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Prevalence and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Atlantic Azores islands: predominance of SCCmec types IV, V and VI

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Abstract In order to obtain insights into the methicillin-resistant *Staphylococcus aureus* (MRSA) population structure in the Azores archipelago, 106 MRSA isolates were collected from patients attending an Azorean central hospital between January 2007 and February 2008. Antimicrobial resistance was determined for all isolates. Molecular typing was performed by pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST), staphylococcal chromosome cassette *mec* (SCCmec) typing and the presence of Pantone–Valentine leukocidin (PVL). The majority of the isolates (87%, $n=92$) belonged to the EMRSA-15 clone (ST22, SCCmec-IVh), followed by the Pediatric clone (ST5-VI/IVc) (11%, $n=12$). The Berlin clone (ST45-IVa) and a new clone (*spa* type t1839, ST1339 and SCCmec V variant) were represented by single isolates. All of the isolates carried SCCmec types IV, V or VI and a non-multiresistant antibiotic profile, resembling the currently emerging community MRSA. Moreover, PVL was described for the first time to be associated with the

Pediatric clone carrying SCCmec type VI. We provided the first description of the population structure of MRSA in the Azores islands, which seems to be shaped by genetic events occurring locally, as well as by the regular population exchange between the islands, continental Portugal, the United Kingdom and the United States.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen associated with both nosocomial- and community-acquired infections (hospital-acquired [HA] MRSA and CA-MRSA, respectively) [1]. MRSA are believed to have emerged by the acquisition of the staphylococcal cassette chromosome *mec* (SCCmec), the mobile genetic element carrying the determinant of methicillin resistance (*mecA*) into methicillin-susceptible *S. aureus* (MSSA). Eight different SCCmec types (I to VIII) have been described so far in *S. aureus*, differing in their structure and size. Epidemiological studies using molecular typing methods showed that the massive geographical spread of MRSA in hospitals results from the dissemination of a few highly epidemic clones. These major epidemic clones resulted from a limited number of independent acquisitions of SCCmec into well-defined genetic backgrounds: (i) New York/Japan clone, sequence type (ST) 5, SCCmec II; (ii) Pediatric clone, ST5-IV/VI; (iii) Brazilian clone, ST239-III/IIIA; (iv) Iberian clone, ST247-IA; (v) EMRSA-15, ST22-IV; (vi) EMRSA-16, ST36-II; and (vii) Berlin clone, ST45-IV [2–4].

The MRSA prevalence in European hospitals varies from less than 3.0% in the Nordic Countries and The Netherlands to over 50% in southern European countries and the United Kingdom. Despite the significant decrease in MRSA incidence in a few countries (France, Slovenia,

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Belgium, Cyprus and Turkey) due to improved infection control, the incidence has increased in other countries, including Portugal, where the MRSA proportion is, nowadays, over 50%, being one of the highest in Europe [1, 5].

Surveillance studies have been conducted in Portugal since the early 1990s, showing temporal waves of MRSA clonal prevalence. In the most recent survey conducted in 2006, EMRSA-15 (ST22-IVh) was found to be the most prevalent clone in Portuguese hospitals, followed by the New York/Japan clone (ST5-II) [5, 6].

Information about the epidemiology of CA-MRSA is still scarce and non-existent in Portugal. Unlike HA-MRSA, CA-MRSA are usually only resistant to beta-lactams and one or two additional antimicrobial classes, and carry mainly SCC*mec* types IV and V, the smallest in size, which are believed to be more mobile. CA-MRSA frequently harbour Panton–Valentine leukocidin (PVL), but the toxin has also been detected in the hospital environment [7, 8]. Similarly to HA-MRSA, CA-MRSA epidemic clones have been described [9]. However, a much larger genetic diversity in the genetic backgrounds of CA-MRSA has been observed, suggesting a high number of SCC*mec* acquisitions in the community. Recent data indicates that MRSA epidemiology is suffering a new change and that the boundaries between the hospital and the community are blurring. As a result, clones traditionally linked to the hospital, like the EMRSA-15 clone, were found to be aetiologic agents of infection in the community [10] and clones specific to the community were observed as causing infections in hospitals, as is the case of the USA300 strain [11, 12]. A common trait to these clones that can survive in both environments appears to be the presence of SCC*mec* type IV that is, nowadays, prevalent among the current overall MRSA population [5, 6, 13–15].

Whereas the epidemiology of nosocomial MRSA has been very well characterised in the Portuguese continental territory, there is no information concerning the prevalence and clonality of MRSA in the Portuguese islands, namely in the Azores archipelago.

The aim of the present study was to identify the MRSA clonal types currently circulating in the Azores archipelago and compare them with the major MRSA clones described in continental Portugal, Europe and the United States.

Materials and methods

Hospital setting

Hospital do Divino Espírito Santo (HDES) is a 390-bed central hospital located in Ponta Delgada, São Miguel island, that services an outpatient population of 138,000 inhabitants, from the two most populous Azorean islands, S. Miguel and Santa Maria. The hospital, the largest in the

archipelago, includes medicine and surgery as principal wards, and a large outpatient ambulatory unit.

Bacterial isolates

Between January 2007 and February 2008, a total of 106 MRSA isolates were collected from both inpatients ($n=93$, 87.7% of the isolates) and outpatients ($n=13$, 12.3% of the isolates) attending HDES. The collection included single-patient isolates recovered from several sources, including sputum ($n=35$, 33%), urine ($n=22$, 21%), swabs from various origins ($n=19$, 18%), exudates from wounds, one abscess and one ear infection ($n=16$, 15%), blood ($n=10$, 9.5%), catheters ($n=2$, 1.9%) and auricular and peritoneal fluids ($n=1$, 0.9% each). Most of the isolates (95%, $n=101$) were from infection. The majority of the patients (73.5%) were inpatients hospitalised in three wards: medicine ($n=42$, 39.6%), surgery ($n=24$, 22.6%) and pneumology ($n=12$, 11.3%). Among the 13 outpatients, three attended the haemodialysis unit.

Antimicrobial susceptibility testing and *mecA* detection

Susceptibility testing was performed with the semi-automatic Vitek system (bioMérieux, SA, France) according to the manufacturer's instructions for a panel of 12 antibiotics: ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, oxacillin, penicillin, rifampicin, trimethoprim-sulfamethoxazole, teicoplanin, tetracycline and vancomycin. Bacterial isolates were considered to belong to different antibiotypes if at least one difference was observed in the antibiotic resistance profile.

The presence of the *mecA* gene was confirmed in all oxacillin-resistant isolates by polymerase chain reaction (PCR) [16].

PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as described by Chung et al. [17] on all 106 isolates. The resulting SmaI restriction patterns were analysed by both visual inspection using the criteria of McDougal et al. [18] and automatically with the BioNumerics software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated as previously described [19] using an optimisation of 0.50% and a tolerance of 1.25%. A similarity coefficient of 80% was used to define the PFGE type clusters [19].

spa typing and MLST

spa typing was carried out on at least one representative of each PFGE subtype ($n=26$) and *spa* types were assigned

through the Ridom web server (<http://spaserver.ridom.de>) [20]. Multilocus sequence typing (MLST) was conducted as described [21] for selected strains previously typed by PFGE and *spa* typing. The allelic profiles and sequence types (ST) were defined using the MLST database (<http://www.mlst.net>).

SCC*mec* and *ccr* typing

SCC*mec* was typed by the multiplex PCR strategy described by Milheiriço et al. [22]. Additionally, all type IV isolates were further subtyped as previously described [23]. Type VI isolates were confirmed by amplification of the cassette chromosome recombinase genes *ccrAB4* [24]. Type V was confirmed by PCR amplification of *ccrC* and *mec* complex C, as previously described [16, 25]. Non-typeable isolates by the methods mentioned above were characterised by *ccrB* sequencing [26].

PVL detection

All 106 MRSA isolates were screened for the presence of PVL *lukS/lukF* genes by PCR, as described previously [27]. Positive results were confirmed by sequencing of the PCR amplicon.

Results

Antimicrobial susceptibility

The 106 isolates were assigned to eight different antibiotic types (Table 1) with a prevalent non-multiresistant pattern.

The majority of the isolates ($n=91$, 85.9%) showed antibiotic type 1 (resistance to oxacillin, penicillin, ciprofloxacin and erythromycin) or 2 (resistance to oxacillin, penicillin and ciprofloxacin). The isolate with antibiotic type 7 was unique in showing resistance to gentamicin. Antibiotic type 8, which showed resistance to six different antimicrobials, included a single isolate.

All 106 isolates harboured the *mecA* gene and, therefore, were classified as MRSA.

Predominance of EMRSA-15 clone

The characterisation of the 106 MRSA strains by PFGE, *spa* typing, MLST and SCC*mec* typing clustered the isolates into four clonal types (Fig. 1). The majority of the isolates ($n=92$, 87%) belonged to PFGE type A. PFGE type B included 11% ($n=12$) of the isolates and types C and D were represented by single isolates only (1% each).

PFGE type A was subdivided into nine subtypes; subtypes A1 ($n=40$) and A2 ($n=22$) represented 67% of all type A isolates. The representative isolate of PFGE type A (subtype A1) showed *spa* type t032, ST22 and SCC*mec* type IVh, which are characteristics of the internationally disseminated EMRSA-15 clone [28]. Interestingly, subtype A4 showed the same restriction profile as the EMRSA-15 clone reference strain, HAR22, but a different *spa* type (t032), including one additional repeat sequence (Fig. 1). All representative isolates of type A shared the same *spa* type t032, except one isolate (t910), which differs from t032 by the deletion of five repeats. HDES85 was the unique isolate of PFGE type A1 defined as non-typeable by *spa* typing, since no PCR product was obtained. Another

Table 1 Resistance profile of the 106 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and associated pulsed-field gel electrophoresis (PFGE) types

Antibiotype	Resistance profile ^a												Total no. of isolates (%)	PFGE types (subtypes)
	OXA	PEN	CIP	ERY	CLI	RIF	GEN	LZD	SXT	TEC	TET	VAN		
1	R	R	R	R	S	S	S	S	S	S	S	S	67 (63.2)	A (1–9); B (1, 2); D (1)
2	R	R	R	S	S	S	S	S	S	S	S	S	24 (22.7)	A (1–6); B (1, 4); C (1)
3	R	R	S	R	S	S	S	S	S	S	S	S	7 (6.6)	A (1, 2, 5); B (1)
4	R	R	R	R	R	S	S	S	S	S	S	S	3 (2.8)	A (1)
5	R	R	S	S	S	S	S	S	S	S	S	S	2 (1.9)	A (4); B (3)
6	R	R	R	S	S	R	S	S	S	S	S	S	1 (0.9)	A (1)
7	R	R	S	S	I	R	R	S	S	S	S	S	1 (0.9)	B (1)
8	R	R	R	R	R	R	S	S	S	S	S	S	1 (0.9)	B (1)

^a S, susceptible; I, intermediate; R, resistant

Antibiotic abbreviations: OXA, oxacillin; PEN, penicillin; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; RIF, rifampicin; GEN, gentamicin; LZD, linezolid; SXT, trimethoprim-sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin

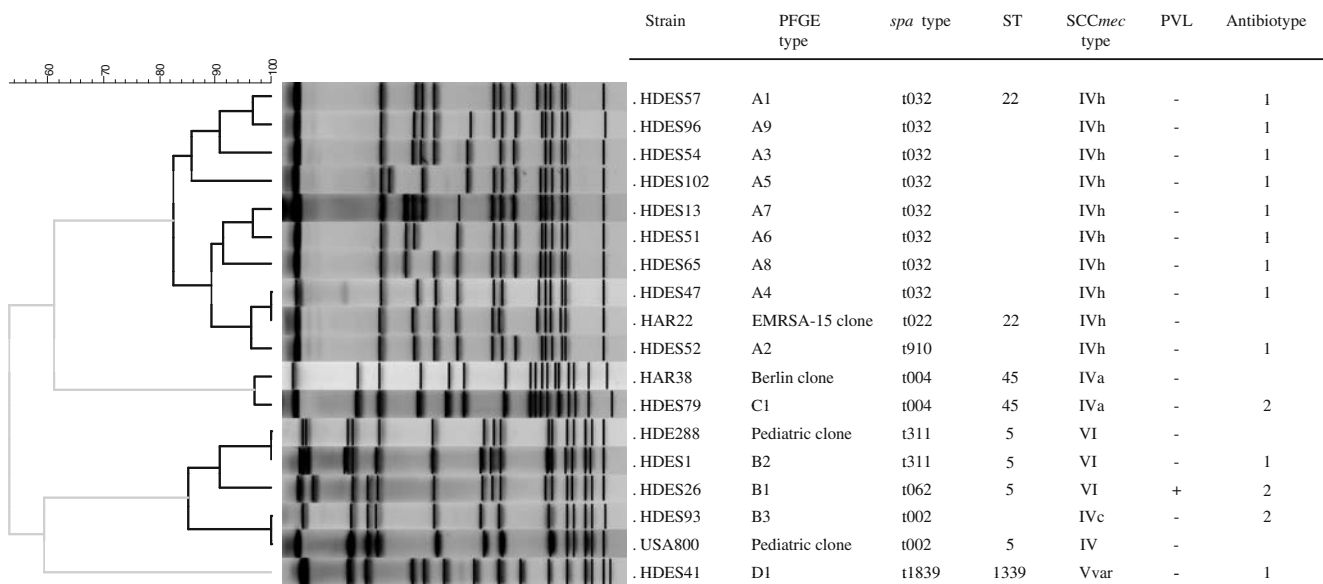


Fig. 1 Molecular characterisation of representative methicillin-resistant *Staphylococcus aureus* (MRSA) isolates of each pulsed-field gel electrophoresis (PFGE) subtype and comparison with MRSA pandemic clones

single type A1 isolate, HDES73, showed a SCC*mec* cassette type IV non-subtypeable. PFGE type A included isolates from six out of the eight antibiotypes described, but 95% ($n=53$) of the isolates belonged to antibiotypes 1 and 2 (Table 1).

Evidence of horizontal gene transfer among strains of the Pediatric clone

PFGE type B was the second most common PFGE type and included three subtypes, of which B1 represented 75% ($n=9$) of type B isolates (Fig. 1). Subtypes B1 and B2 showed ST5, SCC*mec* VI and *spa* types t062, t2049, t2724 (B1)/t311 (B2), which are typical of the Pediatric clone. SCC*mec* type VI was confirmed in five isolates by amplification of the characteristic *ccrAB4* genes. The remaining type VI isolates did not amplify the *ccrAB4* genes by uniplex PCR but showed a *ccrB* sequence with 100% homology with the Pediatric clone reference strain HDE288 [29]. Interestingly, subtype B3 carried SCC*mec* type IVc instead of the SCC*mec* type VI, which may indicate the occurrence of independent acquisition of SCC*mec* in the Pediatric clone background.

Moreover, we found that three isolates belonging to PFGE subtype B1 (ST5-VI) carried the PVL genes.

The remaining two isolates belonged to PFGE types C and D and were, respectively, recovered from an outpatient and an inpatient. PFGE C isolate, *spa* type t004 and ST45-IVa showed a single band difference relative to HAR38, the prototype isolate of the Berlin clone (*spa* type t004, ST45-IVa) [30, 31]. PFGE type D isolate showed *spa* type t1839, a novel sequence type (ST1339) not related to any major clonal complex (CC) and a variant of SCC*mec* type V.

High genetic diversity and high frequency of SCC*mec* types IV, V and VI

SCC*mec* type IV was found to be the prevalent type ($n = 13$) among the 24 isolates tested. Three different SCC*mec* IV subtypes were found in this collection, associated to three unrelated genetic backgrounds (ST5: SCC*mec* IVc, ST22: SCC*mec* IVh/IVnon-subtypeable, and ST45: SCC*mec* IVa). SCC*mec* type VI was the second most prevalent cassette type, which appeared to be associated to isolates with PFGE types B1 and B2. A variant of SCC*mec* type V was shown on a single isolate that harboured the *mec* complex C and *ccrC* genes, but missed the specific type V J1 region. No isolates carrying SCC*mec* types I, II or III were identified in this collection.

Discussion

In order to describe the MRSA population structure in the Atlantic Azores islands, a collection of 106 isolates recovered during a 13-month period was characterised by a combination of up-to-date molecular typing methods. The analysis showed the predominance of two major international MRSA clones: the EMRSA-15 clone and the Pediatric clone.

Massive dominance of the EMRSA-15 clone

In the present study, 87% of the isolates showed molecular characteristics of the highly internationally disseminated EMRSA-15 clone (ST22-IVh) [28], including a common

spa type t032. In addition, a single PFGE type A2 isolate showed t910, a *spa* type already reported in a CC22 isolate from Germany [32]. This fact is not surprising, since the *spa* gene is a variable repeat region prone to the introduction or deletion of repeats. Regional *spa* clusters frequently include highly related *spa* types characterising clonal related isolates, which translates a local evolution of the *spa* locus [8, 33]. Unusually, a non-typeable isolate was found in the collection, which should be due to a mutation or insertion/deletion in *spa* rather than non-existence of the gene [34]. Interestingly, the majority of the EMRSA-15 isolates described in this study were recovered from sources other than blood (sputum, swabs, exudates and urine), corresponding to the major pathologies presented by the patients attending HDES (respiratory tract and skin related). The non-multiresistant EMRSA-15 clone emerged in the United Kingdom in 1991 and rapidly became a dominant clone worldwide, particularly in Europe [8, 35]. Recently, an EMRSA-15 variant was reported in central United States [36]. In the Azores islands, the clear dominance of the EMRSA-15 clone is, in part, in agreement with the situation in continental Portugal [5, 6]. In fact, in a study involving isolates recovered in the same period from 11 hospitals scattered over continental Portugal, EMRSA-15 was found to be the most prevalent clone. However, and in contrast with the present study, the New York/Japan clone (ST5-II) was shown to represent a new wave of MRSA in the country, whereas the Pediatric clone (ST5-VI/IV) was exceedingly rare [5]. In the Portuguese neighbouring country Spain, EMRSA-15 was found in continental territory in co-existence with EMRSA-16 and two CC5 clones (ST125-IV and ST146-IV), but not as a major clone as in the Spanish islands (Majorca and Canarias) and as in our study [14, 37, 38]. The frequent touristic flux from the United Kingdom, hypothesised as the cause of the importation of EMRSA-15 to the Mediterranean island of Malta [39], could also support, in part, the emergence of this clone in the most touristic Azores islands. In parallel, the regular exchange of inhabitants between the islands and the continent seem to be an important vehicle of clonal importation.

Emergence of PVL and *SCCmec* variability: evidence for local evolution

Recent studies revealed that the Pediatric MRSA clone, isolated for the first time in 1992 in a Portuguese hospital [29], was no longer present in that hospital in 2006 [5]. However, it appeared in the present collection as the second major clone.

Interestingly, in this study, we observed that strains of the Pediatric clone differing in three to five bands in PFGE pattern, isolated in the same hospital, carried either the

typical *SCCmec* types VI or IVc. ST5-VI MRSA isolates, which had been restricted to isolates from continental Portugal, were recently found in a collection of nosocomial isolates recovered from 23 hospitals located throughout France [24, 40]. The hypothesis of a recent introduction of this clone from continental Portugal seems to be quite remote, since, in a recent national study, this clone was isolated from a single patient only in a hospital in the South of Portugal [5]. On the other hand, the Azores islands have a high emigrant community in the United States, who frequently travel to the national territory, constituting also possible vehicles of dissemination of the Pediatric clone strains already reported in this country.

ST5 lineage is known to frequently acquire mobile genetic elements, such as different *SCCmec*, antibiotic resistance and toxin genes [4, 41]. In a recent study, Nübel et al. showed that at least 23 *SCCmec* acquisitions events have occurred within ST5 lineage, on multiple and independent occasions, showing evidence that the MRSA emerged in numerous circumstances and in distinct locations rather than by the geographic dispersal of isolates [4]. Moreover, besides being originally identified in a community-associated USA400 strain, the first introduction of *SCCmec*IV in *S. aureus* might have occurred in the ST5 Pediatric clone background that was circulating in hospitals in the 1990s [9]. Similarly, our results suggest that *SCCmec* cassettes VI and IVc might have been introduced de novo and recently in these ST5 strains and that the Pediatric clone may be evolving locally. Another observation that supports the hypothesis of recent local evolution of the Pediatric clone is the presence of PVL-positive and PVL-negative strains with closely related PFGE profiles. Noteworthy, this is the first report of PVL among isolates belonging to the *SCCmec* type VI Pediatric clone. The introduction of the PVL genes in a genetic background with such a high epidemic potential is of particular concern. However, we cannot exclude the reverse hypothesis of a local evolution through the acquisition of *SCCmec* type VI by a PVL-positive MSSA. Only the analysis of the genetic background of susceptible *S. aureus* in the Azores islands would clarify the chronological acquisition of PVL and *SCCmec* in these Pediatric clone strains.

The Berlin MRSA clone (ST45-IVa), first observed in Berlin hospitals in 1993, is currently spread in some European countries [8, 42], showing a high epidemic potential not only in the hospital environment but also in the community [43, 44]. This clone was found in a single MRSA isolate in the Azores island collection. The same genetic background (ST45) had been previously described in Portuguese hospitals, but exclusively among MSSA isolates [45]. The collection of a single MRSA isolate carrying *SCCmec* IVa suggests another de novo *SCCmec* acquisition, probably in an already established MSSA clone.

Likewise, the introduction of a variant of SCCmec type V seems to have occurred in an isolate with *spa* type t1839 and a novel sequence type (ST1339) that is a singleton. This strain is not related to the single SCCmec type V variant isolate (ST45) previously reported in Portugal [5]. Interestingly, this isolate was recovered from a patient within the first 48 h of hospitalisation, from a skin and soft tissue infection (erysipelas), the main source of CA-MRSA isolates. However, the isolate could not be considered as having a community origin, since no additional information regarding patient risk factors was available.

Emergence of HA-MRSA isolates with community traits

SCCmec type IV is one of the most frequent SCCmec types found in the community and is, nowadays, becoming the predominant type also in the nosocomial setting [5, 6, 9, 13–15, 46]. Moreover, this SCCmec type has been usually associated to a pattern of susceptibility to almost all antimicrobial classes. These same characteristics were observed in isolates from our study: SCCmec type IV was found to be the most frequent SCCmec type and all isolates were highly susceptible. At least three different SCCmec subtypes were identified (IVh: EMRSA-15, IVc: Pediatric clone, IVa: Berlin clone), showing evidence of a high genetic diversity among the pool of SCCmec type IV in the Azores islands. Interestingly, the detection of one isolate belonging to the EMRSA-15 clone, SCCmec type IV non-subtypeable (only positive for the *ccrB2* allele in the subtyping multiplex), suggests the presence of a new uncharacterised SCCmec type IV subtype in addition to the ones already described [23].

Besides SCCmec type IV, the only SCCmec types found among the whole collection were the small SCCmec V and VI, reflecting the actual tendency for the dominance of low resistant and fitness cost clones within the hospital setting, which is parallel with what is observed in the community environment [9]. The introduction of well-established community isolates into the hospital setting is not new, as observed in Taiwan, where the ST59 clonal type, highly prevalent in the community, emerged in the hospital setting as the second most prevalent clone [38, 47, 48]. Although ST22-IV EMRSA-15-related isolates as well as ST5-IV were already reported in the community [10, 46], no conclusions can be drawn for the flow direction in the present study. Even if a high percentage (30%) of the isolates studied was recovered within the first 48 h of admission or in an outpatient unit, no data of previous hospital contact was available for the patients.

In the present study, we provided the first description of the population structure of MRSA in the Azores islands and showed a prevalence of the epidemic EMRSA-15 clone in co-existence with the Pediatric clone. Our findings were in

line with recent reports from continental Portuguese and European hospitals. The horizontal gene transfer and the de novo acquisition of mobile genetic elements seem to have contributed to the local genetic diversity and clonal evolution. Moreover, the population exchange between the islands and continental Portugal or the United States, in addition to the high flux of tourists, particularly from the United Kingdom, may explain the dominance and spread of the EMRSA-15 and Pediatric clones. Further surveillance studies concerning both HA-MRSA and MSSA populations and CA-MRSA in the geographically restricted Azores islands will be fundamental to support the *S. aureus* localised evolution, as well as to understand the links between the hospital and the community.

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