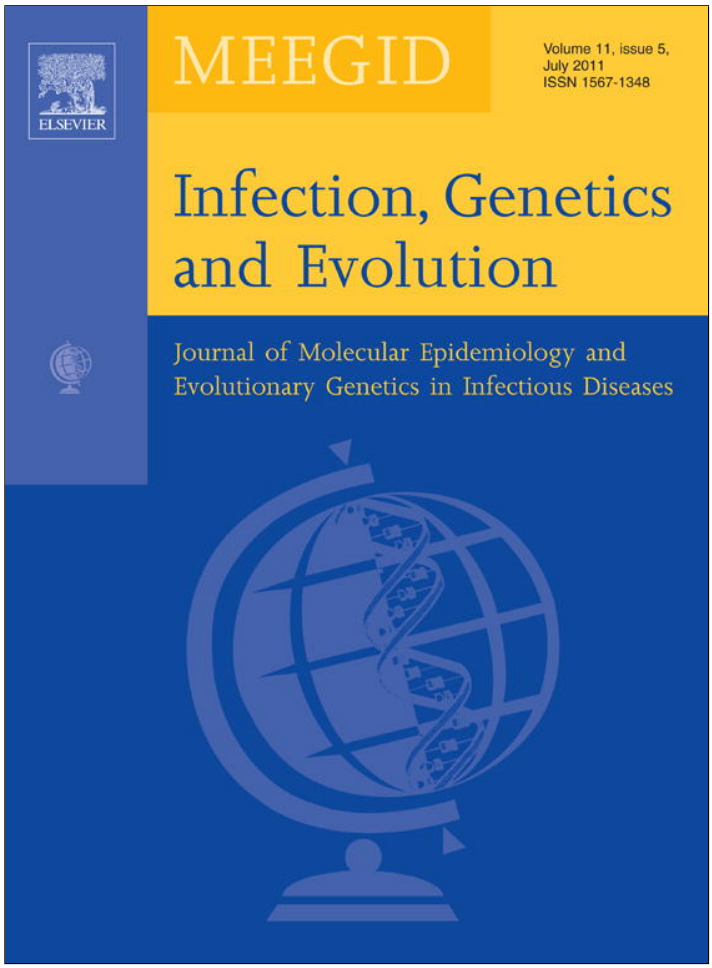

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Research paper

Prevalence and characterization of methicillin-resistant *Staphylococcus aureus* among healthy children in a city of Argentina

N. Gardella [a], S. Murzicato [b], S. Di Gregorio [a], A. Cuirolo [a], J. Desse [c], F. Crudo [b], G. Gutkind [a], M. Mollerach [a,*]

[a] *Cátedra de Microbiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina*
[b] *Hospital Municipal de San Antonio de Areco, Provincia de Buenos Aires, Argentina*
[c] *Hospital Paroissien, Provincia de Buenos Aires, Argentina*

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ABSTRACT

Community acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is a major global problem. Healthy carriers of *S. aureus* strains have an important role in the dissemination of this bacterium. The aim of this study was to estimate the prevalence of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) carriage among healthy children in a city of Buenos Aires province, Argentina, and to determine the potential risk factors for its acquisition. We also described the molecular features of MRSA strains circulating in this population. *S. aureus* carriage was investigated in all children attending the last year of kindergarten during the 2008 school- year period. Household contacts of MRSA carriers were also screened. Of 316 healthy children, 98 (31.0%) carried *S. aureus*, including 14 MRSA carriers (4.4%) and 84 methicillin susceptible *S. aureus* (MSSA) carriers (26.6%). All MRSA isolates carried the SCC*mec* type IV cassette. Eight of the fourteen isolates were closely related to the clone responsible for most severe community-acquired MRSA infections caused in our country (CAA: PFGE A, SCC*mec* IV, *spa* t311, ST5). Two subtypes (A_1 and A_2) were distinguished in this group by PFGE. Both had *agr* type II and presented the same virulence determinants, except for PVL coding genes and *sea* that were only harbored by subtype A_1. Our results, based on the analysis of MRSA isolates recovered in the screening of healthy children, provide evidence of a community reservoir of the major CA-MRSA clone described in Argentina.

1. Introduction

Staphylococcus aureus is an important human pathogen causing a wide range of infections. According to the Sistema Informático de Resistencia (Asociación Argentina de Microbiología) in Argentina, methicillin-resistant *S. aureus* (MRSA) strains are among the most prevalent nosocomial pathogens (HA-MRSA) (http://www.aam.org.ar). Historically, patients who developed MRSA infections in the community had traditional risk factors associated with treatment in nosocomial settings. With the recent emergence of MRSA infections in patients lacking contact with a hospital setting the term community-associated MRSA (CA-MRSA) has been introduced (Herold et al., 1998; Hussain et al., 2001; Suggs et al., 1999). Definitions based on epidemiological origin have been generated and genotype testing, and antibiotic susceptibility testing, have also been proposed to distinguish between HA-MRSA and CA-MRSA (Centers for Disease Control and Prevention, 2009; Salgado et al., 2003).

The nasopharyngeal tract is the primary reservoir of *S. aureus* in both adults and children, although it may be found in other body sites as well (Kluytmans et al., 1997). Carriage of *S. aureus* seems to be important because most *S. aureus* infections occur in persons who are previously colonized with this microorganism and may act as vectors for spreading *S. aureus* and MRSA to both community and hospital environments (Cardoso-Lamaro et al., 2009; Torres et al., 2001; Von Specht et al., 2006).

High prevalence of MRSA colonization among hospital patients or in long-term care facilities has been well documented all over the world (Eveillard et al., 2008; Kluytmans et al., 1997). Reports of carriage on healthy people outside the hospital environment are still scarce (Hisata et al., 2005; Lu et al., 2005; Rim and Bacon, 2007; Torano et al., 2001), especially from South America; moreover, there is little information on factors associated with MRSA colonization and transmission of MRSA to household contacts. The purpose of this study was to determine the prevalence of *S. aureus* and MRSA carriage among all the healthy children attending the last year of kindergarten in a small city of Argentina. We also sought to characterize these MRSA colonizing isolates to compare

* Corresponding author at: Universidad de Buenos Aires. Facultad de Farmacia y Bioquímica, Departamento de Microbiología, Inmunología y Biotecnología, Junin 956, Buenos Aires, Argentina. Tel.: +54 11 4964 8285; fax: +54 11 4964 8274.
E-mail address: mmollera@ffyb.uba.ar (M. Mollerach).

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them with the main HA-MRSA and CA-MRSA clones causing infections in our country.

2. Materials and methods

2.1. Study design

We conducted a cross sectional study on the total population of healthy children attending the last year of the all eight kindergartens in San Antonio de Areco, Buenos Aires, Argentina, during the 2008 school- year period. A total of 316 kids were sampled during 6 months (May–October). Household contacts were sampled in 39 of them. Written informed consent was obtained from each child's parents before specimen collection. The total of the population accepted to participate.

2.2. Specimens

Samples were obtained from children by using sterile dry-cotton swabs from nasal, inguinal, axial and pharyngeal sites. Samples were immediately inoculated onto mannitol-salt agar (Biokar Diagnostic, Beauvais, France) and Chromagar MRSA plates (bioMérieux, Marcy-l'Etoile, France) and incubated at 37 °C for 48 h, after which morphological and Gram stain examinations were conducted. The isolates were identified using standard biochemical tests.

2.3. Antimicrobial susceptibility testing

In vitro susceptibility testing was performed using disk diffusion tests according to the CLSI guidelines (Clinical Laboratory Standards Institute, 2005). The following twelve antimicrobial agents were tested: oxacillin, cefoxitin, gentamicin, ciprofloxacin, clindamycin, erythromycin, rifampin, vancomycin, teicoplanin, minociclin, levofloxacin and trimethoprim-sulfamethoxazole (Laboratorios Britania, Buenos Aires, Argentina). Susceptibility interpretative criteria used were those established by the CLSI (Clinical Laboratory Standards Institute, 2005).

2.4. PCR amplification of mecA and SCCmec typing

Detection of *mecA* coding gene was performed after extraction of genomic DNA as previously described (Von Specht et al., 2006). Typing of staphylococcal cassette chromosome (SCC*mec*) was performed for all MRSA isolates by the multiplex PCR strategy developed by Oliveira and de Lencastre (Oliveira and De Lencastre, 2002).

2.5. PCR amplification of virulence determinants and agr typing

Detection of PVL coding gene was performed as previously described (Lina et al., 1999). Investigation of toxins and adhesins coding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seh*, *sej*, *seg*, *sei*, *hlg*, *ica*, *fnbA*,*fnbB*, *fib*, *clfA* and *clfB*) was carried out by PCR strategies described by Nashev and Tristan (Nashev et al., 2004; Tristan et al., 2003). Multiplex PCR was applied to determine *agr* group (Gilot et al., 2002).

2.6. PFGE, spa typing, MLST and RAPD-PCR typing

A single MRSA isolate per child was genotyped by using *spa* typing (Harmsen et al., 2003) and pulsed-field gel electrophoresis (PFGE) with SmaI as previously described (Chang et al., 2000). PFGE profiles were clustered by the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity), followed by tree inference (Treecon for Windows 1.3b; Y. van de Peer). The following epidemic MRSA clones previously described in Argentina were included in PFGE pattern analysis: the pediatric clone (ST5, SCC*mec* IV), the Brazilian clone (ST239, SCC*mec* IIIa), and the Cordobes clone (ST5, SCC*mec* I). The prevalent clone, named CAA clone (PGFE type A, ST5, t311, SCC*mec* type IV), causing community-associated infections in Argentina, was also included. Representative isolates of major pulsotypes were typed by Multilocus Sequence Typing (MLST).

MRSA isolates from different colonization sites in each kid and their house contacts were compared by Random Amplified Polymorphic DNA PCR (RAPD-PCR), using three different primers (RAPD-1, RAPD-7, ERIC-2) (Gardella et al., 2005).

2.7. Questionnaire survey

Individual variables as well as sociodemographic and family characteristics were obtained prior to sample collection by interviews with the parents, using a standardized questionnaire.

Table 1
Characterization of MRSA recovered from healthy children.

Isolates	Kindergarten	SCC*mec*	PVL[a]	Resistance	*spa*		PFGE Patterns	MLST
					Profile	Type		
1[b]	K1	IV	–	ERY, CLIN	4-20-16-34	t2365	B	nd
2[c]	K2	IV	–	GEN	26-23-17-34-17-20-17-12-17-16	t002	C	nd
3[d]	K2	IV	–		–	nd	D	nd
4[b]	K3	IV	–		07-23-21-16-34-33-13	t127	E	nd
5[e]	K3	IV	–		15-12-16-02-16-02-25-17-24-24	t012	F	nd
6[b]	K4	IV	–	RIF	15-12-16-02-16-02-25-17-24-24	t012	G	nd
7[b]	K5	IV	–		26-23-17-34-17-20-17-12-17-16	t002	A_2	ST5
8[f]	K5	IV	–	ERY, CLIN	26-23-17-34-17-20-17-12-17-16	t002	A_2	nd
9[b]	K6	IV	–	GEN	26-23-17-34-17-20-17-12-17-16	t002	A_2	nd
10[c]	K7	IV	–	GEN	26-23-17-34-17-20-17-12-17-16	t002	A_2	nd
11[e]	K8	IV	+		26-23-17-34-20-17-12-17-16	t311	A_1	nd
12[f]	K8	IV	+		26-23-17-34-20-17-12-17-16	t311	A_1	ST5
13[f]	K8	IV	+	GEN	26-23-17-34-20-17-17-16	t2121	A_1	ST5
14[b]	K8	IV	+		26-23-17-34-20-17-12-17-16	t311	A_1	nd

ERY, erythromycin; CLIN, clindamycin; GEN, gentamicin; RIF, rifampin; nd, not determined.
[a] PVL was assessed by PCR amplification of *luk*-PVL genes.
[b] Nasal and pharyngeal.
[c] Pharyngeal.
[d] Axial.
[e] Nasal, pharyngeal and inguinal.
[f] Nasal.

The participant's age, gender, city or countryside residence, presence and number of pets, number of household cohabitants, medical history, including previous hospitalization, medication and underlying diseases (heart, renal, gastrointestinal, liver and pulmonary disease, diabetes and HIV) were then correlated with *S. aureus* colonization status.

2.8. Statistical analysis

Comparison of categorical variables and percentages between groups was done by the Fisher's exact test. Odds Ratio (OR) and 95% confidence intervals (CIs) were also calculated. The threshold for a significant difference was defined for a P value of <0.05. The values were calculated using the EPI-INFO statistical software (Version EPI-6).

3. Results

3.1. Prevalence of S. aureus and MRSA carriage

S. aureus was recovered in 98 children (31.0%) from a total of 316 healthy kids, including 14 MRSA carriers (4.4%) and 84 methicillin susceptible *S. aureus* (MSSA) carriers (26.6%). MRSA was isolated from nasal, pharyngeal, axial and inguinal sites in 11/

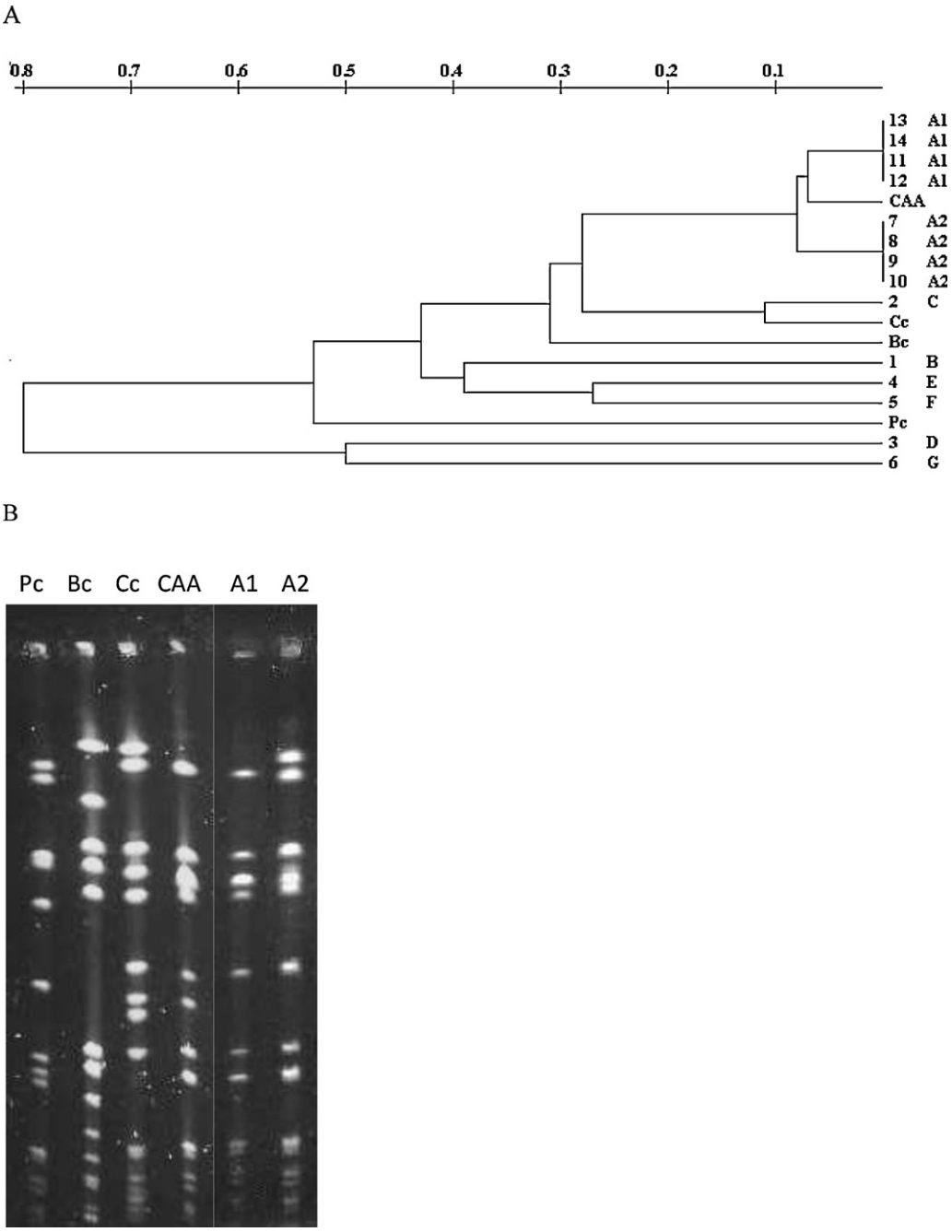

Fig. 1. (A) Dendrogram showing genetic distances based on Dice distance coefficient measurements of PFGE banding patterns of MRSA isolates recovered from healthy children and most prevalent clonal types in Argentina: the Pediatric clone (Pc), the Brazilian clone (Bc), the Cordobes clone (Cc), the clone responsible for most severe community-acquired MRSA infections (CAA). Patterns were coded with capital letters; A_1 and A_2 were defined as subtypes of the major group (Type A). (B) SmaI restriction patterns of representative isolates of the two major pulsotypes (A_1 and A_2) and main HA-MRSA and CA-MRSA clones causing infections in Argentina. Lane 1: Pc, lane 2: Bc, lane 3: Cc, lane 4: CAA, lane 5: Pulsotype A_1, lane 6: Pulsotype A_2.

14 (78.6%), 9/14 (64.3%), 1/14 (7.1%) and 1/14 (7.1%) carriers, respectively. Combination of nasal and pharyngeal swabs allowed a detection of 92.9%.

3.2. Antimicrobial susceptibility

Antibiotic susceptibility testing revealed that 1/14, 2/14, 2/14 and 4/14 of MRSA isolates were resistant to rifampin, erythromycin, clindamycin, and gentamicin, respectively (Table 1).

3.3. Molecular characterization of MRSA isolates

All MRSA isolates harbored *mecA* gene in SCC*mec* type IV cassette. Eight of fourteen isolates were closely related by PFGE patterns (Type A) to the clone responsible for most severe community-acquired MRSA infection in our country (CAA) (Gardella et al., 2008). In this group, two subtypes (A_1 and A_2) were distinguished (Fig. 1). Both subtypes had *agr* type II and presented the same virulence determinants, *seg*, *sei*, *hlg*, *ica*, *fnbA*, *fib*, *clfA* and *clfB*, except for PVL coding genes and *sea* that were only harbored by subtype A_1. Within this subtype A_1, 3/4 of isolates were *spa* type t311 and one *spa* type t2121. All 4 isolates belonging to subtype A_2 showed *spa* type t002 (Table 1).

Household contacts of all the 14 children colonized by MRSA were included, in 5 of these families we detected any other MRSA carrier. RAPD-PCR analysis demonstrated a potential intrafamily transmission in the 5 cases, but different families had different clones. Isolates recovered from different colonization sites (nasal, pharyngeal, axial and inguinal) from each child were indistinguishable. One half of MRSA- positive children had pets. MRSA was not recovered in any of them.

3.4. Risk factors for S. aureus colonization

Univariate analysis revealed that chronic diseases (odds ratio [OR], 9.4194; 95% confidence interval, 1.4609–60.7312) and rural housing (OR, 3.3526; 95% confidence interval, 1.6084–6.9885) were significant risk factors for *S. aureus* colonization (MRSA and MSSA combined) (Table 2).

The other variables examined showed no statistically significant association with *S. aureus* carriage. Despite the high frequency of antimicrobial use (27.8%), this variable was not epidemiologically associated with *S. aureus* carriage (*P*: 0.2206). The same situation was observed with the "presence of pets" variable, which was not associated with *S. aureus* carriage (P: 0.6002), even though it had a frequency of 68.6%. Notwithstanding the non- statistical association between MRSA carriage and the variables currently recognized as risk factors for infection, we detected that 4 out of 14 children colonized with MRSA had registered antibiotic use during the previous year, 2/14 had had a hospitalized cohabitant in that period, and 1/14 had a healthcare worker in the family.

4. Discussion

Several reports have described prevalence of and risk factors for MRSA colonization at hospital admission, but the literature on healthy population is limited. The increasing prevalence of MRSA as a cause of community-associated infection has led to the assumption that colonization with MRSA must be widespread in the general community.

This is the first epidemiological study of *S. aureus* carriage conducted in Argentina. In this study, we investigated the prevalence of *S. aureus* and MRSA colonization in healthy children,

Table 2
Risk factors associated with *S. aureus* carriage in infants from San Antonio de Areco, Argentina.

Risk factors	Carriage (*n* = 97) No. (%)	No-carriage (*n* = 220) No. (%)	*P* value	OR (CI 95)
Gender				
Female	53 (54.6)	106 (66.7)	0.3299	1.2955 (0.8038–2.0878)
Male	44 (45.4)	114 (51.8)		
School				
Urban	92 (94.8)	217 (98.6)	0.0603	3.3912 (1.0069–15.3485)
Countryside	5 (5.2)	3 (1.4)		
House				
Countryside	18 (18.6)	14 (6.4)	0.0014	3.3526 (1.6084–6.9885)
Urban	79 (81.4)	206 (93.6)		
Presence of pets				
No	33 (34.0)	68 (30.9)	0.6002	1.1709 (0.7056–1.9428)
Yes	64 (66.0)	152 (69.1)		
Hospitalization[a]				
Yes	0 (0.0)	9 (4.1)	0.0616	Indeterminable
No	97 (100.0)	211 (95.9)		
Use of an ATB[b]				
No	75 (77.3)	154 (70.0)	0.2206	1.4610 (0.8416–2.5364)
Yes	22 (22.7)	66 (30.0)		
Inmunocompromised				
Yes	0 (0.0)	2 (0.9)	0.5739	Indeterminable
No	97 (100)	218 (99.1)		
Health-worker cohabitant				
Yes	6 (6.2)	9 (4.1)	0.5665	1.5458 (0.5533–4.3186)
No	91 (93.8)	211 (95.9)		
Hospitalizaded cohabitant[a]				
No	81 (63.5)	182 (98.6)	0.8737	1.0570 (0.5612–1.9908)
Yes	16 (16.5)	38 (17.3)		
Previous MRSA infection				
Yes	0 (0.0)	3 (1.4)	0.5557	Indeterminable
No	97 (100.0)	217 (98.6)		
Having or suffering chronic disease[c]				
Yes	4 (4.1)	1 (0.5)	0.0320	9.4194 (1.4609–60.7312)
No	93 (95.9)	219 (99.5)		

[a] One year before the study.
[b] Six months before the study, ATB: antibiotic.
[c] Chronic diseases included in the questionnaire were heart, renal, gastrointestinal, liver and pulmonary disease, diabetes and HIV.

and we determined the molecular features of organisms recovered from kindergarten attendees. Even though the use of nasal swabs is the most common strategy for detection of *S. aureus* carriers (Cardoso-Lamaro et al., 2009; Hisata et al., 2005; Pan et al., 2005), we found that a better strategy was the study of the nasopharyngeal site with a detection rate that reached 93%, as was showed before in the study conducted by Widmer et al. (2008).

The prevalence of MSSA carriage in our population (healthy children) was 26.6%, a similar value to that reported in other studies, which represents between 15 and 40% of the population (Kluytmans et al., 1997; Ruimy et al., 2008). On the other hand, MRSA carriage rate was 4.4%, which is in close agreement with that reported by Hisata et al. (2005), accounting for 2.3% more than the values mentioned in the study carried out by Torano et al. (2001) in La Habana, Cuba, 3.1% more than those of a study conducted in Brazil (Cardoso-Lamaro et al., 2009). A recent study by Tavares and coworkers found a very low prevalence (<0.5%) of MRSA among healthy children aged up to 6 years attending day-care centers in Portugal. Favoring the exclusion of this group as a reservoir of MRSA isolates (Tavares et al., 2010).

Chronic disease was associated with increased risk of *S. aureus* colonization; in fact only one of them had been hospitalized in the previous year, but the five patients had been attending health care facilities connected with their underlying disease. No association between MRSA carriage and variables currently recognized as risk factors for infection could be found, perhaps because the number of MRSA carriers was not enough for detecting such an expected association.

The recognition of MRSA in animals has raised concern over their role as potential reservoirs or vectors for human MRSA infection in the community (Loeffler and Lloyd, 2010); however, in this study, MRSA was not detected in domestic animals of MRSA colonized children, suggesting a low probability of their participation in the spread of these strains.

The proportion of children colonized by MRSA who carried PFGE type A (8/14) indicates that the main clone causing community-acquired MRSA infections in different regions of Argentina, (CAA clone) is also widely distributed in colonization, suggesting high epidemicity, perhaps associated to an enhanced capacity of these subtypes to survive on human skin and mucosa. Further surveillance in the region will be required to determine whether colonization by these strains is becoming prevalent.

We noticed that subtype A_2 was distributed in three different institutions, but all subtype A_1 isolates were recovered from children attending the same class in the same institution. This finding suggests a potential epidemiological link for subtype A_1 isolates. This CAA clone variant circulating among healthy children represents a latent risk, considering the high epidemic potential and virulence attributed to this clone. A completely different scenario occurs in the USA, where the main clones (USA300 and MW2) responsible for causing infection in that region, are not prevalent in colonization (Gorwitz et al., 2008).

Our approach to define subtypes was based in currently used typing procedures (macrorestricton, spa typing, and MLST); however new sequence-based approaches provide a complete inventory of microevolutionary changes producing higher-resolution phylogenetic analyses. The knowledge about the evolution of MRSA is increasing through investigations implementing genome-wide single-nucleotide polymorphisms (SNPs). The general perception that MRSA epidemic clones have spread globally was challenged by strong evidence provided by genome based population analysis. A frequent emergence and a limited geographic dispersal of MRSA was proposed within ST5 by Nübel and coworkers (Nübel et al., 2008). Furthermore, Harris et al. (2010), by analyzing whole-genome in ST239 lineage proposed a geographic structure, including a limited number of successful intercontinental transmission events.

None of the children colonized by MRSA had a documented infection up to a year after the time of this study according to surveillance data given by healthcare workers. Parents of MRSA colonized children were informed about potential risks for MRSA carriage and instructed to report this colonization in the case of eventual hospitalization.

Limitations of this work should be mentioned. Firstly, the survey design was cross-sectional. Therefore, patients who were only intermittently colonized may not have been detected, and in addition, seasonal variation in *S. aureus* colonization could not be explored. Finally, the small number of MRSA strains isolated in this study resulted in insufficient statistical power for assessing association with recognized risk factors for MRSA infection.

Our findings add more evidence regarding transmission among healthy children attending kindergarten, which may contribute to the spread of this pathogen in the community. Continued monitoring of the ecology of colonization patterns among children will be essential to improve our understanding of risk for disease due to *S. aureus* and MRSA in the community.

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