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## High prevalence of ST121 in community-associated methicillin-susceptible lineages responsible for skin and soft tissue infections in Portuguese children

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1 **High prevalence of ST121 in community associated methicillin-susceptible**  
2 ***Staphylococcus aureus* lineages responsible for skin and soft tissue infections in**  
3 **Portuguese children**

4

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23 **ABSTRACT**

24 In order to evaluate the incidence of CA-MRSA in Portugal, we analyzed a collection  
25 of 38 *S. aureus* isolates recovered from 30 children attending the paediatric  
26 emergency department of a central hospital in Lisbon due to skin and soft tissue  
27 infections. Molecular characterization identified seven clonal lineages among the 35  
28 MSSA isolates, out of which the major lineage PFGE A/t159/ST121 included 63% of  
29 the isolates. The three MRSA isolates belonged to the Paediatric clone - PFGE  
30 D/t535/ ST5-IV (n = 2) and to the European CA-MRSA clone - PFGE G/ t044/ ST80-  
31 IVc. All isolates harboured several virulence factors, namely leukocidins. PVL was  
32 produced by isolates from five MSSA lineages and by the ST80 MRSA. Of interest  
33 this is the first reported isolation of CA-MRSA ST80 in Portugal.

34 **(127 words, max 200)**

35

36 (Body text: **1386 words**)

37

38 Methicillin resistant *Staphylococcus aureus* (MRSA) infections in the community  
39 have been increasing worldwide and are mainly reported as skin and soft tissues  
40 infections (SSTI) in otherwise healthy young individuals [1-3]. The Centers for  
41 Disease Control and Prevention (CDC) consider a community associated MRSA (CA-  
42 MRSA) infection when the patient has no previous history of MRSA infection or  
43 colonization, surgery, dialysis, hospitalization, residence in a long-term care facility  
44 within the year before infection, presence of a percutaneous device and indwelling  
45 catheter and hospitalization >48 h before MRSA cultures [4, 5].

46 CA-MRSA isolates differ phenotypically and genotypically from hospital-associated  
47 (HA-) MRSA, namely in its non-multiresistant antibiotic patterns, enhanced virulent  
48 gene content, including the acquisition of the necrotizing Pantone Valentine leukocidin  
49 (PVL) genes or expression of  $\alpha$ -type phenol-soluble modulins (PSMs) [1, 3]. The  
50 staphylococcal cassette chromosome *mec* (SCC*mec*) type and the accessory gene  
51 regulator (*agr*) alleles are also differentially distributed: SCC*mec* types IV or V and  
52 *agr* type III are more commonly present in CA-MRSA, while SCC*mec* types I-III and  
53 *agr* type II are more typical of HA-MRSA [1]. In contrast, community-acquired  
54 methicillin susceptible *S. aureus* (CA-MSSA) isolates, responsible for a significant  
55 number of mild SSTI, do not differ from HA-MSSA [6, 7].

56 Although reports of CA-MRSA prevalence are increasing worldwide, there is no  
57 description of the actual scenario in Portugal, a country with >50% of HA-MRSA  
58 infection, currently the highest in Europe [8]. Previous studies dated from 1996 to  
59 2009, including isolates from nasal swabs of young healthy individuals and  
60 nasopharyngeal swabs of children attending day care centres, reported a MRSA

61 prevalence lower than 0.25% in the Portuguese healthy community (0.24% in 1996-  
62 1998 and 0.13% in 2006-2009) [6, 9, 10].

63 It is conceivable that the incidence of CA-MRSA in Portugal is underestimated  
64 partially because skin infection samples are not routinely cultured.

65 The aim of the present study was to assess the prevalence and molecular  
66 characterization of *S. aureus* in children attending the paediatric emergency  
67 department of a central hospital due to SSTI.

68

69 The paediatric emergency department of Hospital Fernando Fonseca, a large tertiary  
70 care hospital (670 beds), is the second largest paediatric urgency unit in the urban area  
71 of Lisbon, Portugal, and receives approximately 180 children per day. Between  
72 August 2005 and October 2006 all children attending this unit due to SSTI were  
73 enrolled in the study. Samples were recovered by swabbing the largest area of skin  
74 infection or wounds with spontaneous or surgical drainage, or in case of severe SSTI  
75 from hemoculture. Whenever possible, nasal swabs were also performed. A  
76 questionnaire was filled in order to assemble data on basic socio-demographic patient  
77 information, risk factors associated with skin infection, and description of the  
78 infection.

79 *S. aureus* isolates were first identified by conventional coagulase and catalase tests.  
80 Additional identification and susceptibility testing with a panel of 10 antibiotics (Fig.  
81 1) were performed by the semi-automatic VITEK2 system (bioMérieux, SA, France)  
82 and disk diffusion method for clindamycin [11]. All isolates were tested by PCR for  
83 the presence of *mecA* [12] and also characterized by PFGE [13]. The resulting SmaI

84 patterns were analyzed by both visual inspection and automatically with BioNumerics  
85 software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium) [14, 15].  
86 Characterization by *spa* typing, multilocus sequence typing (MLST) and the *agr* allele  
87 type were performed on representative isolates of each PFGE type [16-18]. The  
88 *SCCmec* was typed for all MRSA isolates [19, 20]. Specific staphylococcal virulence  
89 determinants, including leukocidins, hemolysins and super-antigenic toxins of each  
90 isolate were determined by PCR [21, 22] (Fig. 2). Categorical variables were  
91 compared using the  $\chi^2$  or the Fisher's exact test when appropriated, considering *P*  
92 values of  $\leq 0.05$  statistically significant, and Odds Ratio (OD) estimates using SPSS  
93 package version 11.5 (SPSS Inc., Chicago, USA).

94 During the 15-months study period, 30 (73.2%) out of the 41 children that attended  
95 the emergency department due to SSTI were infected by *S. aureus*. The major clinical  
96 infection presentations were cutaneous abscesses ( $n = 12$ , 40%) and cellulitis ( $n = 10$ ,  
97 33.33%). A total of 38 isolates was recovered, out of which three (7.9%) were MRSA.  
98 Although eight clonal types (Table 1) were identified among the isolates, 63% ( $n =$   
99 19) belonged to a single type: PFGE A, *spa* type t159, sequence type (ST) 121, *agr*  
100 type IV. ST121 lineage was also the predominant lineage found in MSSA isolates  
101 through Europe and Russia [23-25] and an identical scenario has been reported among  
102 Asiatic children in both carriage and disease isolates, and frequently associated to  
103 PVL. Of major concern was the local emergence of methicillin resistance in ST121  
104 isolates in these paediatric communities: one MRSA isolate, PVL negative  
105 responsible for staphylococcal scalded skin syndrome in China and two MRSA, PVL  
106 positive, *SCCmec* type V isolates responsible for osteomyelitis and soft tissue abscess,  
107 in Cambodia [26, 27]. Moreover, single ST121 MRSA isolates were reported in China  
108 (<http://saureus.mlst.net/>) and the United States [28].

109 Retrospectively, all STs found in the present study (with the exception of ST152)  
110 have already been described in carriage in the Portuguese community in 1996,  
111 although with a different prevalence [6]. Despite the small size of the collection, the  
112 major CA-MSSA clones described in Europe, ST1, ST5, ST30 and ST45 [29], were  
113 found in the paediatric Portuguese community (Table 1). Moreover, all clonal  
114 lineages, with the exception of PFGE H/t084/ST582/*agrIV*, were recently reported  
115 among a geographical and temporal diverse collection of 211 PVL-positive CA-  
116 MSSA [23]. ST1/t127, the sixth more frequent type among MSSA isolates recovered  
117 in 26 European countries [29] was also found in the present collection. In the same  
118 work, Grundmann et al. showed that European MSSA isolates belonged to more  
119 diverse genetic backgrounds and have a wide geographical distribution comparing to  
120 MRSA, which have a predominantly regional spread of few pandemic clones [29].

121 A non-multiresistant antibiotic pattern is common in the present collection (Fig. 1).  
122 Clindamycin and trimethoprim-sulfamethoxazole (SXT) are rational empiric choices  
123 for mild-to-moderate CA-MRSA infections [30]. However, positive D-test in  
124 erythromycin resistant isolates indicates that clindamycin resistance may emerge  
125 during therapy and therefore should not be prescribed [31, 32]. The detection of  
126 clindamycin inducible resistance in all erythromycin resistant isolates (n=10) raises  
127 some concerns about the antibiotherapy available for children treatment, namely since  
128 resistance to STX was found in three isolates from children with no previous  
129 hospitalization.

130 As far as we know, the single tetracycline resistant strain, HFF189, was the first  
131 MRSA ST80 (known as the European CA-MRSA clone), PVL-positive described in  
132 Portugal. Nevertheless, since it was recovered from a periumbilical exudate of a  
133 neonate, the connection to the nosocomial setting could not be discarded, as ST80

134 PVL-positive isolates were already described as nosocomial isolates in the late 1990s  
135 [33]. Regular surveillance studies in Portuguese hospitals [34] together with the  
136 present study seem to indicate that CA-MRSA-ST80 is not widely spread in Portugal  
137 in contrast to what was described in several other European countries [21, 35].

138 Interestingly, the remaining two MRSA isolates, PVL-negative, showed ST5-SCC*mec*  
139 type IV typical of the Paediatric clone, described for the first time in a Portuguese  
140 paediatric hospital [36]. The spread in the community of a typical HA-MRSA lineage  
141 raises some concern about the changing epidemiology of MRSA, and the blurring of  
142 the boundaries between the hospital and the community.

143 All the isolates presented virulence determinants, namely leukocidins (Fig. 2).  
144 Recently, a mechanism of PVL-induced acute lung injury and lung inflammation in  
145 rabbit models resolved the controversial about the role of PVL as a key factor in *S.*  
146 *aureus* infection [37]. In our study, isolates producing PVL or  $\gamma$ -hemolysin were  
147 mainly associated to cutaneous abscesses ( $P = 0.003$  and  $P = 0.000$ , respectively) as  
148 already reported in recent studies in children infections [38, 39]. Conversely,  
149 production of  $\beta$ -hemolysin, ETA or ETB seemed to be associated with cellulitis ( $P =$   
150  $0.010$ ,  $P = 0.003$  and  $P = 0.038$ , respectively) (data not shown).

151 No significant positive association was found between socio-demographic data or  
152 possible risk factors for *S. aureus* infection, possibly due to the small dimension of the  
153 collection (data not shown).

154

155 MSSA infections, independently of the PVL content, frequently show similar  
156 epidemiological and clinical characteristics as MRSA, but specific PVL-positive

157 MSSA lineages, are dynamically interrelated and recently reported as reservoirs of  
158 CA-MRSA [23, 40]. Therefore, a regular surveillance of SSTI, namely in children, is  
159 critical to predict and control the emergence of methicillin resistance and spread of  
160 staphylococcal infections in the community.

161

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166

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330

331 **FIGURE LEGENDS:**

332 **Fig. 1** Antimicrobial susceptibility of the 38 *S. aureus* isolates to a panel of 10  
 333 antibiotics. Abbreviations: S, susceptible; R, resistant; na, not available; OXA,  
 334 oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin;  
 335 ERY, erythromycin; CLI, clindamycin; TET, tetracycline; GEN, gentamicin; SXT,  
 336 trimethoprim-sulfamethoxazole; VAN, vancomycin. All the resistant phenotypes  
 337 observed for clindamycin mean inducible resistance, determined by D-test [11].

338

339 **Fig. 2** Virulence gene determinants of the eight clonal lineages. The totality of the  
 340 isolates was tested for 11 virulence genes. Abbreviations: PVL, Panton Valentine  
 341 leukocidin; LukM, leukocidin M; LukDE, leukocidins D and E;  $\beta$ -hemol,  $\beta$ -  
 342 hemolysin;  $\gamma$ -hemol,  $\gamma$ -hemolysin;  $\gamma$ -hemol var,  $\gamma$ -hemolysin variant; ETA, ETB,  
 343 ETD, exfoliative toxins A, B and D; SEL, SEP, staphylococcal enterotoxins L and P.

**Table 1** Molecular characterization and clinical presentation of the clonal lineages found among the 38 *S. aureus* isolates recovered during the 15-month study period.

No. of isolates	PFGE types (No. of subtypes)	<i>spa</i> type <sup>a</sup>	MLST <sup>b</sup>		SCC <sub>mec</sub> type	PVL	<i>agr</i> type	Related clone <sup>c</sup>	Clinical presentation (No. of
			ST	CC					

									isolates)
19	A (8)	t159	121	121	MSSA	-	IV		cellulitis (9), cutaneous abscess (3), impetigo (1), wound (1), nasal swab (5)
6	B (4)	t318	30	30	MSSA	+	III	South Westh Pacific	cutaneous abcess (6)
3	C (3)	t576	45	45	MSSA	-	I	Berlin	cutaneous abscess (2), celullitis (1)
2	D (2)	t535	5	5	IVc	-	II	Paediatric	impetigo (1), bacteremia (1)
1	D (1)	t311	5	5	MSSA	+	II	Paediatric	cutaneous abscess (1),
3	E (2)	t355	152	152	MSSA	+	I		cutaneous abscess (1), cellulitis (1), adenophlegmon (1)
2	F (2)	t127	1	1	MSSA	+	III		cutaneous abscess (1), pustule (1)
1	G	t044	80	80	IVc	+	III	European	pustule (1)
1	H	t084	582	15	MSSA	-	IV		impetigo (1)

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345 <sup>a</sup> Ridom nomenclature (<http://spaserver.ridom.de/>).346 <sup>b</sup> MLST – multilocus sequence typing, ST – sequence type; CC – clonal complex, defined by  
347 eBurst v3 assessed on 12 April 2010.348 <sup>d</sup> Common designation of community or hospital associated MRSA or MSSA clones.

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