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Nasal carriage of Panton Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* in healthy preschool children

Colonización nasal de *Staphylococcus aureus* Meticilino Resistente
portador de la Leucocidina Panton-Valentine en preescolares

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ABSTRACT

Objective Determining the prevalence of nasal carriage of *S. aureus*, both sensitive to methicillin and resistant to it, in preschool children and evaluating the presence of Panton-Valentine leukocidin genes in the isolates.

Methods This was a cross-sectional study in which cultures from anterior nares were obtained from healthy preschool children. Isolates were identified as *S. aureus* based on morphological and biochemical tests. Antibiotic susceptibility profiles were determined by the disk diffusion method. All the isolates were further analyzed by multiplex PCR to determine the presence of *mecA* and PVL genes; methicillin-resistant isolates were also SCCmec typed by multiplex PCR.

Results Overall *S. aureus* nasal colonization prevalence was 38.5 % and 4.8 % for methicillin-resistant strains. All the methicillin-resistant isolates carried the genes for PVL; two isolates possessed the SCCmec type IV, two were SCCmec type I and one was SCCmec type II.

Conclusion This study revealed high PVL-positive, methicillin-resistant *S. aureus* colonization prevalence in healthy preschool children from Cartagena, which may play a key role in the epidemiology of community-associated infection by methicillin-resistant *S. aureus* in healthy children from this particular geographical area.

Key words: Nasal carriage, *Staphylococcus aureus*, methicillin resistance, healthy children (source: MeSH, NLM).

RESUMEN

Objetivo Determinar la prevalencia de colonización nasal de *S. aureus*, tanto sensible como resistente a meticilina, en niños preescolares y evaluar la presencia de los genes de la leucocidina Panton-Valentine en estos aislamientos.

Métodos Estudio de corte transversal en el que se realizaron cultivos de flora nasal de niños preescolares. Los aislamientos fueron identificados como *S. aureus* con base en su morfología y pruebas bioquímicas. La susceptibilidad a antibióticos se determinó por el método de difusión en disco. Todos los aislamientos fueron analizados por PCR múltiple para determinar la presencia de los genes *mecA* y *PVL*, y para la tipificación del casete cromosómico *SCCmec* de los aislamientos resistentes a meticilina.

Resultados La colonización nasal por *S. aureus* fue 38,5 %, y la de cepas meticilino-resistentes fue 4,8 %. Todos los aislamientos SARM portaban los genes para *PVL*, dos portaban el elemento *SCCmec* tipo IV, dos fueron tipo I y uno fue tipo II.

Conclusión Encontramos una alta prevalencia de colonización por cepas meticilino-resistentes, *PVL*-positivos en la población estudiada, lo que podría jugar un papel clave en la epidemiología de las infecciones por *S. aureus* meticilino-resistente en esta área geográfica.

Palabras Clave: Colonización nasal, *Staphylococcus aureus*, resistencia a meticilina, niños sanos (fuente: DeCS, BIREME).

Staphylococcus aureus is a bacterium that behaves both as human commensal and as a pathogen frequently causing clinically important infections ranging from skin abscesses to life-threatening infections such as bacteremia and pneumonia (1,2). The anterior nares represent the most common site for staphylococcal colonization (3,4), longitudinal studies having shown that about 30 % of individuals are *S. aureus* nasal carriers (5). Nasal carriage of this bacterium has been identified as a risk factor for developing community-acquired and nosocomial infections, more than 80 % of isolates originating from the nose; therefore, it has been widely accepted that the bacterium plays a key role in the infection's pathogenesis and epidemiology (6,7).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as an important pathogen since 1969; it was first considered to be confined to hospitals and medical centres and nowadays as being presented in community settings. Community-acquired MRSA (CA-MRSA) strains have been identified as being highly clonal and virulent, responsible for around 30 % of *S. aureus* infections (8) and are probably the most important challenge to routine clinical practice regarding managing infectious diseases. Although the nasal carriage of MRSA has been reported to have a low frequency in healthy pediatric populations (0.2 % - 2.2 %), several recent reports have documented increasing MRSA nasal colonization prevalence in healthy children (9-11). A cross-sectional study was carried out in preschool institutions to ascertain the prevalence of nasal carriage of *S. aureus* and MRSA in preschool children and to evaluate the possible risk factors involved in such carriage. A high

PVL-positive MRSA colonization prevalence was found in healthy preschool children in the study population.

MATERIALS AND METHODS

Study design and population

This cross-sectional study was conducted from June to November, 2009. Anterior nares from 104 children aged 2 to 6 years were sampled. Written questionnaires regarding their demographics and medical history (antibiotic use and the presence of respiratory infection, skin infection or allergies) were completed by the children's parents; these parents gave their written consent. This study was approved by the University of Cartagena's Ethics Committee.

Specimen collection and microbiological methods

Nasal samples were obtained with a sterile cotton swab and placed in modified Stuart transport medium (OXOID, England); they were then transported to our microbiology lab and processed within 4 hours. *S. aureus* isolates were identified by previously described methods (12). Antibiotic susceptibility was assessed by the disc diffusion method following Clinical Laboratory Standards Institute (CLSI) guidelines (13). The antibiotics tested were: ceftioxylin, erythromycin, clindamycin, gentamycin, vancomycin and rifampin; the double disk diffusion test (D test) was performed in the same plate by placing the clindamycin and erythromycin disks 15 mm apart.

DNA extraction

Genomic DNA from all isolates was extracted using the following protocol. Around 3-5 colonies were suspended and washed with Tris 0.5 M and then homogenized in TE buffer (10 mM Tris, 1 mM EDTA), heated at 100 °C for one hour and immediately frozen at -35 °C for 20 minutes, thawed at 65 °C and finally centrifuged at 13,000 rpm for 15 minutes. The supernatant containing the bacterial DNA was deposited in a fresh tube and stored at -20 °C for further analysis.

Detecting *mecA*, *nuc* and *LuK*-PV genes by multiplex PCR

Multiplex PCR of all the *S. aureus* isolates was carried out using three sets of oligonucleotides: MecA1F - MecA2R (14) which amplifies a 147 bp fragment from the *mecA* gene, Nuc1F - Nuc2R (15) which amplifies an *S. aureus*-specific fragment of about 300 bp from the *nuc* gene and LukPV1F - LukPV2R (16) which amplifies a 437 bp fragment from the *lukS/lukF*-PV genes.

DNA was amplified in a 25 µL reaction volume containing 12.5 µL PCR mix (PCR Master Mix, Promega), 0.2 µM of each primer and 5 µL of template DNA. PCR reactions were carried out in a Perkin-Elmer thermocycler in the following conditions: an initial denaturing cycle at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with a final 10 min extension step at 72 °C .

The *S. aureus* reference strains ATCC 33591 (*mecA* positive; *nuc* positive, PVL negative) and ATCC 25923 (*mecA* negative; *nuc* positive, PVL positive) were used as control strains. DNA template was replaced by ultrapure water in negative PCR reaction controls. All PCR products were analyzed on 2 % agarose gel and visualized with ethidium bromide staining using a UV light transilluminator.

SCCmec typing by multiplex PCR

All MRSA isolates were SCCmec typed using a multiplex PCR strategy, according to previously described protocols (17) which included 3 sets of primers amplifying different sized fragments for each SCCmec type.

Statistical analysis

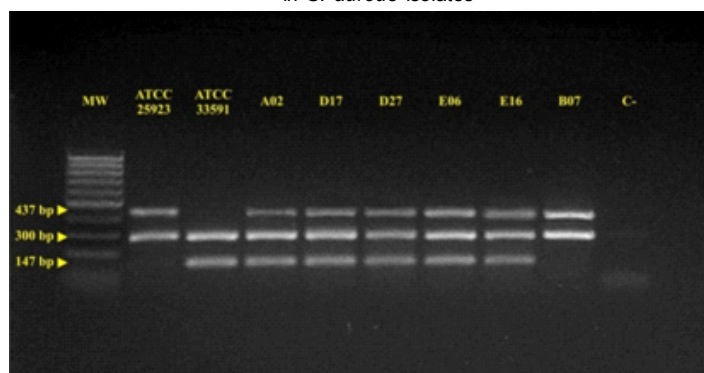
Microsoft Office Excel was used for recording data and exported to SPSS statistical software (version 18.0) which was used for final data analysis. Univariate analysis was applied to determine colonization association with potential risk factors using Fisher's exact test. A < 0.05 p value was defined as being statistically significant.

RESULTS

A total of 104 preschool children from three different preschool institutions, ages ranging from 2 to 6 years, participated in the study. Participating children's average age was 4.01 years (SD: 1.15) and 48 of them (46.2 %) were male.

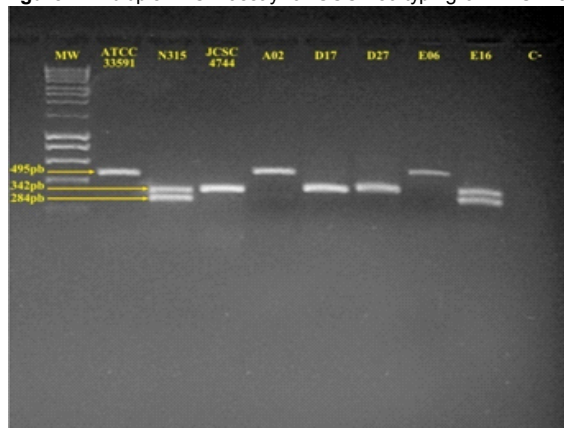
S. aureus nasal colonization prevalence was 38.5 % and 5 children (4.8 %) were colonized with MRSA strains. MRSA isolates' frequency amongst all *S. aureus* isolates was 12.5 %. PVL genes were present in all the MRSA strains, while just one MSSA strain had the PVL genes (Figure 1), for an overall 5.8 % PVL-positive *S. aureus* prevalence. SCCmec typing showed that two isolates carried SCCmec type 4, two carried SCCmec type 1 and one carried SCCmec type 2 (Figure 2).

Figure 1. Multiplex PCR for detecting *nuc*, *mecA* and PVL genes in *S. aureus* isolates



Multiplex PCR was carried out to detect the presence of *nuc*, *mecA* and PVL genes in isolates from nasal carriers to confirm *Staphylococcus aureus* species (*nuc* gene), methicillin resistance (*mecA* gene) and PVL gene presence. Lanes 1: MW (DNA molecular weight marker). Lanes 2 and 3 : ATCC 25923 reference strain (*nuc*+, *mecA*-, PVL+) and ATCC 33591 reference strain (*nuc*+, *mecA*+, PVL-) respectively. Lanes 4 to 9: representative isolates from the study. Lane 10: negative PCR reaction control.

Figure 2. Multiplex PCR assay for SCCmec typing of MRSA isolates



Multiplex PCR was carried out to determine SCCmec types of MRSA isolates. Lane 1: MW (DNA molecular weight marker). Lane 2: *S. aureus* reference strain ATCC 33591 (SCCmec type 1). Lane 3: *S. aureus* reference strain N315 (SCCmec type 2). Lane 4: *S. aureus* reference strain JCSC4744 (SCCmec type 4). Lanes 5 to 9: SCCmec typing results for MRSA isolates from the study. Two MRSA isolates were SCCmec type 1 (Lanes 5 and 8), one MRSA isolate was SCCmec type 2 (Lane 9), two isolates were SCCmec type 4 (Lanes 6 and 7). Lane 10 shows the results for a negative PCR reaction control.

No association was found between *S. aureus* nasal carriage and gender, ethnicity, visit to a doctor, household contact with hospital staff, having a skin or respiratory infection and household number. However, there was an association between *S. aureus* nasal carriage and antibiotic use during the past 3 months (5.13 odds ratio, 2.18- 12.06 confidence interval, $p = 0.00011$) (Table 1).

Table 1. Univariate analysis of risk factors for *S. aureus* nasal carriage amongst preschool children

Variable	Carriage status ($n = 104$)		P^*	OR (95% CI)
	Carrier (n)	Non-carrier (n)		
Antibiotic use	Absent	14	< 0.001*	5.13 (2.18-12.06)
	Present	26		
Respiratory infection	Absent	32	0.116	0.512 (0.20-1.30)
	Present	8		
Healthcare worker in household	Absent	38	0.251	0.429 (0.08-2.17)
	Present	2		
Skin infection	Absent	25	0.216	1.53 (0.66-3.55)
	Present	15		
Visit to a doctor	Absent	17	0.243	1.44 (0.65-3.19)
	Present	23		
Gender	Male	18	0.5	0.92 (0.42-0.20)
	Female	22		
Allergy	Absent	34	0.375	1.437 (0.44-4.63)
	Present	6		

*p values for each category refer to *S. aureus* carriage risk factors

Antibiotic susceptibility tests indicated that 20 % of the isolates were erythromycin-resistant, one isolate (2.5 %) was clindamycin resistant and the D test showed no inducible clindamycin resistance. All the isolates were sensitive to vancomycin and five isolates were resistant to 2 or more antibiotics. Table 2 shows the isolates' antibiotic sensitivity pattern.

Table 2. Antibiotic susceptibility profiles for *S. aureus* isolates from nasal carriers ($n = 40$)

Antibiotic	S	I	R
	n (%)	n (%)	n (%)
Cefoxitin	35 (87.5)	0	5 (12.5)
Erythromycin	25 (62.5)	7 (17.5)	8 (20)
Clindamycin	37 (92.5)	2 (5)	1 (2.5)
Gentamycin	39 (97.5)	0	1 (2.5)
Vancomycin	40 (100)	0	0
Rifampin	39 (97.5)	0	1 (2.5)

S = susceptible I = intermediate R = resistant

A total of 104 preschool children aged 2 to 6 years (average 4.01 years, SD + 1.15) were enrolled in the study. Five children were carriers of MRSA strains (4.8 %) and 35 children were carriers of MSSA strains (33.6 %).

DISCUSSION

S. aureus is an important pathogen associated with nosocomial and community-acquired infection. Several reports have documented an increase in infections caused by methicillin-resistant *S. aureus*, mostly affecting children in several geographic regions. Nasal colonization by *S. aureus* has been considered an important risk factor for infections that could threaten a carrier's life.

High MSSA and PVL-positive MRSA nasal carriage rates were found (38.5 % and 4.8 %, respectively) which may represent a risk for colonized children and the rest of the community, taking into account that these types of strain are implicated in invasive *S. aureus* infections (18,19). Because *S. aureus* is spread through contact, school institutions, where children are in close contact with each other, may play a role in these strains' transmission and spread in the community and preschool children may be an important reservoir and source for the transmission of strains causing skin and soft tissue infections.

The MRSA strains found in this study carried the three SCCmec types, revealing the diversity of MRSA strains dispersed in our community. Although CA-MRSA strains have been reported as mainly carrying SCCmec type 4, two isolates were also found carrying the type 1 SCCmec and one carrying the type 2 SCCmec which have been previously associated with nosocomial settings (20); this may represent evidence of *S. aureus* strains' changing epidemiology and distribution.

Previous studies have suggested that environmental factors like prior antibiotic use, contact with a healthcare facility, poor socio-economic conditions and overcrowding are involved in the increase of CA-MRSA nasal carriage (21,22). However, an association was only found between prior antibiotic use and carriage state; there was no association between contact with a healthcare facility and MRSA carriage in this study, according to statistical analysis.

There was a high erythromycin resistance rate in this study and five isolates were resistant to more than 2 antibiotics; three of them were MRSA strains. This is an important finding because multi-resistant strains which are now dispersed in the community represent a challenge to routine clinical practice in the management of infectious diseases. Our results support the view that

MRSA strains may spread from a hospital setting to the community, because three of the 5 MRSA isolates found in this study had features pertinent to nosocomial strains, such as SCCmec types 1 and 2 along with multi-drug resistance. Therefore, studies addressed at tracking *S. aureus* strains' epidemiology and distribution are needed and may serve as the basis for designing strategies for controlling their spread and dissemination from (or to) a hospital or community setting ♣

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Conflicts of interest: The authors declare that they do not have any conflict of interest related to this study.

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