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High frequency of Panton-Valentine leukocidin in *Staphylococcus aureus* causing pediatric infections in the city of Cartagena-Colombia

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Summary Panton-Valentine leucocidin (PVL) is a pore-forming toxin that has been epidemiologically associated with CA-MRSA infections. However, its role in the pathogenicity of *Staphylococcus aureus* is still unclear. We evaluated the prevalence of PVL-coding genes in methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) isolates that cause infections in pediatric patients in the city of Cartagena, Colombia. We obtained *S. aureus* isolates from patients at the Napoleon Franco Pareja Children's Hospital in Cartagena. Then, we evaluated the presence of the *nuc*, *mecA*, and PVL genes in these isolates by multiplex PCR and determined the antibiotic susceptibility profiles using CLSI standards. We further correlated methicillin susceptibility and the presence of PVL genes with clinical variables. Overall PVL prevalence in *S. aureus* isolates was 73.91%, with a frequency of 80.92% among MRSA isolates and 67.59% among MSSA. We found a correlation between erythromycin resistance and lack of PVL and found that PVL+ cases were more common in older patients. We found a high PVL prevalence in both MRSA and MSSA isolates, in concordance with previous regional reports.

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Introduction

Staphylococcus aureus is a major human pathogen that causes both nosocomial and community-acquired infections, which range from skin and soft tissue infections to osteomyelitis, pneumonia, bacteremia and infections associated with medical devices [1]. Additionally, *S. aureus* behaves as a commensal bacterium, colonizing up to 60% of healthy populations [2]. In Cartagena, Colombia, *S. aureus* colonization ranges from 15.9 to 38.5% [3,4], depending on the study population.

Both colonizing and pathogenic potentials of *S. aureus* are related to the virulence factors carried by circulating strains [5—7]. High prevalence of Panton-Valentine leucocidin (PVL) has been documented in methicillin-resistant isolates of community origin (CA-MRSA) [6]. PVL is a pore-forming leukotoxin composed of two components, S and F, which are encoded by the *Luk*S-PV and *Luk*F-PV genes in the lysogenic phage *phi*SLT [8]. Due to the epidemiological association between PVL and CA-MRSA isolates, many efforts have been directed toward identifying the pathogenic role of this toxin, but the results have been controversial. Several *in vitro* studies have shown that PVL induces cell lysis by forming pores in the membranes of polymorphonuclear cells (PMNs), and it induces apoptosis by interacting with the mitochondrial membrane [9] and activating downstream TLR-2 signaling pathways, leading to an inflammatory response [10] and complement receptor-mediated cytotoxicity [11]. However, animal model studies have failed to demonstrate a pathogenic role for PVL in staphylococcal infections [12,13]. Despite this discrepancy, PVL has been increasingly associated with severe clinical manifestations of *S. aureus* infections [14—16]; thus, more research is still needed to identify subjects at risk for developing severe forms of infection [17].

The prevalence of PVL has not been as well-studied in MSSA isolates as in MRSA isolates [18]. However, it has been determined that the presence of genes encoding PVL varies by region, and there is a low prevalence in countries such as Spain and Portugal and higher prevalence reported in African countries [18] and Argentina [19]. This information is important for regions in which most cases of infections by PVL-positive isolates correspond to MSSA strains [20].

In this study, we evaluated the prevalence of PVL in both MRSA and MSSA *S. aureus* isolates that were obtained from pediatric infection patients in the regional children's hospital, and we correlated the presence of the PVL virulence factor with the type of infection and other clinical variables.

Materials and methods

Design and study population

This was an observational cross-sectional study that was carried out at the Napoleon Franco Pareja Children's Hospital in Cartagena; this is a University-affiliated pediatric hospital of tertiary care that receives patients from rural and urban areas, and it is the regional referral hospital. The ethics committee of the University of Cartagena approved this study. From October 2009 to June 2012, *S. aureus* isolates that caused laboratory-confirmed *S. aureus* infections were analyzed in the microbiology laboratory of the Genetics and Molecular Biology Research Group of the University of Cartagena. One isolate per patient was included in the study, and we reviewed the clinical data for each case to obtain sociodemographic information and infection type. Children's guardians signed an informed consent allowing us to use the information for research.

Bacterial isolates

Only one isolate per case was included in the study; isolates were confirmed by standard microbiological methods. Using the disk diffusion method, antibiotic susceptibility was determined according to CLSI standards for rifampicin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg); for vancomycin, the minimum inhibitory concentration was determined using the agar dilution test. The D test was used to detect macrolide-inducible resistance to clindamycin.

DNA extraction and multiplex PCR assay

Genomic DNA from each isolate was extracted using a modification of the method developed by Millar et al. [21], as we have previously described [22]. Genomic DNA from each isolate was used to determine the presence of *nuc*, *mecA*, and *lukS/F-PV* genes by multiplex polymerase chain reaction assay, according to protocols that we have previously described [22]. The PCR products were subjected to electrophoresis in a 1.5% agarose gel, followed by ethidium bromide staining and visualization using UV trans-illumination.

Data analysis

Statistical analysis was performed using the IBM SPSS software for Windows, version 20. According to *mec*A gene presence, the isolates were classified

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Table 1 General characteristics of the study cases.

Characteristics		Number (%) $N = 276$
Gender	Female	104 (37.68)
	Male	172 (62.32)
Age[a]	Neonates	14 (5.43)
	Infants	34 (11.96)
	Toddlers	38 (13.77)
	Pre-schoolers	35 (12.68)
	School age	72 (26.09)
	Adolescents	82 (29.71)
Type	SSTI	196 (71.01)
	MSI	38 (13.77)
	UTI	3 (1.09)
	SSI	4 (1.45)
	Pneumonia	7 (2.54)
	Bacteremia	28 (10.14)
LukF-PV	Positive	204 (73.91)
	Negative	72 (26.09)
MecA	Positive	131 (47.46)
	Negative	145 (52.54)

[a] Neonates: ≤1 month old; infants: >1 month old to <1 year old; toddlers: ≥1 year old to 2 years old; pre-schoolers: >2 years old to <5 years old; school age: 5 years old to <10 years old; adolescents ≥10 years old to <18 years old.

as MRSA or MSSA and further sub-classified based on the presence of *lukS/F*-PV genes as PVL-positive or negative. The frequency of each type of isolate was determined. Descriptive statistics, including distribution, central tendency and dispersion, were calculated. For categorical variables, the Fisher exact test or Pearson's Chi Square test were used for comparisons. We considered p values <0.05 to be statistically significant.

Results

A total of 276 cases of *S. aureus* infections were identified during the study period. Of these, 131 were classified as MRSA infections, and 145 were MSSA. Table 1 summarizes the general characteristics of the cases included in the study. Most of the study population consisted of school-age and adolescent patients. Skin and soft tissue infections were the most frequent, followed by musculoskeletal infections. Overall, 204 isolates (73.91%) had PVL genes; 80.92% of MRSA isolates but only 67.59% of MSSA isolates were PVL-positive ($p = 0.0135$). Table 2 summarizes the differences between MRSA and MSSA isolates. When we perform the same analysis for cases of MRSA infections with or without PVL, we found no statistically significant differences in gender, age or type of infection (data not shown). In contrast, we found that patients with MSSA infections due to PVL+ strains were older ($p = 0.0013$, data not shown).

Resistance profiles are shown in Table 3. Resistance to erythromycin was associated with a lack of PVL in the isolates; 23.61% of the PVL-negative isolates were resistant to this antibiotic compared with 9.31% PVL-positive isolates. When we compared methicillin resistance in PVL-positive and negative strains (see Table 4), we confirmed the relationship between erythromycin resistance and the lack of PVL genes in the MSSA isolates ($p = 0.0007$). No other association between antibiotic resistance and PVL presence was found. Only one isolate was positive in the D-test, and a different isolate showed resistance to trimethoprim-sulfamethoxazole.

Discussion

One of the most important determinants of the severity and outcome of any infection is the presence of virulence factors in the infectious agent. Multiple virulence factors have been associated with the pathogenic potential of *S. aureus* [6,7]. Of these factors, PVL has been the most studied. There is historical, epidemiological and biochemical evidence that supports the implication of this toxin in the pathogenesis of *S. aureus*; however, it is unclear whether PVL affects the presentation or severity of infection [17]. Most studies have found a strong epidemiological association between PVL and infections caused by CA-MRSA. In this study, the prevalence of MRSA isolates carrying PVL genes was 80.92%, which is similar to data previously described in different geographical latitudes [23].

In Colombia, the reported prevalence of PVL in MRSA isolates ranges from 73 to 98.7%. Jimenez et al. found that 73% of MRSA isolates obtained from pediatric infections in Medellin were positive for PVL [24]. Meanwhile, it was reported in the city of Bucaramanga that 88% of MRSA isolates obtained from patients of the same age were positive for PVL [25]. In Bogota, Marquez-Ortiz et al. determined that 98.7% of the community-acquired MRSA isolates that caused pediatric infections were positive for PVL [26]. One plausible explanation for this higher frequency than that reported by our group is that our study population was larger, and researchers only focused on community-acquired MRSA isolates.

Less data are available on PVL prevalence in MSSA isolates compared with MRSA isolates. There are reports of frequencies lower than 1% in northern Spain [27], lower than 20% in Turkey [28], Greece

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Table 2 Differences between MRSA and MSSA isolates.

		MRSA Number (%) $N = 131$	MSSA Number (%) $N = 145$	p value
Gender	Female	53 (40.46)	51 (35.17)	0.3859
	Male	78 (59.54)	94 (64.83)	
Age[a]	Neonates	8 (6.11)	6 (4.83)	0.2097
	Infants	15 (11.45)	19 (12.41)	
	Toddlers	25 (19.08)	13 (8.96)	
	Pre-School	16 (12.21)	19 (13.10)	
	School age	31 (23.66)	41 (28.27)	
	Adolescents	35 (27.72)	47 (32.41)	
Type	SSTI[b]	95 (72.52)	105 (72.41)	0.8997
	MSI	19 (14.50)	19 (13.10)	
	Invasive	17 (12.98)	21 (14.48)	
LukF-PV	Positive	106 (80.92)	98 (67.59)	0.0135
	Negative	25 (19.08)	47 (32.41)	

[a] Neonates: ≤1 month old; infants: >1 month old to <1 year old; toddlers: ≥1 year old to 2 years old; pre-schoolers: >2 years old to <5 years old; school age: 5 years old to <10 years old; adolescents ≥10 years old to <18 years old.

[b] For analysis purposes, SSTI also included surgical wound infections, and invasive infections included pneumonia, urinary tract infections and bacteremia. p values from Fisher's exact test.

Table 3 Antimicrobial resistance profiles.

	General $N = 276$	PVL+ $N = 204$	PVL− $N = 72$	p
Gentamicin	5 (1.81)	4 (1.96)	1 (1.39)	1.0000
Erythromycin	36 (13.04)	19 (9.31)	17 (23.61)	0.0038
Clindamycin	14 (5.07)	11 (5.39)	3 (4.17)	1.0000
D test	1 (0.36)	1 (0.49)	0	
Trimethoprim/Sulfamethoxazole	1 (0.36)	0	1 (1.39)	
Rifampicin	0	0	0	
Vancomycin	0	0	0	

p values were obtained from Fisher's exact test where appropriate.

[29] and Lebanon [30]; and above 40% in India [31], Nepal [32] and Africa [33]. More recently, Bazzi et al. described a 30% prevalence of PVL genes in MSSA isolates [18].

In this study, we report a 67.59% prevalence of PVL genes in MSSA isolates. To our knowledge, this is one of the highest reported frequencies of PVL genes in this type of isolate, higher than any

Table 4 Antimicrobial resistance differences between MRSA and MSSA isolates.

	General	MRSA			MSSA		
	$N = 276$	PVL+ $N = 106$	PVL− $N = 25$	p	PVL+ $N = 98$	PVL− $N = 47$	p
Gentamicin	5 (1.81)	3 (2.83)	1 (4.00)	0.5760	1 (1.02)	0 (0)	
Erythromycin	36 (13.04)	17 (16.04)	8 (32.00)	0.0888	2 (2.04)	9 (19.14)	0.0007
Clindamycin	14 (5.07)	8 (7.55)	0 (0)		3 (3.06)	3 (6.38)	0.3895
D test	1 (0.36)	1 (0.94)	0 (0)		0 (0)	0 (0)	
TMP/SMX	1 (0.36)	0 (0)	1 (4.00)		0 (0)	0 (0)	
Rifampicin	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
Vancomycin	0 (0)	0 (0)	0 (0)		NE	NE	

p values were obtained using Fisher's exact test where appropriate. TMP/SMX: trimethoprim/sulfamethoxazole. The minimum inhibitory concentration for vancomycin was not evaluated in MSSA isolates.

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previous reports in our country [24] and only exceeded by the 81.1% reported in Mozambique by van der Meeren et al. [34]. Finding a high prevalence of PVL genes among MSSA isolates lends support to the hypothesis that the origin of the predominant MRSA clone in Colombia is from a MSSA-derived clone [35] that already harbored the PVL genes.

An association between PVL presence in MSSA isolates and resistance to trimethoprim sulfamethoxazole has been described [18]. However, when we tested antibiotic susceptibility, we only found an association between PVL and lack of resistance to erythromycin in MSSA isolates.

Conclusions

In this study, we found a high prevalence of PVL genes in MRSA and MSSA isolates. The observed prevalence of PVL genes in MSSA isolates is one of the highest reported. This finding supports the hypothesis that the major circulating MRSA clone in Colombia may have originated from a MSSA isolate. To test this hypothesis, it will be necessary to study these isolates phylogenetically to determine the genetic relatedness between MRSA and MSSA isolates. Additionally, we found no association between the type of infection and presence of PVL genes in the isolates. Longitudinal studies with close clinical monitoring will be needed to identify such relationships.

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Competing interests

None declared.

Ethical approval

Approved by the Ethics Committee of the University of Cartagena.

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