Original Article

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) clones from Paraguayan children

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Abstract

Introduction: *Staphylococcus aureus* is considered one of the most important human pathogens, and its levels of resistance to methicillin have increased even in strains isolated from people without nosocomial risk factors. Molecular analysis is essential for understanding the patterns of dissemination. The objective of this study was to identify community-acquired methicillin-resistant *S. aureus* (CA-MRSA) clones that infected Paraguayan children patients in two periods of time.

Methodology: An observational, descriptive study was designed to determine the genetic variability of 115 isolates of CA-MRSA recovered from children who attended four reference centers in Paraguay between 2009-2010 and 2012-2013.

Results: The combined use of Pulsed Field Gel Electrophoresis (PFGE), Multi-Locus Sequencing Typing, Multi-Locus Variable Analysis (MLVA) and *Spa typing* techniques allowed the identification of two dominant clones: ST30-IV-t019 (77%) and ST5-IV-t311 (10%), and the establishment of the former as the leading cause of CA-MRSA infections in children during the study period.

Conclusions: This is the first study that provides epidemiological information as well as microbiological and molecular characteristics of CA-MRSA isolates recovered from children from Asunción and the Central Department of Paraguay.

Key words: Methicillin-resistant *Staphylococcus aureus*; MRSA; children; community-associated.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were once confined mostly to hospitals, healthcare environments, and their patients. Since the 1990s, virulent community-acquired MRSA (CA-MRSA) clones spread worldwide, first in the community in healthy young patients, but later also in healthcare facilities [1]. They have been associated predominantly with skin and soft tissue infections (SSTIs), but due to the invasive nature of this pathogen and the carriage of antibiotic resistance and virulence factors genes (mainly the toxin Panton-Valentine Leukocidin, PVL), they have also been linked to severe clinical syndromes such as necrotizing pneumonia, osteomyelitis, meningitis, sepsis, and death. However, the frontier between CA-MRSA and healthcare-associated MRSA (HA-MRSA) has begun to fade [1,2].

Different CA-MRSA lineages have been described worldwide: ST1 reported in Asia, Europe, and the United States; ST8 in Europe, and the United States; ST30 in Australia, Europe, and South America; ST59 in Asia and the United States and ST80 in Asia, Europe, the Middle East, and Africa. Some african studies also reveal other frequent sequence types in addition to the ST80, such as ST22 and ST239. This global ST239 was also common in other countries, such as China and

Taiwan. Clones ST8-IV, also known as USA300, and ST30-IV, Southwest Pacific clone (SWP), have spread globally and are considered pandemic [3–5].

In South America, the MRSA lineages found most frequently (including CA-MRSA and HA-MRSA) are the Pediatric clone (CC5-ST5-IV), the Cordobés/Chilean clone (CC5-ST5-I), the SWP clone (CC30-ST30-IV), the Brazilian clone (CC8-ST239-III) and the New York/Japan (CC5-ST5-II) clone [6].

In Argentina, the main clone recovered from patients with CA-MRSA invasive infections between 2010 and 2011 was CC30-ST30-IV-t019-PVL positive, which has become predominant and replaced the previously described CA-MRSA clone: CC5-ST5-IV-t311-PVL positive [7].

Information about MRSA clones circulating in Paraguay is limited to one report of two HA-MRSA clones, but there is no information about those circulating in the community. The objective of this study was to identify the CA-MRSA clones that infected Paraguayan children patients in two periods of time: 2009-2010 and 2012-2013.

Methodology

An observational, descriptive, and cross-sectional study was designed to analyze phenotypic and molecular characteristics of CA-MRSA isolates recovered from Paraguayan children.

S. aureus isolates recovered in two phases; phase one between 2009-2010 and phase two between 2012-2013, from children under sixteen years old attending four reference hospitals from Asunción and the Central Department of Paraguay, were collected. The isolates were recovered from different clinical specimens, including skin and soft tissue secretions, pleural, cerebrospinal, peritoneal body fluids and blood, and stored at -80ºC at the Health Sciences Research Institute (IICS in Spanish). Identification data, epidemiology files, and records of antimicrobial susceptibility were provided by the microbiology laboratories of the participating hospitals.

Only CA-MRSA recovered from both study phases were characterized molecularly. CA-MRSA infection was defined according to the CDC criteria by a positive MRSA culture at the time of hospital admission or within the 48 h after hospital admission and the absence of HA-MRSA associated risk factors [8].

Identification of the isolates was based on standard bacteriological methods, including cultural characteristics, Gram's stain and biochemical tests such as oxidative/fermentative, catalase and coagulase tube tests. The phenotypic identification process was carried out too by testing the beta hemolysis and pigment production and analyzing the morphology of the colony on sheep blood agar. *In vitro* antimicrobial susceptibility testing was done by the Kirby-Bauer diffusion method, using Oxoid/BBI discs following the criteria recommended by the CLSI (Clinical and Laboratory Standards Institute) from 2009 to 2013, according to the strain collection date or by automated systems using Vitek®2 (BioMérieux, La Balme, French) following the manufacturer's instructions. D-test was also performed. Results were interpreted based on CLSI breakpoints [9,10].

The strains were cultured on TSA (Tryptic Soy Agar, Difco, Le Pont de Claix, France) medium and incubated at 35°C for 24-48 hours under 5% CO_2 for subsequent extraction using a commercial genomic DNA extraction kit (Wizard Genomic, Promega, Madison, USA), following the manufacturer's instructions. The amplification of 16S rRNA genes was performed according to conditions described by Manfredi *et al.* to recognize genus *Staphylococcus* [11].

The detection of *mecA* and Panton-Valentine leukocidin (PVL) coding genes (*lukS/F-PV*) were carried out as previously described [12]. Genes encoding enterotoxins: *sea, seb, sec, sed, seh* were detected as described elsewhere [11,13]. Detection of genes encoding hemolysins (*hla* and *hlb*) and exfoliative toxins (*eta* and *etb*) was carried out using oligonucleotides and protocols described by Wang *et al.* and Johnson *et al.* respectively [14,15].

The *SCCmec* types were determined by PCR with a simplified version of Kondo's typing system [16].

The polymorphic region of the s*pa* gene was amplified by PCR with primers described by Shopsin *et al.*, 1999 [17]. DNA sequences (Macrogen, Seoul, Korea) were analyzed, and *spa*-types were determined with the Ridom Spa Server [18].

Multiple-Locus Variable Analysis (MLVA) was performed using five pairs of oligonucleotides amplifying seven VNTR loci (*clfA, clfB, sdrC, sdrD, sdrE, spA,* and *sspA*), followed by polyacrylamide gel electrophoresis (7.5% separator, 5% concentrator) staining with silver nitrate to visualize the products [19]. Gels were digitized using UN SCAN IT GEL software, and the data was analyzed. All the strains with bands of equal size or up to 1 band of difference ($\geq$ 85% similarity) were included in the same cluster, following the criterion used by Malachowa *et al.* 2005. Clusters were numbered for identification as recommended by Sabat *et al.* 2003 [19,20].

All the CA-MRSA were studied by MLVA, spatyping, and virulence profile. Isolates that presented

differences in *Spa* typing and virulence profile were chosen as representatives of each MLVA cluster for PFGE (Pulse Field Gel Electrophoresis), performed with *Sma*I as previously described [18,21]. In the process, the clones ST5-IV-t311-PVL positive, ST30-IV-t019-PVL positive, and ST8-IV-t008-PVL positive (USA 300) were included as references [7,21,22]. PFGE analysis was carried out by visual inspection, and the dendrogram was constructed (FigTree v1.4.0 software) applying the algorithm for cluster analysis (UPGMA, *applying unweighted pair-group method clustering analysis*) and the Dice's coefficient. Isolates with similar band patterns (> 80%) were considered to belong to the same pulsotype.

Representative isolates of the major pulsotypes were studied by multilocus sequence typing (MLST), by amplification and posterior sequencing (Macrogen, Seoul, Korea) of seven constitutive loci (*arcC, aroE, glpF, gmk, pta, tpi, yqi)* performed as previously described [23]. Sequences were analyzed with the MLST database (http://saureus.mlst.net).

The statistical analyses were carried out with the Epi Info software (v 7.2.) and the calculations with Fisher's Exact or Chi-Square Test as appropriate.

Ethical considerations

The study was approved by the Scientific and Ethics Committee of the IICS, Universidad Nacional de Asunción (UNA) (P29/2011 and P44/2012). The samples were strictly managed by codes, and the results obtained were exclusively used for epidemiological purposes.

Results

In this study, a total of 281 community-acquired *S. aureus* (CA-SA) isolates were collected (113 CA-SA from phase one and 168 CA-SA from phase two): 115 (41%) were MRSA. The prevalence of CA-MRSA was 21% (24/113) between 2009-2010 and 54% (91/168) between 2012-2013. Eighty-five CA-MRSA isolates (74%) caused skin and soft tissue infections (SSTI), and 30 (26%), invasive diseases (sepsis, osteomyelitis, and pneumonia).

Antimicrobial resistance analysis of CA-MRSA isolates (N = 115) showed susceptibility to tetracycline (100%), tigecycline (100%), teicoplanin (100%), and vancomycin (100%) in all tested isolates. Most CA-MRSA isolates (95%) were resistant to penicillin. Chloramphenicol was the second antibiotic with the highest level of global resistance (21%: 38% phase one and 16% phase two), followed by erythromycin (17%: 21% phase one and 16% phase two), inducible

Table 1. Molecular characteristics of CA-MRSA isolates (N = 115).

PT	ST	CC	*spa* type	MLVA	Virulence genes	CA-MRSA isolates (N = 115)
A	ST30	30			-	44
			t019	M1	*lukS/F-PV*	41
					lukS/F-PV, hlB	1
					lukS/F-PV, hlA, hlB	2
			t975	M2	*lukS/F-PV*	1
			t975	M3	-	1
B	ST30	30	t363	M4	*lukS/F-PV*	1
C	ST30	30	t021	M5	-	1
D	ST8	8	t11770	M6	*lukS/F-PV, hlA, hlB, seB*	1
E	ST25	No data	t081	M7	-	1
F	ST435	30	t021	M8	*lukS/F-PV*	1
G	ST5	5			-	1
			t311	M10	*lukS/F-PV*	6
					lukS/F-PV , hlA	3
					hlA	1
					lukS/F-PV, hlA, seA	1
			t002	M11	-	1
			t1791	M9	*hlA*	1
H	ST5	5	t7078	M12	*lukS/F-PV*	1
					-	1
	ST100	5	t002	M13	*hlA*	2
I	ST669	97	t359	M14	-	1
J	ST72	8	t148	M15	*hlA*	1

PT: Pulsotype; ST: Sequence type; CC: Clonal complex; -: No virulence factor gene detected.

clindamycin resistance (15%: 8% phase one and 16% phase two) and gentamicin (7%: 17% phase one and 4% phase two). Global resistance levels to ciprofloxacin, rifampicin, and trimethoprim-sulfamethoxazole were lower than 3% (N = 4).

All CA-MRSA isolates (N = 115) were *mec*A positive and harbored SCC*mec* type IV. Genes encoding PVL and hemolysin A were detected in 51% (79% phase one and 44% phase two), and 10% (29% phase one and 5% phase two) of the CA-MRSA isolates analyzed, respectively. Hemolysin B, enterotoxin A, and B were detected in less than 3% of the isolates. Enterotoxin C, D, H, and exfoliative toxins A and B were not detected in this study.

The most frequent *spa* type was t019 (77%), followed by *spa* type t311 (10%) and others representing less than 3% of the 115 CA-MRSA isolates (Table 1). MLVA analysis recognized fifteen different profiles (Table 1). Congruently, all samples with a certain *spa*-type were grouped within the same MLVA profile. MLVA 1 (M1) profile with t019 *spa* type was the most frequent (M1/t019, N = 88), followed by profile 10 (M10/t311, N = 12) and profile 12 (M12/t7078, N = 2). Other profiles included only one isolate each.

Representative isolates of each MLVA profile, sequence types and, virulence genes profile were chosen for PFGE analysis, revealing ten different PFGE types, and the dendrogram was constructed (Figure 1) Pulsotype A was the most frequent band pattern obtained (78%). Eight different sequence types (ST) were determined within the isolates analyzed; ST30 was the most common (80%), followed by ST5 (14%). Overall, two significant clones were identified among

Figure 1. Dendrogram of representative CA-MRSA isolates (N = 26), revealing ten different PFGE types using FigTree v1.4.0 software. The ST5-IV-t311-PVL positive, ST30-IV-t019-PVL positive, and ST8-IV-t008-PVL positive (USA 300) clones were included as references. The Pulsotype A includes the following isolates: SIP48, SIP49, SIP83, SIP88, SCM65, SCM66, SCM68, SGP68, SGP95, SGP191, SHN55 and SHN63. The Pulsotype G includes the following isolates: SHN18, SHN31, SHN59, SCM 73 and SIP54. The Pulsotype H includes the isolates SCM71 and SGP167. And the following pulsotypes are represented by a single isolated: Pulsotype B-SGP166, Pulsotype C-SHN80, Pulsotype D-SGP63, Pulsotype E-SGP215, Pulsotype F-SGP22, Pulsotype I-SGP199, Pulsotype J-SCM47.
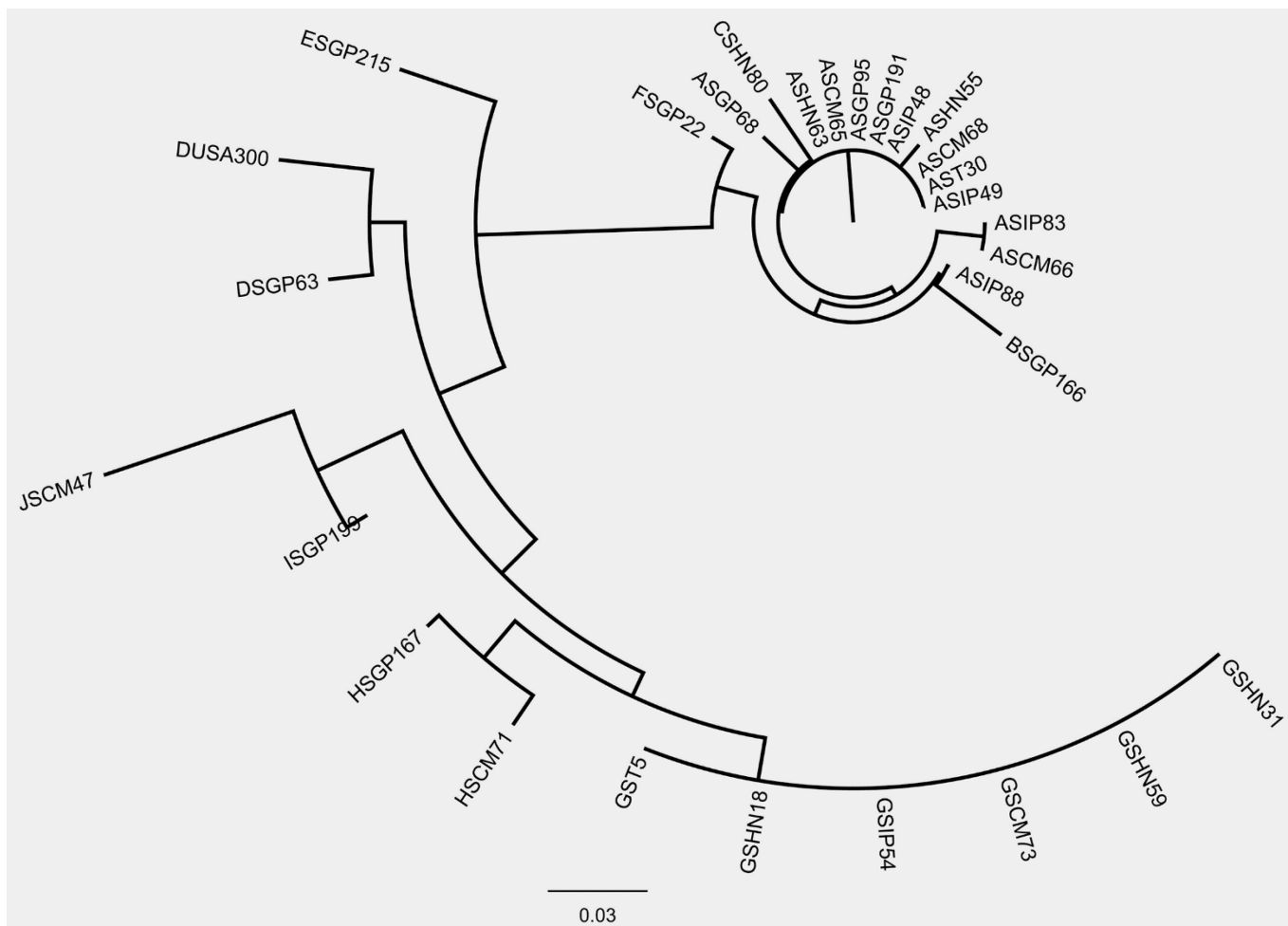
the isolates: ST30-IV-t019 77% (88/115) and ST5-IV-t311 10% (12/115). Table 1 shows the molecular characteristics of all CA-MRSA isolates.

In the phase one of the study (2009-2010) a predominance of ST30-IV-t019 (54%) over ST5-IV-t311 clone (17%) was observed, this difference increased even more in phase two (65% ST30-IV-t019 to 5% ST5-IV-t311 clone, respectively), being statistically significant (p = 0.01). ST30-IV-t019 (N = 88) was the main clone circulating in the four hospitals included and was persistent throughout the study time. Sixty-nine isolates belonging to this clone (78%) were associated with SSTIs, while the remaining 22% with invasive type infections. Furthermore, eight isolates (67%) belonging to the ST5-IV-t311 clone were responsible for SSTIs, and the other 33% (N = 4) associated with invasive infections. Interestingly, one isolate presented the ST8-IV-t11770-PVL positive profile, similar to ST8-USA300-LV (Latinoamerican Variant).

Discussion

In Paraguay, this study constitutes the first epidemiological report that includes phenotypic and genotypic data of CA-MRSA isolates recovered from children from Asunción and the Central Department. The limited number of reports that refer to molecular data and genetic variability of *S. aureus* include only one description of two nosocomial clones recovered from adults: most isolates analyzed were CC5-ST221-I-t049, similar to the Cordobés/Chilean clone that expanded through South America in the past decade and the minority belonged to CC8-ST239-III-t037 clone (related to the Brazilian clone) [24].

The increase in the prevalence of CA-MRSA observed between these two phases is in accordance with the regional trend of an increase in the incidence rate of infections caused by community isolates and with the methicillin-resistant phenotype among the infections caused by this pathogen. Increases of up to 12 times in this rate are reported in a study conducted over ten years in Argentina [25,26].

The dominant clone identified in this study was CC30-ST30-IV-t019-PVL positive (77%). Although some isolates belonging to this major clonal complex shared the same pulsotype, there were differences in the presence of virulence factors and the MLVA analysis. Two ST30-IV isolates were *spa* type t975; this *spa* type differs from the main found in this study, t019, in the deletion of one repeat. The loss of repeated regions is suggested as a novel mechanism of microevolution during chronic diseases [27]. In this case, one of the

isolates was recovered from a child with a chronic infection that started with a skin abscess and evolved to bilateral pneumonia and sepsis. The second isolate was recovered from an abscess in the nasal region.

ST30-IV-t019 has also been described as the main CA-MRSA clone found in other countries of South America [7,28]. This clone emerged initially in the SWP region (Australia, New Zealand); however, given the reports of its circulation in Europe, the USA, and South America, it is now considered a pandemic clone. The SWP clone is related to other virulent CC30 clones, such as the penicillin-resistant phage-type 80/81 clone and the HA-MRSA clone EMRSA-16 [29]. In 2008, the SWP clone was described as a minor community-associated clone causing skin and soft tissue infections and invasive diseases in Argentina [30,31]. Later, ST30-IV clone has replaced the previously prevalent CA-MRSA clone in this region, ST5-IV-t311-PVL positive, and it is well adapted to the community environment infecting both adult and adolescent patients [7,32,33].

The second most frequent clone identified in this study (ST5-IV-t311), is closely related to other CA-MRSA clones circulating in our region [6,22,30]. Interestingly, the SWP clone is the prevalent clone found in this study and the ST5-IV-t311 clone in second place. This observation reinforces the successful adaptation of the SWP clone to this region of South America.

In Paraguay, we observe a similar trend: during 2009-2010 ST30-IV-t019 (54%) was predominant over ST5-IV-t311 (17%) in the second phase of this study (2012-2013), the difference was higher (65% and 5%, respectively) suggesting the replacement of ST5-IV-t311 by ST30-IV-t019 again. This replacement could be due to the presence or increased expression of certain virulence and adhesion factors, such as fibrinogen binding proteins (FnbPs), in the ST30 clone that determine greater success in establishing in certain niches and the subsequent dissemination to other tissues [34].

In this study, we also described for the first time in Paraguay the CC5-ST100-IV-t002 clone closely related to the Argentinian pediatric clone [35]. Moreover, two other minor clones were also identified: CC30-ST435-IV-t021-PVL positive and CC8-ST72-IV-t148, both reported previously in other countries of the region such as Argentina, Brazil, and Uruguay [6,7,28,35–38].

One clone MRSA ST8-IV-t11770 (additionally resistant to tetracycline) was recovered from an SSTI in a ten-year-old child with no comorbidities (starting spot on foot) that was complicated by sepsis with multiple

foci. This is the first report in Paraguay of a strain related to the ST8-USA300-LV (Latin American Variant) clone. This epidemic and hypervirulent clone was determined to be frequent in the northern region of South America and is associated with the carrying of a pathogenicity island that encodes the genes of enterotoxins sek and seq (SaPI5), as well as mobile genetic elements such as COMER (Copper and mercury resistance mobile element), which promotes bacterial survival by providing resistance against certain defense mechanisms used by the innate immune system [39,40].

The absence of vancomycin, tetracycline, tigecycline, and teicoplanin-resistant isolates reveals the still intact potential of these antibiotics for the treatment of CA-MRSA infections in Paraguay. Other reports also indicate that CA-MRSA isolates are usually susceptible to other families of antibiotics, in contrast to HA-MRSA [41–43]. However, due to the appearance of vancomycin-resistant *S. aureus* (VRSA) strains in EEUU in 2002, after the increase in vancomycin treatment in hospitalized patients infected with MRSA, it is necessary to maintain an efficient surveillance system that could alert in real-time changes in the susceptibility for vancomycin [44–48].

The levels of resistance to chloramphenicol (21%), erythromycin (17%), clindamycin (15%), and gentamicin (7%) are similar to the reported by other authors in CA-MRSA isolates [49,50]. The increasing frequency of CA-MRSA infections has forced the incorporation of clindamycin and other antibiotics as part of the antimicrobial treatment schemes in cases of staphylococcal SSTIs. The clindamycin resistance levels detected in this study have exceeded the cutoff point of 10%, considered a reasonable limit for the use of clindamycin in the empirical treatment of mild or moderate skin and soft tissue infection produced by MRSA (or by MSSA in patients allergic to beta-lactams) [42]. This fact increases the possibilities of therapeutic failure, being able to have a direct impact on the empirical scheme that should be taken into account in daily pediatric practice [43,51].

The extraordinary genetic success of CA-MRSA clones is now well known. The spread of CA-MRSA virulent clones in the hospital setting is currently a major cause of concern worldwide [52].

Conclusion

The ST30-IV-t019 clone was identified as the dominant CA-MRSA clone that infected Paraguayan children patients in the studied period. This is the first report of CA-MRSA clones isolated from children in Paraguay, and it demonstrates the imperative need for a better understanding of the transmission dynamics of this microorganism to establish an effective epidemiological surveillance system.

Acknowledgements

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