².

Patient Data and Case Definitions

Permission to review patient charts was granted by the MCH institutional review board and the study was

performed in accordance to the instructions of the MCH ethics committee.

A researcher blinded to the results of phage typing, PFGE and PCR performed the clinical chart review. Standard data extraction sheets were used to collect the following variables: patient demographics (date of birth, sex) and past medical history (use of antibiotics in past 3 months; underlying medical conditions including skin disease; surgery or hospitalization other than birth in the past year), clinical features (fever $\geq 38.0^{\circ}\text{C}$ by any route, diagnosis), microbiological and laboratory data (site of SA isolate, SA antibiotic susceptibilities, peripheral blood neutrophil count, erythrocyte sedimentation rate, C-reactive protein, diagnostic medical imaging), clinical management (admission to hospital, length of hospital stay, antibiotic use and route of administration, surgical intervention), and outcomes (complete recovery, recurrent or chronic infection).

Invasive infection was defined as SA isolated from a normally sterile body fluid. Superficial skin infection was defined as infection of the superficial skin structures (cellulitis, impetigo, bullous impetigo, pustules, folliculitis, paronychia, superinfected eczema). Abscess was defined as a pus-filled lesion measuring more than 1 cm in diameter.

Statistical Analysis

Statistical analysis was performed using the software R 2.6.2. Descriptive statistics calculated included mean, standard deviation, median and interquartile range (percentile 25 to percentile 75). Categorical variables were presented with their frequency distribution. Means were compared using the two-tailed Student t-test. Categorical variables were compared using the χ^2 test. A *P* value of less than 0.05 was considered significant.

Univariate logistic analysis was initially performed to explore the relation between *pvl*⁺ and the other collected variables. Subsequently, we used multivariate logistic regression to evaluate the relation

between *pvl*⁺ (outcome) and its possible risk factors (exposure), and also the relation between *pvl*⁺ (exposure) and different clinical findings (outcomes). Adjusted odds ratios (OR) were reported with their 95% confidence intervals (CI).

Results

We identified 90 potential CA-SA infections from July 1 to November 15, 2006. Fifteen cases were excluded after clinical chart review: 8 infections did not meet criteria for being CA, 5 samples had been performed for colonization screening, and 2 isolates had no clinical data available (specimens sent to MCH by outside clinics). In addition, one isolate was not available for molecular analysis. Consequently, 74 CA-SA infections were included in our study. Nineteen strains (25.7%) were *pvl*⁺.

Baseline patient characteristics (Table 1) were similar in children infected with *pvl*⁺ CA-SA compared to those infected with *pvl*⁻ strains.

Table 2 presents the Clinical manifestations, laboratory characteristics, patient management and outcomes in *pvl*⁺ and *pvl*⁻ infections are presented in Table 2. Four isolates were resistant to methicillin (5.4%). Three of these four strains were *pvl*⁺.

Overall, there were 46 superficial skin infections (62.2%) and 15 abscesses (20.3%). Five other non-invasive infections were identified (1 acute otitis media, 1 lymphadenitis, 1 infected septal hematoma, 1

TABLE 1. Baseline patient characteristics

Variable	PVL (+) n = 19 (%)	PVL (-) n = 55 (%)	OR	95% CI
Age, yr (median and interquartile range)	7.8 (1.5 – 9.0)	4.1 (0.6 – 9.7)	1.04	0.94 – 1.15
Female	10 (53)	25 (46)	1.33	0.47 – 3.79
Recent antibiotics	4 (21)	6 (11)	2.18	0.54 – 8.75
Hospitalization or surgery past year	3 (16)	3 (6)	3.25	0.60 – 17.71
Underlying skin condition	4 (21)	12 (22)	0.96	0.27 – 3.42
Other medical condition	2 (10.5)	4 (7)	1.5	0.25 – 8.93

TABLE 2. Clinical presentation, laboratory features, management, outcomes and univariate logistic regression results.

Variable	PVL (+) n = 19 (%)	PVL (-) n = 55 (%)	OR	95% CI
Fever	7 (37)	10 (18)	2.63	0.83 - 8.35
Superficial skin infection	7 (37)	39 (71)	0.24	0.08 - 0.72
Abscess	11 (58)	4 (7)	17.53	4.47 - 68.69
Invasive infection	1 (5)	7 (13)	0.38	0.04 - 3.32
Erythromycin resistance	1 (5)	21 (38)	0.08	0.01 - 0.52
Clindamycin resistance *	1 (5)	20 (36)	0.09	0.01 - 0.72
Admission	6 (32)	18 (33)	0.95	0.31 - 2.91
Hospital length of stay, days (median and interquartile range)	3.5 (3.0 - 4.8)	5.5 (2.3 - 7.8)	0.98	0.87 - 1.11
Intravenous antibiotics	10 (53)	26 (47)	1.24	0.44 - 3.52
Incomplete recovery	5 (26)	10 (18)	1.61	0.47 - 5.50
Lytic group				
Group 1	2 (11)	4 (7)	1.50	0.25 - 7.28
Group 2	4 (21)	18 (33)	0.55	0.16 - 1.89
Group 3	6 (32)	12 (22)	1.65	0.52 - 5.27
Group 5	1 (5)	2 (4)	1.47	0.13 - 17.22
Non-typable	6 (32)	18 (33)	0.95	0.31 - 2.91
Non-grouped	0 (0)	1 (2)	NA	NA

NA = not applicable

*In 20 of 21 (95.2%) clindamycin resistant strains resistance was inducible, detected only by D-test.

conjunctivitis, and 1 bursitis). Eight infections were classified as invasive (10.8%): 4 osteomyelitis, 1 septic arthritis, 1 mastoiditis, 1 orbital cellulitis, and 1 bacteremia of unclear origin. The *pvl*⁺ and *pvl*⁻ groups were similar in their propensity to cause invasive disease. None of the invasive isolates were MRSA.

Peripheral blood neutrophil counts were analysed in 12 *pvl*⁺ and 25 *pvl*⁻ cases, with mean values ($\pm$ SD) of 6.2 ($\pm$ 3.4) and 7.3 ($\pm$ 5.0), respectively (P = 0.46). Erythrocyte sedimentation rate and/or C-reactive protein values were measured in only 2 *pvl*⁺ infections and consequently, were not analyzed. Patient management and outcomes did not differ between *pvl*⁺ and *pvl*⁻ cases. One *pvl*⁺ and one *pvl*⁻ case required ICU admission. There were no deaths.

The results of the multivariate logistic regression analysis are presented in Table 3. The regression model using *pvl*⁺ as the exposure and adjusted for age, underlying skin disease and MRSA status, showed that *pvl*⁺ is associated with abscess formation (OR 20.79; 95% CI = 4.93 - 87.58), and less likely to be

associated with superficial skin infections (OR 0.18; 95% CI = 0.05 - 0.64). Multivariate analysis using *pvl*⁺ as the outcome and adjusted for age, recent hospitalization (other than birth) or surgery, and MRSA status, showed that *pvl*⁺ strains remained less likely to be resistant to erythromycin (OR 0.048; 95% = CI 0.004 - 0.52).

No predominant clone was identified overall by phage typing, and the distribution of *pvl*⁺ and *pvl*⁻ strains across lytic groups was similar (P > 0.5). PFGE analysis of the 20 strains that were *pvl*⁺ and/or MRSA, revealed only two pairs of related strains. One pair was MRSA, and neither pair was related to a

TABLE 3. Multivariate logistic regression analysis

	OR	95% CI
PVL (+) as exposure		
Abscess	20.79	4.93 - 87.58
Superficial skin infection	0.18	0.05 - 0.64
PVL (+) as outcome		
Erythromycin resistance	0.048	0.004 - 0.52

known clonal group. One MRSA isolate was identified as Canadian MRSA epidemic clone CMRSA-4 (USA200). Among methicillin-sensitive SA (MSSA) isolates, one was indistinguishable from CMRSA-7 (USA 400), and one was closely related to CMRSA-10 (USA 300). No other epidemic clones were identified.

Discussion

Despite having been associated with a broad spectrum of clinical manifestations, the importance of PVL as a virulence factor remains controversial. We describe here a prospective cohort of 74 children with CA-SA infections. This is the first Canadian pediatric study on the role of PVL.

We did not observe any risk factor among baseline patient characteristics that might predispose to *pvl*⁺ infections. However, unexpectedly, we observed that isolates resistant to erythromycin were less likely to be *pvl*⁺. Twenty of the 21 clindamycin resistant isolates (95.2%) were detected only by D-test. All such isolates were also erythromycin resistant, indicating inducible resistance to macrolides, lincosamides and streptogramin B via an *erm* gene²³. Transmission of *pvl* to SA by bacteriophages containing *pvl*-encoding genes is not known to be inhibited by the presence of an *erm* gene in SA. Since PVL and susceptibility to erythromycin are not thought to be mechanistically nor genetically linked, the observed association should be interpreted with caution.

We also observed that *pvl*⁺ isolates were more likely to cause abscesses and were less likely to be associated with superficial skin infections. These observations remained significant after adjusting for potential confounders in a multivariate logistic regression model using *pvl*⁺ as the exposure. This is the first cohort study of pediatric CA-SA infections to present this association. These findings confirm previous reports of high rates of *pvl*⁺ strains in abscesses caused by SA.^{5,24-27}

The rates of invasive disease were similar in the *pvl*⁺ (5.3%) and *pvl*⁻ (12.7%) groups. Moreover, we did not observe a difference in patient management (need for hospitalization, requirement of intravenous antibiotics) or outcomes (resolution, recurrence or development of chronic infection) that might suggest a difference in severity of infection between the two groups. These results conflict with previous reports associating PVL to severe invasive disease including complicated bone and joint infections²⁸⁻³⁰, sepsis³¹ and highly lethal necrotizing pneumonia.³² However, other studies did not detect differences between *pvl*⁺ and *pvl*⁻ strains in terms of invasive disease or patient outcomes.³³ It must be noted that our study contained only 8 invasive cases (10.8%), which may have prevented us from detecting an association between PVL and more severe disease.

Of note, we did not observe any cases of necrotizing pneumonia. Indeed, this syndrome is rare. It has been linked to *pvl*⁺ CA-SA by Gillet et al. in a case-control study examining 16 *pvl*⁺ pneumonias with a clonally heterogeneous population of predominantly MSSA isolates infecting French children and adults.³²

The major limitation of our study is its small sample size. Despite this, we were still able to detect significant associations, such as that between *pvl*⁺ and abscess formation. However, our calculated 95% confidence intervals reflect the loss of precision due to the size of our study population. The retrospective collection of clinical data is another potential limitation of our study. In order to minimize this problem we blinded and standardized the chart review, and we collected objective variables whenever possible. Nevertheless, we acknowledge that we may not have been able to capture all treatment failures and recurrences with this design.

A distinguishing feature and strength of our study is the ascertainment of clonal heterogeneity in our population of CA-SA. Phage typing revealed a clonally diverse population, and this was confirmed within the *pvl*⁺ group by PFGE. Consequently, when describing the role of PVL in pediatric CA-SA infec-

tions, our findings are not potentially biased by the presence of a predominant clone that may carry other virulence determinants of significance. Previous reports regarding the role of PVL as a virulence factor in invasive disease may have been confounded by (1) a predominance of MRSA isolates and (2) the emergence of an epidemic clone.³⁴

The incidence of pediatric CA-MRSA infections has increased dramatically in many North American centres in recent years, greatly adding to the overall burden of pediatric SA disease.³⁵ Some centres are reporting MRSA rates of over 75% in their CA-SA infections in children.¹²

PVL has been tightly associated with CA-MRSA clones in the United States^{13, 36}, Canada^{37, 38} and many parts of the world.^{39,40} One particular clone, USA300, nearly uniformly carries the PVL genes.⁴¹

The USA300 epidemic clone has recently emerged as the predominant MSSA and MRSA isolate in purulent skin and soft tissue infections in large parts of the USA⁴¹, and its prevalence in invasive pediatric CA-MSSA disease has increased to > 35% in some reports.⁴² With PVL so tightly linked to USA300, it has become difficult in these settings to extract its effect compared with that of other as-of-yet unstudied virulence determinants. Indeed, in communities where other PVL-containing clones previously circulated, USA300 now predominates, suggesting that it has other important factors contributing to its virulence.³⁴ In addition, a pediatric study by Jaggi *et al* reported that 18 of 18 invasive CA-MRSA infections were *pvl*⁺, whereas only 1 of 10 invasive CA-MSSA infections were *pvl*⁺. This again suggests a role for virulence determinants other than PVL in invasive disease.⁴³

Recent experimental studies have also produced conflicting findings on the role of PVL. Levels of PVL toxin production and of *pvl* gene expression measured in laboratory isolates and directly from clinical samples have been reported not to correlate with MRSA disease severity.^{44,45} Mouse pneumonia models have yielded contradictory results^{9, 10}, and

authors reporting on a mouse sepsis and abscess model concluded that PVL was not a major virulence determinant.⁸

The extent to which PVL specifically contributes to the pathophysiology of CA-SA infections remains to be fully elucidated. Nonetheless, it has already been proposed that the use of antibiotic agents such as clindamycin or linezolid, which shut down ribosomal synthesis of toxins in SA, might improve outcomes in *pvl*⁺ infections.⁴⁶⁻⁴⁸ Consequently, a better understanding of the role of PVL as a virulence factor is needed, as it has important clinical therapeutic implications.

Conclusions

We observed, in a clonally heterogeneous population, that *pvl*⁺ strains in pediatric CA-SA infections are associated with abscess formation, are less likely to cause superficial skin infections, and are less likely to be resistant to erythromycin. We did not observe an association between PVL and invasive disease.

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Correspondence to:

Dr. Caroline Quach,
 Infectious Diseases Division,
 Montreal Children's Hospital,
 2300 Tupper Street, Room C-1246,
 Montreal, Québec, Canada, H3H 1P3..
 E-mail: caroline.quach@muhc.mcgill.ca