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Research Paper

Community-associated methicillin-resistant *Staphylococcus aureus* in non-outbreak skin infections

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Abstract

The aim of this study was to determine the prevalence of *Staphylococcus aureus* and risk factors for the acquisition of MRSA (Methicillin Resistant *Staphylococcus aureus*) as the main cause of skin and soft tissue infections. *S. aureus* were characterized for the presence of PVL, TSST-1 and *mec*A genes. SCC*mec* typing was carried out in *mec*A positive strains and PFGE was performed only in these strains. During the study period, 127 outpatients attending a dermatology clinical the Botucatu Medical School, a regional tertiary hospital in Botucatu, Sao Paulo, Brazil, were diagnosed with active skin infections. A total 66 (56.9%) *S. aureus* strains were isolated. The methicillin resistance gene *mec*A was detected in seven (10.6%) *S. aureus* strains. The SCC*mec* types detected in the seven *mec*A-positive *S. aureus* strains were type Ia in one, type II in three, and type IV in three. The PVL gene was detected in 10 (15.1%) in sensitive strains. Pulsed field gel electrophoresis revealed non-clonal diversity among the isolates. The risk factors associated with MRSA acquisition in this study were previous ciprofloxacin use and working in a healthcare environment. The risk factors indicate plausible routes of CA-MRSA transmission among the subjects studied.

Key words: *Staphylococcus aureus*, resistance, skin infections, virulence, epidemiology.

Introduction

Staphylococcus aureus is a versatile pathogen associated with infections in both hospital and community setting (Miranda *et al.*, 2007, Schuenck *et al.*, 2009, D'Agata *et al.*, 2009). Due to its widespread use, treatment with penicillin became ineffective due to the high rate of resistance; consequently, semi-synthetic penicillin, such as methicillin, was developed as the main alternative against penicillin resistant strains. However soon before its introduction, Methicillin-Resistant *S. aureus* (MRSA) strains emerged in hospital environments (Jevons 1961) and then in the community about 20 years later (Gosbell 2004).

The main methicillin resistance mechanism is the production of PBP2a, a transpeptidase involved in bacterial wall synthesis, codified by the *mec*A gene (Chambers 1997) and carried in a mobile element called the staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* is classified according to the presence or absence of specific DNA regions (Turlej *et al.*, 2011). So far, 11 main SCC*mec* types (I to XI, and subtypes) have been described (IWG-SCC). Usually, SCC*mec* types I, II and III are found in hospital environments (Hospital-Associated Methicillin Resistant *S. aureus*) while types IV, V and VI are associated with community settings (Community-Associated Methicillin

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Resistant *S. aureus*). However, according to recent data, a change in the epidemiology of CA-MRSA has been reported (Cook and Brown 2010).

Community-associated methicillin-resistant *S. aureus* (CA-MRSA)cause a broad range of skin and soft tissue infections such as furuncles, boils, carbuncles (Yao *et al.*, 2010), cellulitis, and necrotizing fasciitis (Miller *et al.*, 2005) in patients without traditional risk factors (Campbell *et al.*, 2004). CA-MRSA strains usually carry the Panton Valentine leukocidin (PVL) gene as the main virulence factor. PVL is a two-component pore-forming cytotoxin that acts on mitochondria and polymorphonuclear leucocytes wich indirectly leads to epithelial necrosis and invasiveness (Boyle-Vavra and Daum 2007). In addition to reports demonstrating the importance of PVL for MRSA infections (David *et al.*, 2008; Goering *et al.*, 2008; Camargo *et al.*, 2013), methicillin-sensitive *S. aureus* (MSSA) strains have also been shown to carry the PVL gene and to be responsible for severe infections (D'Azevedo *et al.*, 2009; Lee *et al.*, 2010; Kreienbuehl *et al.*, 2011).

The objectives of the present study were to determine the prevalence of MRSA from a population attending an outpatient dermatology clinic, to evaluate associated risk factors, and to determine the virulence profile of *S. aureus* strains.

Material and Methods

Study design

This was a prospective cross-sectional study involving a convenience sample of outpatients presenting skin and/or soft tissue infections. The patients were seen at an emergency and/or outpatient dermatology clinic of the Botucatu Medical School, between September 2008 and September 2009. The patients provided written informed consent to participate in this study. The researcher was trained to interview the patients using a questionnaire to collect the following data: age, gender, residence area (urban or rural), working in a healthcare environment, previous institutional admission in the last 12 months (hospital, clinics, prison, sports team facilities), diabetes diagnosis, medical procedure in the last 12 months (surgery, dialysis, catheterization, drainage), illicit drug intake, sexual orientation, length of infection, antibiotic usage, sports practice, work status and income. We defined working in healthcare environment (healthcare worker) as a professional who directly assists patients.

Inclusion criteria

The patients were screened by the dermatologist during a medical consultation. Patients presenting active infections at the time of consultation who met the inclusion criterion for infections were included in the study. Infections included in this criterion were diagnoses for: boils, cellulitis, erysipelas, abscesses, hidradenitis, folliculitis, diabetes-related infected foot, suppurative osteomyelitis, secondarily infected dermatitis, secondarily infected traumatic lesions, and bullous impetigo. We considered patients who were hospitalized and healthcare workers to be at risk.

Collection and bacterial isolation

A moistened (sterile saline) swab was used to collect the clinical material. The cultures were immediately sent to the laboratory and streaked onto Sheep Blood Agar and Mannitol Salt agar and incubated at 37 °C. *Staphylococcus* spp. colonies were identified based on Gram stain, catalase and coagulase tube production. The isolates were stored in nutrient broth with glycerol at -70 °C.

Detection of methicillin resistance and antimicrobial susceptibility testing

The disk diffusion test was performed using oxacillin (1 μg) and cefoxitin (30 μg) disks as described by the Clinical Laboratory Standards Institute (CLSI 2009). Additionally, we used the *mec*A gene detection as the gold standard for evaluating MRSA. The plates were incubated at 35 °C. Susceptibility to penicillin (10 U), erythromycin (15 μg), clindamycin (2 μg), sulfamethoxazole/trimethoprim (125 μg/23.75 μg), gentamicin (10 μg)and linezolid (30 μg) was tested according to CLSI guidelines. The *mec*A gene was detected by polymerase chain reaction (PCR) (Murakami *et al.*, 1991) after bacterial DNA extraction (Pereira *et al.*, 2009), and a positive result defined MRSA. Reference strains were included in all reactions as positive (*S. aureus* ATCC 33591) and negative (*S. aureus* ATCC 25923) controls for PCR reactions.

Characterization of *S. aureus* SCC*mec*

SCC*mec* typing was performed for *mec*A positive isolates only, using the primers and multiplex PCR parameters described by Milheiriço *et al.* (2007). The following reference strains were used: COL for SCC*mec* type I; N315 for SCC*mec* type Ia; PER34 for SCC*mec* type II; AN546 for SCC*mec* type III; HU25 for SCC*mec* type IIIa, and MW2 for SCC*mec* type IV.

PCR detection of l*ukF*-PV and *lukS*-PV and TSST-1 genes

The genes and parameters standardized by Lina *et al.* (1999) were used for detecting the PVL genes (*lukF*-PV and *lukS*-PV). The toxic shock syndrome toxin 1 (TSST-1)gene was detected as proposed by Johnson (1991) and optimized by Cunha *et al.* (2006). *S. aureus* ATCC 49775 and *S. aureus* ATCC 29213 were used as positive and negative controls, respectively, in all reactions.

PFGE of MRSA isolates

MRSA typing was performed by macrorestriction of genomic DNA of the strains, followed by PFGE according to McDougal *et al.* (2003). The gels were stained with the GelRed system (10,000x in water; Biotium, USA) for 1 h and photographed under UV transillumination. The BioNumerics software, version 6.1 (AppliedMaths, Belgium) was used for similarity analysis, calculation of the Dice correlation coefficient, and construction of a dendrogram by UPGMA (unweighted pair group method using arithmetic averages). Band position tolerance and optimization were set at 1.25% and 0.5%, respectively. A similarity coefficient of 80% was chosen for the determination of clusters (McDougal *et al.*, 2003). CA-MRSA representative clones (USA 500, USA400, USA 100, USA 800, USA 300, HU-25, EMRSA 15, JCSC 4469, OSPC, MR 108, NCTC 10422) were used for comparison in PFGE analysis.

Statistical analysis of risk factors

Risk factors were analyzed according to the demographic data collected (described in study design) from the population studied. The data were analyzed using the Epi Info® 3.5.2 (Centers for Disease Control and Prevention, Atlanta, USA) and SPSS 15.0 programs.

The results were submitted to univariate analysis. Dichotomous variables were analyzed by the chi-squared or Fisher's exact test. The Mann-Whitney test was applied to the analysis of numerical variables and to multivariate analysis using a logistic regression model. p values < 0.05 and > 0.1 were used for inclusion and exclusion of the variables in the model, respectively.

Results

A total of 127 swabs,one per patient,were obtained from patients seen in the emergency department and/or dermatology outpatient clinic during the study period, from which *S. aureus* was isolated in 66 (52%). Coagulase-negative *Staphylococcus* strains were isolated in 42 (33%) patients and the remaining 19 (15%) patients presented negative culture for *Staphylococcus*. *S. aureus* were isolated from secondarily infected dermatitis/secondarily infected traumatic lesions (26 strains, 39.4%), ingrown toenail infection (13 strains, 19.7%), boils (8 strains, 12.1%), bullous impetigo (6 strains, 9.1%), diabetes-related infected feet (4 strains, 6.1%) cellulitis, osteomyelitis (3 strains, 4.5%, each), folliculitis, erysipelas and necrotic tissue (1 strain, 1.5%, each).

Identification of MRSA by the disk diffusion method and *mec*A gene detection

Nine (13.6%) *S. aureus* strains were resistant to oxacillin and eight (12.1%) were resistant to cefoxitin. The *mec*A gene was detected in seven (10.6%) isolates. Of these, six (85.7%) were resistant to oxacillin and six (85.7%) to cefoxitin. On the other hand, among the *mec*A negative strains, 3 presented oxacillin resistance and 2 presented cefoxitin resistance.

SCC*mec* typing of *S. aureus* and susceptibility profile of MRSA

Among the seven *S. aureus* strains carrying the *mec*A gene, one (14.2%) was classified as type Ia, three (42.9%) as type II, and three (42.9%) as type IV. *S. aureus* carrying SCC*mec* type Ia was only resistant to penicillin. The three strains carrying type II were resistant to cefoxitin, clindamycin, erythromycin, oxacillin, and penicillin. The three SCC*mec* type-IV isolates were resistant to cefoxitin, oxacillin and penicillin, but only one strain each (33.3%) was resistant to clindamycin and erythromycin.

Detection of the *lukS*-PV/*lukF*-PV genes and TSST-1 gene in *S. aureus*

The TSST-1 gene was not detected in any of the strains studied. The *lukSF*-PV gene was detected in 10 (15.1%) strains, only in methicillin-sensitive strains. Among the 10 *S. aureus* strains carrying the PVL genes, six (60%) were recovered from furuncles, two (20%) from secondary infections, and two (20%) from impetigo.

PFGE of MRSA

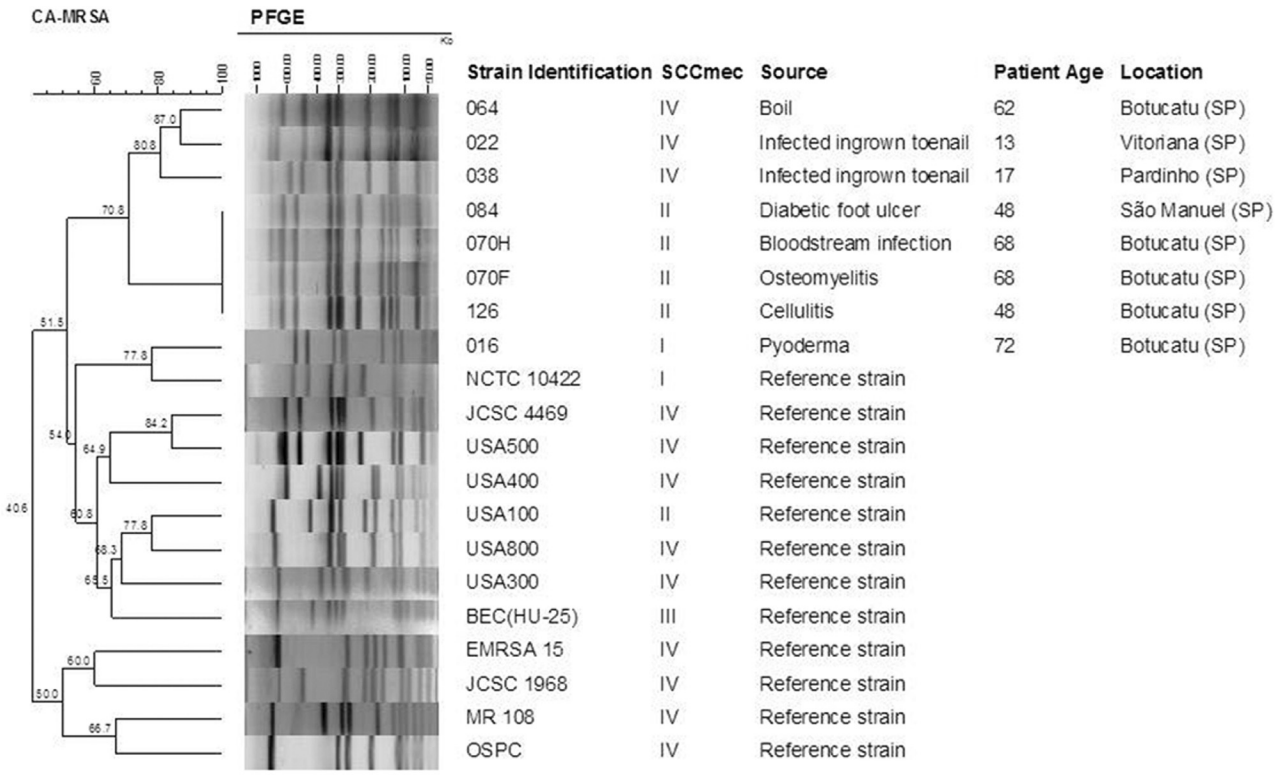
Four isolates containing SCC*mec* II presented the same pulsotype in PFGE, including a bloodstream infection strain (070H) recovered from a patient with suppurative osteomyelites. Strains with SCC*mec* IV presented other band profiles and were grouped in another cluster with similarity of 80.8%. The only SCC*mec* Ia strain was not be allocated to any cluster. None of the evaluated strains were grouped with circulating known clones (Figure 1).

Analysis of the results

One risk factor for MRSA acquisition identified in the present study by univariate analysis was previous use of ciprofloxacin [OR: 8.75 (1.59-48.29), $p = 0.04$]. In addition to ciprofloxacin use, multivariate analysis revealed that working in a healthcare environment [OR:17.5 (1.22-250.36), $p = 0.04$]was also associated with the acquisition of MRSA. However, the latter finding is questionable since only one patient infected with MRSA worked in a healthcare setting (Table 1).

Discussion

In a non-outbreak context of outpatients with skin and soft tissue infection, *S. aureus* was the most frequently isolated *Staphylococcus* species. The presence of *S. aureus* as the main agent of skin and soft tissue infections highlights the importance of identifying this pathogen. The high frequency of isolation of *S. aureus* is a common phenomenon in patients with infections such as cellulitis, impetigo, fu-



Strain Identification	SCCmec	Source	Patient Age	Location
064	IV	Boil	62	Botucatu (SP)
022	IV	Infected ingrown toenail	13	Vitoriana (SP)
038	IV	Infected ingrown toenail	17	Pardinho (SP)
084	II	Diabetic foot ulcer	48	São Manuel (SP)
070H	II	Bloodstream infection	68	Botucatu (SP)
070F	II	Osteomyelitis	68	Botucatu (SP)
126	II	Cellulitis	48	Botucatu (SP)
016	I	Pyoderma	72	Botucatu (SP)
NCTC 10422	I	Reference strain		
JCSC 4469	IV	Reference strain		
USA500	IV	Reference strain		
USA400	IV	Reference strain		
USA100	II	Reference strain		
USA800	IV	Reference strain		
USA300	IV	Reference strain		
BEC(HU-25)	III	Reference strain		
EMRSA 15	IV	Reference strain		
JCSC 1968	IV	Reference strain		
MR 108	IV	Reference strain		
OSPC	IV	Reference strain		

Figure 1 - PFGE analysis of MRSA isolates from skin and soft tissue infections. Three SCC*mec* II samples (070, 064 and 126) had identical profiles, while three SCC*mec* IV samples (064, 022 and 038) grouped with high similarity, and one sample (016) could not be allocated in any group.

runcles, carbuncles, and folliculitis (Stullberg *et al.*, 2002), reaching a frequency of up to 72% (Issartel *et al.*, 2002).

The PVL gene was detected in 15.1% of the *S. aureus* strains studied here. The presence of leukocidin in community-acquired strains has been extensively documented and is associated with methicillin resistance (Francis *et al.*, 2005; Miller *et al.*, 2005). However, in the present study PVL was not detected in methicillin-resistant strains, only in methicillin-sensitive strains. Demir *et al.* (2012) detected the PVL gene in 9% of *S. aureus* strains isolated from skin and soft tissue infections, but in other studies, the gene was absent in MRSA strains (Takizawa *et al.*, 2005; Lee *et al.*, 2010), supporting our findings.

None of the *S. aureus* or MRSA isolates carried the TSST-1 gene. The absence of PVL and TSST-1 gene in CA-MRSA strains isolated from skin and soft tissue infections was also verified in another study (Lee *et al.*, 2010) suggesting involvement of other pathogenicity factors in such isolates.

The *mec*A gene was detected in seven strains isolated from skin infections of outpatients. SCC*mec* typing revealed the presence of types Ia, II and IV. A higher prevalence of types II and IV was also reported by Kikuta *et al.* (2011) in Japanese children with impetigo. In Brazil, detection of SCC*mec* type II was previously shown to be associated with hospital settings (Miranda *et al.*, 2007; Schuenck *et al.*, 2009). However, our findings indicate that patients who presented MRSA with this SCC*mec* type did not present an association with hospital environments nor with the healthcare worker included in our analysis.

The presence of such diversity types of SCC*mec* could be explained by the patient origin. They came from a community previously seen in an ambulatory care center, suggesting that this may constitute an intermediate zone. This zone may have the most variable types of SCC*mec*, characterizing by the prevalence and likely a coexistence of both CA- and HA-MRSA (Kouyos *et al.*, 2013).

In a Brazilian study published in 2005, Ribeiro *et al.*, described the first cases of CA-MRSA infections in Brazil. The MRSA isolates were collected from two patients presenting skin and soft tissue infections, and from another patient presenting septic arthritis. The three patients were from the community and had no risk factors for MRSA acquisition, such as the presence of medical devices, hospitalization or surgery in the last 12 months before MRSA isolation. Due to these characteristics, the origin of infection was considered community acquired. The samples were obtained between June 2002 and September 2003 in the outpatient clinic from two different hospitals in Porto Alegre, Rio Grande do Sul, Brasil. All samples harbored the SCC*mec* IV and presented identical profiles compared to the Ocean Southeast Pacific Clone (OSPC). In another study, Ribeiro and cols (2007) characterized CA-MRSA isolated in Rio de Janeiro and Porto Alegre. Besides the

Table 1 - Statistical analysis ofdemographic data. Univariate and Multivariate analysis reveal the previous use of ciprofloxacin as a risk factor for CA-MRSA acquisition, and multivariate analysis showed that healthcare work was a risk factor. The risk factor in univariate analysis is highlighted in red and the values (number of cases, non-cases, OR and p values) are in bold. We highlighted in yellow the additional risk factors according to multivatiate analysis.

Risk factor	Univariate analysis				Multivariate analysis	
	Cases (n = 7)	Non-cases (n = 119)	OR (95%CI)	p	OR (95%CI)	p
Demographic data						
Female gender	6 (89.7)	57 (47.9)	6.53 (0.76-55.88)	0.052		
Age in years mean	48 (13-72)	46 (0-82)	...	0.64		
Urban residence	7 (100.0)	107 (89.9)	...	1.00		
Working in a rural area	1 (14.3)	16 (13.4)	1.07 (0.12-9.51)	1.00		
Working in a healthcare environment	1 (14.3)	2 (1.7)	9.75 (0.77-123.23)	0.16	**17.5 (1.22-250.36)**	**0.04**
Retired	2 (28.6)	18 (15.1)	2.24 (0.40-12.57)	0.31		
Wage > R$ 1 000.00 per month*	2 (28.6)	35 (29.4)	0.96 (0.18-5.19)	1.00		
Previous hospital admission and procedures						
Previous hospital admission	2 (28.6)	20 (16.8)	1.98 (0.36-10.93)	0.61		
Previous surgery	1 (14.3)	10 (8.4)	1.82 (0.20-16.62)	0.48		
Abscess drainage	0 (0.0)	6 (5.0)	0.0 (...)	1.00		
Underlying comorbidity						
Diabetes mellitus	2 (28.6)	21 (17.6)	1.87 (0.34-10.28)	0.61		
Use of insulin	1 (14.3)	6 (5.0)	3.14 (0.32-30.40)	0.34		
Underlying dermatological disease	0 (0.0)	4 (3.4)	0.0 (...)	1.00		
Characteristics of present infection						
Secondary to local trauma	3 (42.9)	49 (41.2)	1.07 (0.23-5.00)	1.00		
Duration > 30 days	4 (57.1)	56 (47.1)	1.50 (0.32-6.99)	0.78		
History of antibiotic use						
Any antibiotic	4 (57.1)	40 (33.6)	2.63 (0.56-12.34)	0.24		
Penicillin benzathine	0	2 (1.7)	0.0 (...)	1.00		
Amoxicillin	2 (18.6)	16 (13.4)	2.58 (0.46-14.14)	0.26		
Cephalexin	0 (0.0)	26 (21.4)	0.0 (...)	0.34		
Clindamycin	2 (28.6)	11 (9.2)	3.93 (0.68-22.67)	0.15		
Ciprofloxacin	**3 (42.9)**	**12 (10.1)**	**6.69 (1.34-33.41)**	**0.04**	**8.75 (1.59-48.29)**	**0.01**
Macrolides	0 (0.0)	3 (2.5)	0.0 (...)	1.00		
Other						
Participation in sports	0 (0.0)	17 (14.3)	0.0 (...)	0.59		
Use of illicit drugs	0 (0.0)	12 (10.1)	0.0 (...)	1.00		

Results are numbers (percentage), unless otherwise stated.
*Approximately US$ 500.00 per month.
OR, odds ratio; CI, confidence interval.

OSPC clone, they have found the USA300 and USA400 (Ribeiro *et al.*, 2007). Other Brazilian studies verified that most samples carrying SCC*mec* IV presented identical profiles as the pediatric clone/USA800 (Miranda *et al.*, 2007; Scribel *et al.*, 2009; Carmo *et al.*, 2011) and OSPC (Scribel *et al.*, 2009) and strains highly associated with the USA400 clone (Schuenck *et al.*, 2009). In Sao Paulo, Brazil, a new ST1176 strain emerged from a mutation in the yqiL locus, probably originating from the pediatric clone (Carmo *et al.*, 2011). In our study the strains harboring the SCC*mec* IV were highly associated with each other, but lacked association with the most common clones by PFGE. In a study conducted in Europe, a large number of strains could not be related to previously known clones, a finding that might be explained by the wide diversity of CA-MRSA strains (Rolo *et al.*, 2012).

The SCC*mec* II strains presented identical PFGE profiles. The strain SCC*mec* II isolated from one patient (patient number 070), that was isolated from osteomyelitis and bloodstream infection as well. It seems to be an invasive strain that deserves further characterization. The most con-

cerning fact is its high similarity and presence in patients not related to each other.

Risk factor analysis revealed that patients carrying MRSA had a history of ciprofloxacin use and worked in a healthcare environment. The use of fluoroquinolones, particularly ciprofloxacin, has been shown to contribute to colonization with MRSA. *S. aureus* is highly resistant to fluoroquinolones while in sub-inhibitory concentrations can increase the adhesin expression (Bisognano *et al.*, 2004, 1997) thus leading to the success on MRSA colonization.

Healthcare workers play an important role in the dissemination of CA-MRSA in hospital settings and have been responsible for hospital outbreaks(Maltezou *et al.*, 2009, Tang *et al.*, 2007). Healthcare workers are prone to contracting community-associated resistant strains and represent an important source of infection with CA-MRSA for patients (Alia *et al.*, 2012; Maltezou *et al.*, 2009; Wagenlehner *et al.*, 2008; Tang *et al.;* 2007; Raab *et al.*, 2006). In our study, we verified that healthcare working was a risk factor for acquiring MRSA. However, this is a matter that needs to be validated by more specific studies and careful analysis in this regard.

In conclusion, we verified a low prevalence of MRSA in outpatients with skin infections, with strains not related with known MRSA clones, indicating a possible geographic clonal pattern. Risk factors indicate plausible routes of transmission since the use of ciprofloxacin and working in a healthcare environment are associated with the persistence of strains and dynamics of pathogen transmission. These findings have significant importance in CA-MRSA epidemiology.

Conflict of interest

The authors state no conflict of interest.

Acknowledgments

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