Characterisation of virulence genes in methicillin susceptible and resistant *Staphylococcus aureus* isolates from a paediatric population in a university hospital of Medellín, Colombia

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Virulence and antibiotic resistance are significant determinants of the types of infections caused by Staphylococcus aureus *and paediatric groups remain among the most commonly affected populations. The goal of this study was to characterise virulence genes of methicillin-susceptible* S. aureus *(MSSA) and methicillin-resistant* S. aureus *(MRSA) strains isolated from a paediatric population of a Colombian University Hospital during 2009. Sixty MSSA and MRSA isolates were obtained from paediatric patients between zero-14 years. We identified the genes encoding virulence factors, which included Panton-Valentine leucocidine (PVL), staphylococcal enterotoxins A-E, exfoliative toxins A and B and toxic shock syndrome toxin 1. Typing of the staphylococcal chromosome cassette* mec *(SCC*mec*) was performed in MRSA strains. The virulence genes were more diverse and frequent in MSSA than in MRSA isolates (83% vs. 73%). MRSA strains harboured SCC*mec *types IVc (60%), I (30%), IVa (7%) and V (3%). SCC*mec *type IVc isolates frequently carried the PVL encoding genes and harboured virulence determinants resembling susceptible strains while SCC*mec *type I isolates were often negative. PVL was not exclusive to skin and soft tissue infections. As previously suggested, these differences in the distribution of virulence factor genes may be due to the fitness cost associated with methicillin resistance.*

Key words: *Staphylococcus aureus* - MRSA - MSSA - virulence factors

The success of *Staphylococcus aureus* as a pathogen is in part due to its ability to express a variety of virulence factors, both structural and secreted, that mediate host colonisation, tissue invasion and dissemination (Gordon & Lowy 2008). In addition, *S. aureus* has an exceptional ability to develop resistance to antimicrobial agents (Chambers & Deleo 2009). Among the most known secreted virulence factors is a large family of superantigen exotoxins that include the staphylococcal enterotoxins, which cause food poisoning, toxic shock syndrome toxin 1 (*tst*-1) and the exfoliative toxins (*eta* and *etb*), which are implicated in staphylococcal scalded-skin syndrome (John & Schreiber 2006, Tristan et al. 2007). An important virulence factor produced by many methicillin-resistant *S. aureus* (MRSA) strains is the Panton-Valentine leucocidine (PVL), a bicomponent pore-forming cytolytic toxin that targets cell membranes of leukocytes (McClure et al. 2006), which is associated with skin and soft tissue infections (SSTI), necrotising pneumonia and epidemiologically linked to community-associated MRSA (CA-MRSA) (Chambers 2005, DeLeo et al. 2009).

S. aureus and in particular CA-MRSA, has become a successful pathogen within the paediatric population. A dramatic increase in community-MRSA infections has been reported in healthy children (Herold et al. 1998, Bocchini et al. 2006, Stankovic & Mahajan 2006, Faden et al. 2007, Baker 2010, Wu et al. 2010).

This could be explained by the high rates of anterior nares' carriage in the paediatric population, which is associated with subsequent infection (Miller & Diep 2008, Lee et al. 2009).

There is significant controversy on the virulence of methicillin-susceptible *S. aureus* (MSSA) vs. MRSA isolates; both have enormous capacity for virulence and pathogenicity that enables them to reach high rates of infection (Gould 2006). However, they differ in their genotypes, geographical distribution and the infections they cause (Kim et al. 2006).

In Colombia, *S. aureus* represents an important and increasing health problem, yet little is known about the virulence factors present in MSSA and MRSA isolates. Therefore, the aim of this study was to determine the frequencies and profiles of virulence factors in a MSSA and MRSA strain collection obtained from the paediatric population of a Colombian University Hospital in 2009. This information is important as it could facilitate the establishment of more effective infection control measures in this institution.

SUBJECTS, MATERIALS AND METHODS

Study population - An observational cross-sectional study was conducted during 2009 at the Hospital Universitario San Vicente Fundación, a fourth-level care centre located in Medellín, the second largest city of

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Colombia. The sample size was calculated based on the records of paediatric MRSA isolates during 2008, which numbered 30 isolates. Therefore, a sample size of 60 *S. aureus* isolates, 30 MRSA and 30 MSSA, was taken to establish comparison values. The isolates included in the study were randomly selected from children aged zero-14 years in the Paediatric Department of the institution. Clinical and epidemiological information was obtained from the medical records and included data such as gender, age and type of infection. Informed consent to participate in the study was obtained from each patient's parent or guardian.

Bacterial strain identification and molecular confirmation of S. aureus and methicillin resistance - Identification of *S. aureus* was conducted by standard laboratory methods based on colony morphology in sheep blood agar and positive catalase and coagulase tests. Methicillin resistance was assessed in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI 2010).

For molecular confirmation of *S. aureus* and methicillin resistance, the DNA was extracted from the isolates using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions with 10 mg/mL of Chicken Egg White Lysozyme (Sigma Aldrich). The presence of the *mecA* and *femA* genes was verified by polymerase chain reaction (PCR) according to a protocol previously described (Mehrotra et al. 2000).

Staphylococcal chromosome cassette mec (SCCmec) typing and detection of toxin genes - SCC*mec* types and subtypes were determined by using a set of six multiplex PCR reactions according to a previously reported protocol (Kondo et al. 2007). The genes encoding staphylococcal enterotoxins (*sea, seb, sec, sed, see*), *tst*-1, *eta* and *etb* were detected by multiplex PCR (Mehrotra et al. 2000). Amplification of the genes for the PVL (*lukS/F-PV)* was performed as previously reported (McClure et al. 2006).

Statistical analysis - Categorical variables were compared using the chi-square test or Fisher's exact test and p values of ≤ 0.05 were considered statistically significant. Statistical analysis was carried out using the SPSS® v15.0 software package.

Ethics - The study was approved by a bioethics committee at Universidad de Antioquia, Medellín (approval 08036172).

RESULTS

Description of MRSA infections and epidemiologic characteristics - Among the 60 patients included in the study, 44 (73.3%) were males and 39 (65%) were ≥ 1 year old, with an average of 5.4 ± 4.3 years. Patients were distributed into the following services: paediatric ward (37; 62%), paediatric intensive care unit (8; 13%), orthopaedic ward (6; 10%), emergency ward (4; 6.7%), surgery (4; 6.7%) and other services (1, 2%). Isolates were most commonly obtained from SSTI (27; 45%), followed by pneumonia (8; 13.3%), surgical site infection (SSI) (6; 10%), central venous catheter-related bacteraemia (5; 8.3%), primary bacteraemia (3; 5%), arthritis (3; 5%), osteomyelitis (1; 1.7%) and other infections (7; 11.7%), including urinary tract infections, conjunctivitis, meningitis, otitis, tracheitis, orbital cellulitis and infected pulmonary sequestration.

MSSA isolates and virulence gene profiles - Most MSSA isolates (83.3%) carried one or more virulence genes simultaneously. *lukS/F-PV* genes were detected in 11 (37%) of these isolates and staphylococcal enterotoxin genes *sea, seb, sec, sed* and *see* were commonly observed. *seb* was the most frequently detected enterotoxin, which was present in 40% of the MSSA isolates, followed by *sed* (33%), while, *eta* and *etb* were not detected (Fig. 1, Table).

MRSA SCCmec types and virulence gene profiles - Of the 30 MRSA isolates, 60% carried SCC*mec* type IVc (18), 30% type I (9), 7% type IVa (2) and 3% type V (1). Other SCC*mec* types were not detected. Twenty two (73%) MRSA isolates carried one or more virulence genes simultaneously. The SCC*mec* types showed diverse virulence gene patterns (Table). Those carrying SCC*mec* IV had one or more virulence genes, similar to MSSA isolates.

lukS/F-PV genes were present in all SCC*mec* types, but they were more frequently observed in SCC*mec* IVc isolates (94%). The staphylococcal enterotoxin genes *seb*, *sed* and *see* were less common and appeared only in SCC*mec* IV strains, while *sea* and *sec* were not detected in any of the MRSA isolates. *eta* was present at a very low frequency: it was only detected in a SCC*mec* V strain, while *etb* was not detected at all.

Only two (22%) of the nine SCC*mec* type I isolates were positive for *lukS/F-PV* and all nine were negative for any other toxin gene (Fig. 1, Table).

The bivariate analysis showed statistically significant differences ($p < 0.05$) in the presence of the following virulence genes between MSSA-MRSA isolates: *lukS/F-PV* (37% vs. 73%; p = 0.004), *seb* (40% vs. 7%; p = 0.002) and *sed* (33% vs. 7%; p = 0.010) (Table).

Distribution of virulence genes and type of infection - The distribution of virulence genes varied with the type of infection and the strain causing it. In all types

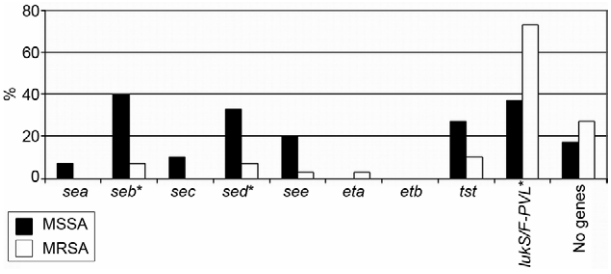

Fig. 1: percentage distribution of virulence genes encoding staphylococcal enterotoxins genes A-E (*sea*, *seb*, *sec*, *sed* and *see*), exfoliative toxins A and B (*eta* and *etb*), toxic shock syndrome toxin 1 (*tst*) and Panton-Valentine leucocidine (*lukS/F-PV*) in methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Asterisk means $p \leq 0.05$, by chi-square or Fisher's exact test.

TABLE

Absolute and relative distribution of staphylococcal virulence genes, enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*) the exfoliative toxins (*eta* and *etb*), toxic shock syndrome toxin 1 (*tst*) and Panton-Valentine leukocidin (*lukS/F-PV*) according to methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolates and staphylococcal chromosome cassette *mec* (SCC*mec*) types

					MRSA SCC*mec* types			
Gene	Total (n = 60) n (%)	MSSA (n = 30) n (%)	MRSA (n = 30) n (%)	p[a]	I (n = 9) n (%)	IVa (n = 2) n (%)	IVc (n = 18) n (%)	V (n = 1) n (%)
One or more	47 (78)	25 (83)	22 (73)	NS	2 (22)	2 (100)	17 (94)	1 (100)
sea	2 (3)	2 (7)	0 (0)	NS	0 (0)	0 (0)	0 (0)	0 (0)
seb	14 (23)	12 (40)	2 (7)	0.002	0 (0)	0 (0)	2 (11)	0 (0)
sec	3 (5)	3 (10)	0 (0)	NS	0 (0)	0 (0)	0 (0)	0 (0)
sed	12 (20)	10 (33)	2 (7)	0.010	0 (0)	2 (100)	0 (0)	0 (0)
see	7 (12)	6 (20)	1 (3)	NS	0 (0)	1 (50)	0 (0)	0 (0)
eta	1 (2)	0 (0)	1 (3)	NS	0 (0)	0 (0)	0 (0)	1 (100)
etb	0 (0)	0 (0)	0 (0)	-	0 (0)	0 (0)	0 (0)	0 (0)
tst	11 (18)	8 (27)	3 (10)	NS	0	1 (50)	2 (11)	0 (0)
lukS/F-PV	33 (55)	11 (37)	22 (73)	0.004	2 (22)	2 (100)	17 (94)	1 (100)
No genes	13 (22)	5 (17)	8 (27)	NS	7 (78)	0 (0)	1(6)	0 (0)

a: by chi-square or Fisher's exact test. NS: not significant.

of MRSA infections, the *lukS/F-PV* genes were present. Pneumonia infections were predominantly caused by MRSA strains, with *PVL* genes detected in 67% of them. Skin and soft tissue and SSI were mainly due to MSSA strains that harboured most of the virulence genes assessed, except for the exfoliative toxin genes. MSSA strains causing blood stream infections (BSI) and arthritis simultaneously carried several virulence genes. MSSA from BSI harboured *sed* (100%), *tst* (100%) and *lukS/F-PV* (100%) and arthritis-associated MSSA presented *sed* (100%) and *see* (100%). The resistant strains causing the same type of infections carried only PVL genes present in 50% and 100% of the isolates, respectively. *eta* was only detected in 50% of MRSA isolates that caused catheter-related BSI. A single case of osteomyelitis was detected, caused by a SCC*mec* IVc isolate carrying only PVL genes (Figs 2, 3).

DISCUSSION

The clinical outcome of *S. aureus* infections is influenced by both the presence of antimicrobial resistance and virulence factors. It has been suggested that the acquisition of antibiotic resistance in *S. aureus* involves changes in virulence factor secretion due to the fitness cost associated with the expression of resistance (Sakoulas et al. 2003, Gill et al. 2005) and it is reflected in decreased toxin expression (Collins et al. 2010, Otto 2010). Therefore, it is not surprising that the results of the present study confirmed this by showing that virulence factor gene carriage was more diverse and abundant in MSSA than in MRSA strains. However, due to the fact that most studies on virulence factor carriage are conducted on resistant strains, there is a need for information in susceptible strains that allows a more thorough comparison. In addition, Collins et al. (2010) indicated that the fitness cost associated with resistance varies depending on the SCC*mec* type and antibiotic resistance levels. These authors found that in MRSA strains carrying the larger cassette SCC*mec* II, there was a reduction in virulence factor secretion, while strains carrying the smaller size SCC*mec* IV secreted a more diverse range of factors. The findings of the present study are consistent with those of Collins et al. (2010) because isolates harbouring SCC*mec* type I presented no or up to two virulence genes, whereas SCC*mec* type IV strains

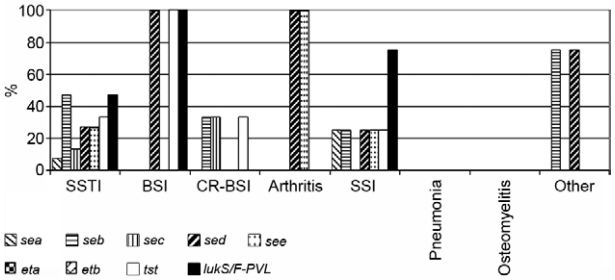

Fig. 2: percentage distribution of virulence genes encoding staphylococcal enterotoxins genes A-E (*sea*, *seb*, *sec*, *sed* and *see*), exfoliative toxins A and B (*eta* and *etb*) , toxic shock syndrome toxin 1 (*tst*) and Panton-Valentine leucocidine (*lukS/F-PVL*) in methicillin-susceptible *Staphylococcus aureus* (MSSA) according to infection types. BSI: blood stream infections; CR-BSI: catheter-related blood stream infections; other: includes urinary tract infections, conjunctivitis, meningitis and otitis; SSI: surgical site infection; SSTI: skin and soft tissue infections.

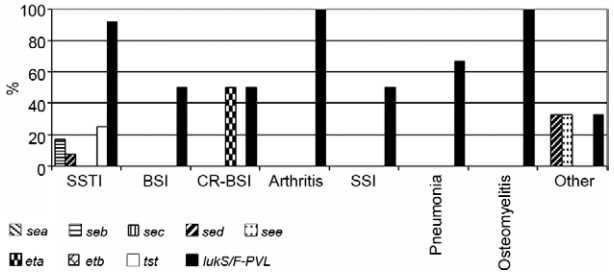

Fig. 3: percentage distribution of virulence genes encoding staphylococcal enterotoxins genes A-E (*sea*, *seb*, *sec*, *sed* and *see*), exfoliative toxins A and B (*eta* and *etb*), toxic shock syndrome toxin 1 (*tst*) and Panton-Valentine leukocidin (*lukS/F-PV*) in methicillin-resistant *Staphylococcus aureus* (MRSA) according to infection types. BSI: blood stream infections; CR-BSI: catheter-related blood stream infections; other: includes tracheitis, orbital cellulitis and infected pulmonary sequestration; SSI: surgical site infection; SSTI: skin and soft tissue infections.

carried one or more virulence genes simultaneously, as observed in susceptible strains. The fitness cost on the growth and cell yield of *S. aureus* containing the SCC*mec* elements has also been experimentally demonstrated; the higher energy demand and decreased cell yield observed in SCC*mec* type I strains has not been observed in the SCC*mec* type IV strains (Lee et al. 2007, Collins et al. 2010).

In the paediatric population evaluated, the *lukS/F-PV* genes were detected more commonly in MRSA isolates, whereas the enterotoxin genes were more frequently observed in MSSA strains. These results agree with those of studies that found lower frequencies of the genes encoding PVL in MSSA clinical isolates (Kuehnert et al. 2006), but contrast with data reported from the Czech Republic, where it was found that the *lukS/F-PV*, *tst* and *sec* genes were more frequent in MSSA isolates and enterotoxins genes *sea*, *seb*, *sed* and *eta* were most prevalent in MRSA strains (Sila et al. 2009). Also, in this study, *lukS/F-PV* were more frequently observed in SCC*mec* IVc isolates (94%), but they were not restricted to this cassette type and they were also present in SCC*mec* types I, IVa and V. Other studies conducted in China (Wu et al. 2010) and in USA (Abdel-Haq et al. 2009) that have evaluated the epidemiological and molecular features of MRSA and MSSA isolates from children found that the most frequent SCC*mec* type was IVa and all of these strains carried the *lukS/F-PV* genes. Also, similar to our findings, results of a study conducted in Lebanon revealed that the PVL genes were present mainly in SCC*mec* IVc isolates (Tokajian et al. 2010).

The low frequency of the *eta* and *etb* genes coincides with results of studies that found that 5% of the isolates harboured one or both toxins and of these, *eta* and *etb* were present in 88% and 4% of them, respectively (Ladhani et al. 1999). The low frequency of the *eta* and *etb* genes in clinical MSSA isolates was also documented in a more recent study (van Trijp et al. 2010) in which *lukS/F-PV* were detected in the skin and soft tissue and in other infections, such as arthritis, SSI, pneumonia and osteomyelitis. In the latter two, only the *lukS/F-PV* genes were detected. Although there is controversy about the role of PVL in the spread and severity of *S. aureus* infections (Oliveira et al. 2002, Chambers & Deleo 2009, DeLeo et al. 2009, Otto 2010), the results of this study show the importance of this virulence factor among resistant strains and in different infections.

In Latin-American countries, specially Argentina and Brazil, other authors have reported the presence of PVL-producer and non-producer MRSA strains carrying SCC*mec* IV, which cause both community and health-care associated infections (mainly severe SSTI, bacteraemia, osteomyelitis and pneumonia or chronic lung infection) and colonise healthy adults and children (Trindade et al. 2005, de Miranda et al. 2007, Rozenbaum et al. 2009, Scribel et al. 2009, Gardella et al. 2011, Mimica et al. 2011). Nevertheless, there is little information available about the virulence factors present in the isolates and most studies do not include SCC*mec* typing or any molecular characterisation of the strains. In Colombia, a study conducted in Bogotá reported three paediatric cases of infections caused by PVL-positive MRSA strains carrying SCC*mec* IVc belonging to clonal complex 8 (CC8) with 75% similarity to USA 300 (Alvarez-Olmos et al. 2009). Remarkably, USA 300 is one of the most prevalent CA-MRSA strains in the USA and it has disseminated into Canada and Europe and is implicated in SSTI with invasive disease, including severe septicaemia, necrotising pneumonia and necrotising fasciitis (Diep et al. 2006). The above suggests the possibility that the PVL-positive SCC*mec* IVc MRSA isolates detected in this study will be related to this CC8.

The findings of this study also show that MSSA strains remain an important source of infection, suggesting that MRSA has not replaced MSSA strains. Therefore, susceptible strains should continue to be monitored and controlled, as resistant strains are, due to their high pathogenic potential. In general, we observed that distinct staphylococcal toxin gene profiles were present according to methicillin resistance/susceptibility. However, future analyses of these strains should include identification of their lineage, which is necessary for better comprehension of their dynamics. This may help to determine to what extent the pathogenicity of *S. aureus* infections is influenced by the presence of staphylococcal toxins.

This is the first study conducted in a child population at the local level that shows the differences in virulence gene profiles between MRSA-MSSA hospital isolates. This information is useful for establishing effective control measures and management of infections caused by this organism. Furthermore, it is important that more comprehensive studies be conducted to evaluate the participation of microbiologic factors, such as virulence genes, in the pathogenicity of MRSA and MSSA in the community. The present study showed specific differences in the distribution of virulence genes in the *S. aureus* strains obtained from the paediatric population of a hospital in Medellín from those of other countries. This suggests that these features may vary according to geographic location and clinical-epidemiological factors, underscoring the importance of characterising the relationships between toxin genes and methicillin resistance in each region.

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