Characterization of *Staphylococcus aureus* small colony variant strains isolated from Italian patients attending a regional cystic fibrosis care centre

Patrizia Morelli[1], Alessandra De Alessandri[1], Graziana Manno[2], Anna Marchese[3], Roberto Bandettini[1], Marta Bassi[1], Raffaele Lobello[1], Laura Minicucci[1,2]

[1]Microbiology Laboratory of Giannina Gaslini Institute, Genoa, Italy;
[2]DINOGMI Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics and Mother-Infant Sciences, Genoa, Italy;
[3]Microbiology Section, DISC, University of Genoa, Genoa, Italy

SUMMARY

Small colony variant (SCV) *Staphylococcus aureus* are a subpopulation of auxotroph, slow-growing strains causing persisting and relapsing infections in cystic fibrosis (CF) patients.
Twenty-eight SCV and 29 normal *S. aureus* strains were isolated from 42 out of 222 Italian CF patients. The isolates were characterized for: susceptibility to antibiotics, methicillin-resistance (MR), Panton Valentine leukocidin, auxotrophy, hypermutability and biofilm formation. Clonal identity of SCV and normal strains was determined by pulsed-field gel electrophoresis. We found that 27 out of 28 SCVs were thymidine-dependent. Furthermore, in contrast to normal phenotype, SCVs were characterized by antibiotic resistance. We also found that 39.3% SCV vs 20.7% normal strains were strong mutators. Moreover, SCVs showed a higher capability to form biofilm compared to normal strains (100% vs 59%). Importantly, we found evidence of clonal spread of SCV strain among CF patients. Using molecular typing, we found that five patients shared the same type A and five out of seven MR-SCV belonged to the same clone (Clone C).
The particular virulence and spreading ability of MR-SCV observed highlights the importance of accurate identification and susceptibility testing of these strains. It is important to adopt the optimal approach to treat patients and to prevent cross-infection in CF centres.

KEY WORDS: Staphylococcus aureus, Small colony variants, hypermutability, Cystic fibrosis, Antibiotic resistance, Biofilm.

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INTRODUCTION

Small colony variant (SCV) is a slow-growing auxotrophic subpopulation of different clinically relevant bacteria, including *Staphylococcus aureus*, which phenotypic and pathogenic traits are distinguishable from those of the wild type strain (Proctor *et al*., 2006). Groups at risk of *S. aureus* SCV infections are typically patients subjected to long-term antibiotic therapy (Melter *et al*., 2010), such as patients with cystic fibrosis (CF), soft-tissue infections, arthritis, sinusitis and osteomyelitis.
CF is a genetic disorder caused by mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator, a transmembrane chloride channel whose functional anomalies result in abnormally thick mucus overstocking in different organs, mainly the digestive tract and lungs. CF lung disease is characterized by chronic bacterial infections and obstructive progressive damage. *S. aureus* and *Haemophilus influenzae* are frequently the first pathogens infecting the lower respiratory tract

Corresponding author
Patrizia Morelli
Giannina Gaslini Institute
Largo G. Gaslini, 5 - 16147 Genoa, Italy
E-mail: patriziamorelli@ospedale-gaslini.ge.it

of CF patients. Some of these patients initially harbour both wild-type and SCV strains. Subsequently they might lose the wild type strain while the SCV persists (Von 2008).

S. aureus SCV is characterised by high resistance rates against the antimicrobial agents routinely used in CF therapy. Reduced susceptibility to aminoglycosides has been related to alterations in the proton motive force on which they depend for drug uptake (Melter *et al.*, 2010). In addition, their ability to survive intracellularly provides a niche where they are protected against host defence mechanisms and antibiotics like β-lactams (Goerke *et al.*, 2012; Besier *et al.*, 2007a). Methicillin-resistant *S. aureus* (MRSA) with SCV phenotype has also been isolated; the combination of this resistance phenotype with the SCV represents a real threat for infected patients (Seifert *et al.*, 1999). A virulence factor often associated with methicillin resistance is an exoprotein defined as Panton Valentine leukocidin (PVL). PVL positive MRSA strains were associated with the development of invasive lung infection including lung abscess in CF patients (Elizur *et al.*, 2007).

Hypermutable SCV strains, characterised by mutation frequencies higher than those found in wild-type bacteria, due to a defective DNA mismatch repair system, have also been isolated from CF patients (Oliver 2012; Besier *et al.*, 2008).

Chronic infection with *S. aureus* is due to the ability of this microorganism to persist within the CF lung. SCV phenotype and biofilm formation are characteristics that play an important role in the persistence of *S. aureus*.

A recent study from Maduka *et al*. has suggested that menadione-dependent SCV strains are more prone to form biofilm in vitro than thymidine-auxotrophic strains (Maduka-Ezeh *et al.*, 2012). This is consistent with the fact that menadione-dependent strains are mainly recovered from osteomyelitis or in device-associated infections, which are often biofilm-related. The situation may however be different *in vivo*, since biofilms are also frequent in CF patients who are more frequently infected by thymidine-dependent SCVs. In this case, the switch to a high biofilm producer SCV phenotype could be induced by the presence of quorum sensing molecules produced by *P. aeruginosa,* which is also present in the respiratory tract of these patients (Mitchell *et al.*, 2010).

In general, studies on *S. aureus* SCV have focused on a single or a pair of virulence factors. This study aims to provide a wide characterization of CF strains' SCV analysing different virulence factors and the relationship between them.

MATERIALS AND METHODS

Bacterial strains and phenotypic identification

CF respiratory samples were cultured as described in Wong *et al.* (Wong *et al.*, 1984). Briefly: after 48h of incubation, non-haemolytic non-pigmented fried-egg colonies on blood agar (BA, Becton Dickinson) and very small pinpoint colonies on mannitol salt agar (MSA, Becton Dickinson) were subbed on BA. After 24 h of incubation at 35°C they were identified as *S. aureus* SCV using catalase test, Slidex Staph Plus latex agglutination (bioMérieux) and Api ID 32 Staph (bioMérieux).

S. aureus strains with stable SCV and normal phenotype were recovered from respiratory specimens of 42 out of 222 patients attending the regional CF care centre at Giannina Gaslini Institute (Genoa, Italy). These 42 patients were chronically colonized (at least 3 consecutive positive cultures) by *S. aureus*. One strain (first isolate) for each patient was included in this study. Data on antibiotic treatments administered to patients in the year before the first isolation of SCV and normal *S. aureus* strains were collected.

The stability of the SCV phenotype was confirmed by periodical subculture of the strains for at least 40 passages under the same conditions and media (in Blood Agar) (Melter *et al.*, 2010). Auxotrophy was assessed by disc diffusion on Mueller Hinton (MH) II agar (Becton Dickinson) using 5.4 µg of hemin (Sigma), 1.5 µg thymidine (Sigma) or 1.5 µg menadione (Sigma) respectively. A strain was considered to be auxotrophic to one of the three tested compounds if a clear zone of growth surrounding the disks was detected after 24 h of incubation at 35°C (Lannergard *et al.*, 2008).

Molecular confirmatory tests and clonal typing

Species identification and methicillin resistance were confirmed by *nucA* PCR and *mecA* PCR respectively as described elsewhere (Brakstad *et al*., 1992; Kipp *et al*., 2004). Clonal identity and relatedness of SCV and 15 non-SCV isolates were analysed by pulsed-field gel electrophoresis (PFGE) after SmaI (Sigma) restriction of whole chromosomal DNA (Goering *et al*., 1992). The obtained PFGE patterns were analysed according to Tenover *et al.* criteria (Tenover *et al.,* 1995).

Detection of PVL

The gene coding for virulence factors lukS and lukF in MRSA strains (SCV and Normal) was detected by PCR reaction, as previously described (Lina *et al*., 1999). *S.aureus* ATCC 49775 strain was used as positive control.

Determination of antibiotic susceptibility

MICs to vancomycin, linezolid, tigecycline, rifampicin, daptomycin and moxifloxacin (active compounds on the MRSA (Seifert *et al*., 1999)) were determined using the Etest® method according to the manufacturer's instructions (bioMérieux, Italy). Susceptibility criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied to evaluate MIC values (http:// www.eucast.org/ clinical_breakpoint/). *S. aureus* ATCC 29213 was used as control strain.

Mutation frequency

One single colony was inoculated in 20 ml of brain heart infusion (BHI) broth (Becton Dickinson) and incubated overnight at 37°C with shaking (200 rpm) to test the mutation frequency as described by Besier *et al*. (Besier *et al*., 2008). All experiments were performed in triplicate; mean values ± standard deviations were computed. All the tested strains were originally susceptible to the concentration of rifampin used. For the isolates with elevated rifampin MICs (16 and 32 µg/mL), the mutation frequency measurement was confirmed with streptomycin (50 µg/mL) as above.

Biofilm formation assay

The biofilm formation assay was performed as previously described (Stepanovic *et al*., 2007). *Staphylococcus aureus* ATCC 12598 and *Staphylococcus epidermidis* ATCC 12228 were used as positive and negative controls, respectively, for biofilm formation. All assays were performed in four replicates on three separate experiments. The cut-off value for optical density (OD) measurements was defined as 3x the standard deviations above the mean OD of the negative control (Stepanovic *et al*., 2007) and the final OD values were expressed as average OD value reduced by the cut-off value. According to the biofilm formation results, strains were divided into the following four categories: OD≤OD = no biofilm producer; ODc<OD≤2xODc = weak biofilm producer; 2xODc<OD≤4xODc = moderate biofilm producer; 4xODc<OD = strong biofilm producer.

Statistical analysis

Two-tailed Fisher's exact test was used to analyse the categorical variables, while metric variables were evaluated with the non-parametric Kruskal-Wallis test. *P* values of <0.05 were considered statistically significant for all analyses. Statistical analysis was performed with SPSS software.

RESULTS

A total of 134 out of 222 (60.4%) patients followed at the Genoa CF centre harboured *S. aureus,* but only 42 patients were chronically colonized by *S. aureus*: 14 patients with normal phenotype and 13 with SCV phenotype and 15 both phenotypes. Of 28 SCV positive patients, 15 were males and 13 females, while of 29 normal phenotype positive patients, 12 were males and 17 females ($P>0.05$).

Median age of enrolled patients was 24 years (range: 5-53 years) for SCV colonized patients and 23 years (range: 1-48) for patients with normal *S. aureus* ($P>0.05$); 75% of patients with *S. aureus* SCV and 37.9% with normal *S. aureus* were also colonized by *P. aeruginosa* ($P=0.007$). Phenotypic characterization data revealed that among 28 SCV strains, 27 were thymidine-dependent and only one haemin-dependent. No menadione-dependent strains were found. PFGE analysis (Table 2) showed 31 different

TABLE 1A - *Antibiotic susceptibility of SCV S. aureus isolates from CF patients (SCV strains n=28).*

Antibiotic	*MIC range (mg/L)*	*MIC50 (mg/L)*	*MIC90 (mg/L)*	*R (%)*
Daptomycin	0,19-1	0,25	0,50	1 (3,6)
Vancomycin	1,0-2,0	1,5	2	0
Linezolid	0,50-4	1,5	4	0
Rifampicin	0,002-32	0,008	32	9 (32,14)
Moxifloxacin	0,016-3	0,25	1	10 (35,7)
Tigecycline	0,094-256	0,50	8	10 (35,7)

Note: Breakpoints were used according to EUCAST.

TABLE 1B - *Antibiotic susceptibility of normal S. aureus isolates from CF patients (normal strains n=29).*

Antibiotic	*MIC range (mg/L)*	*MIC50 (mg/L)*	*MIC90 (mg/L)*	*R (%)*
Daptomycin	0,50-2	0,50	1	3(10,3)
Vancomycin	0,50-2	1	1	0
Linezolid	1,0-4	2	4	0
Rifampicin	0,008-32	0,25	0,25	5 (17,2)
Moxifloxacin	0,19-4	0,50	1	7 (24,1)
Tigecycline	0,25-16	0,50	1	6(20,7)

Note: Breakpoints were used according to EUCAST.

types of banding pattern (defined by alphabet letters). Data on antibiotic therapy in the year before the sputum collection are not statistically significant but showed that of 14 patients with normal *S. aureus* only 42.7% were treated with trimethoprim/sulfamethoxazole and 100% with aminoglycosides, 100% of the 13 patients colonized only by *S. aureus* SCV were treated with trimethoprim/sulfamethoxazole and aminoglycosides, while of the 15 patients with both phenotypes 26.7% were treated with trimethoprim/sulfamethoxazole and 66.7% aminoglycosides In addition, 61.5% of patients with *S. aureus* SCV were treated with linezolid whereas no patients with normal phenotype took that drug. We did not find a difference in the frequency of antibiotic usage between the patients with and without SCV strains. The dominant type C was present in six SCV isolates, type A in five SCV and one normal, type E in three SCV and four normal, while other types had double or single occurrence. Interestingly, the study of the 15 patients harbouring both phenotypes revealed that in nine cases the two types of bacteria had the same clonal lineage (Table 2).

Antibiotic susceptibility

Resistance profiles of the SCV isolates are presented in Table 1a and 1b. Resistance rates were as follows: daptomycin 3.6%; rifampicin 32.1%; moxifloxacin 35.7%; tigecycline 35.7% for SCV strains, while daptomycin 10.3%; rifampicin 17.2%; moxifloxacin 24.1%; tigecycline 20.7% for Normal strains. No resistance was detected for vancomycin and linezolid. MR was found in 25.0% of SCV and 31% of normal isolates and confirmed by *mecA* PCR. Five among the seven MRSA SCVs belonged to the dominant PFGE type C (Tables 2-3).

PVL

The gene coding for virulence factors lukS and lukF was not detected in the MRSA strains (SCV and Normal) tested in this study.

Mutator phenotypes

Nearly 40% of SCV and 20.7% normal strains were strong hypermutators, defined as isolates with mutation rates of $\geq 10^{-7}$ (Oliver *et al.*, 2012). The mean mutation frequency of strong hypermutables was significantly higher than other strains tested ($P<0.01$, Kruskall-Wallis test). In other isolates the mutation frequency (mean ± standard deviation) was $[2.13\pm3.9] \times 10^{-8}$ for SCV and $[2.13\pm3.5] \times 10^{-8}$ for Normal, range from 2.7×10^{-8} to 8.8×10^{-10}. *S. aureus* type strain (ATCC 29213) showed a mutation rate of $[6.6\pm3.3] \times 10^{-10}$.

TABLE 2 - *Different banding pattern of pulsed filed gel electrophoresis of S. aureus SCV and normal isolates from CF patients. The methicillin-resistant (MR) strains are prevalent in genotype C for SCV phenotype and in P and T for normal phenotype.*

CF patient	*Phenotype*	*PFGE type*	*Meticillin resistance*	*CF patient*	*Phenotype*	*PFGE type*	*Meticillin resistance*
1	SCV	*A*	MS	18	SCV	***C***	**MR**
2	SCV	*A*	MS		NORMAL	*Q*	MR
3	SCV	*A*	MS	19	SCV	***C***	**MR**
4	SCV	*A*	MS	20	SCV	***C***	**MR**
5	SCV	*A*	MS	21	SCV	***C***	**MR**
	NORMAL	*A*	MS		NORMAL	***P***	**MR**
6	SCV	*L*	MS	22	SCV	***C***	**MR**
	NORMAL	*L*	MS	23	SCV	*C*	MS
7	SCV	*M*	MS	24	SCV	*G*	MR
	NORMAL	*M*	MS		NORMAL	*U*	MS
8	SCV	*G*	MS	25	SCV	*N*	MS
	NORMAL	*G*	MS	26	SCV	*D*	MR
9	SCV	*H*	MS	27	NORMAL	*T*	MS
	NORMAL	*H*	MS		SCV	*O*	MS
10	SCV	*B*	MS	28	SCV	*O*	MS
	NORMAL	*B*	MS	29	NORMAL	***P***	**MR**
11	SCV	*I*	MS	30	NORMAL	*K*	MS
	NORMAL	*I*	MS	31	NORMAL	*R*	MS
12	SCV	*E*	MS	32	NORMAL	*E*	MS
	NORMAL	*E*	MS	33	NORMAL	***T***	**MR**
13	SCV	*E*	MS	34	NORMAL	***T***	**MR**
	NORMAL	*E*	MS	35	NORMAL	*L*	MS
14	SCV	*E*	MS	36	NORMAL	*Y*	MR
15	SCV	*H*	MS	37	NORMAL	*V*	MS
16	SCV	*B*	MS	38	NORMAL	*E*	MS
	NORMAL	*R*	MS	39	NORMAL	*R*	MS
17	SCV	*F*	MS	40	NORMAL	*W*	MR
	NORMAL	*S*	MS	41	NORMAL	*X*	MR
				42	NORMAL	*Z*	MS

Biofilm

All tested SCV isolates were showed to form biofilm. On the other hand, only 58.6% of normal isolates were able to form biofilm. In particular, six SCV and two normal isolates were strong biofilm producers (1.08±0.47-SCV; 2.11±0.13-Normal), 15 SCV and 11 normal moderate (0.477±0.067 SCV; 1.49±0.20 Normal) and seven SCV and five normal (0.219±0.045 SCV; 0.214±0.31 Normal) weak producers. The difference observed between strong and weak producers and between strong and moderate producers was statistically significant ($P<0.01$, Kruskall-Wallis test). In particular, focusing on 16 MRSA strains, only one was strong producer (SCV), ten were moderate (four SCV and six normal) and five were weak producers (two SCV and three normal).

Four out of seven (57.2%) MR strains were moderate biofilm producers, two out of seven (28.6%) weak producers and one out of seven (14.3%) a strong producer; 66.6% of strong biofilm producers showed resistance to moxifloxacin. Although the strong hypermutators showed an greater ability to form biofilm than non-mutator strains, the observed difference was not statistically significant ($P=0.17$, Fisher's exact test).

TABLE 3 - *MRSA. Characteristics found in 7 MRSA-SCV and 9 MRSA normal strains recovered in this study.*

CF Strain	*MIC (R/S)*						*Phenotype*	*Biofilm*	*Mutation Frequency*
	TCG	*LNZ*	*VA*	*DPC*	*RIF*	*MXF*			
18	0,50 (S)	0,50 (S)	1 (S)	0,19 (S)	32 (R)	0,75 (R)	SCV	Moderate	Nonmutator
18	0,50 (S)	1 (S)	1 (S)	1 (S)	32 (R)	2 (R)	Normal	Weak	Hypermutator
19	0,50 (S)	1 (S)	1 (S)	0,125 (S)	32 (R)	1,5 (R)	SCV	Moderate	Nonmutator
20	0,38 (S)	1 (S)	1,5 (S)	0,5 (S)	0,012 (S)	0,064 (S)	SCV	Weak	Hypermutator
21	0,38 (S)	1,5 (S)	1,5 (S)	0,125 (S)	0,012 (S)	0,75 (S)	SCV	Moderate	Nonmutator
21	0,50 (S)	1 (S)	1 (S)	1 (R)	32 (R)	2 (R)	Normal	Weak	Hypermutator
22	0,50 (S)	1 (S)	1,5 (S)	1 (R)	32 (R)	3 (R)	SCV	Strong	Nonmutator
24	0,25 (S)	1,5 (S)	1 (S)	0,25 (S)	0,008 (S)	0,004 (S)	SCV	Moderate	Hypermutator
26	0,25 (S)	1,5 (S)	1,5 (S)	0,25 (S)	0,008 (S)	0,032 (S)	SCV	Weak	Nonmutator
22	0,50 (S)	1 (S)	1,5 (S)	1 (R)	32 (R)	3 (R)	SCV	Strong	Nonmutator
33	0,50 (S)	2 (S)	2 (S)	2 (R)	32 (R)	4 (R)	Normal	Moderate	Hypermutator
34	1 (R)	2 (S)	2 (S)	2 (R)	32 (R)	2 (R)	Normal	Moderate	Hypermutator
40	0,50 (S)	4 (S)	1 (S)	0,50 (S)	0,25 (S)	0,25 (S)	Normal	Weak	Hypermutator
41	8 (R)	2 (S)	1 (S)	0,50 (S)	0,25 (S)	0,25 (S)	Normal	Moderate	Nonmutator
39	0,50 (S)	2 (S)	0,25 (S)	1 (R)	0,25 (S)	1 (R)	Normal	Moderate	Nonmutator
29	1 (R)	2 (S)	1 (S)	0,50 (S)	0,25 (S)	4 (R)	Normal	Moderate	Nonmutator
36	0,50 (S)	2 (S)	1 (S)	0,50 (S)	0,25 (S)	2 (R)	Normal	Moderate	Nonmutaror

DISCUSSION

Bacterial populations within CF airways develop resistance to several antibiotics, enhance the ability to form biofilm and display a wide spectrum of different morphotypes, including *S. aureus* SCV (Proctor *et al.*, 2006; Melter *et al.*, 2010; Besier *et al.*, 2007a; Smyth 2005; Davies *et al.*, 2009). In this study, the relationship between antibiotic resistance, hypermutability and biofilm growth of SCV isolates was investigated for the first time to improve our knowledge of the SCV role in *S. aureus* persistence in the airways of CF patients.

A total of 222 patients in follow-up at the CF centre in Genoa (Italy) were tested for the prevalence of *S. aureus* SCV which resulted as 12.61%. In agreement with our data, recent studies of adults and children with CF in Europe (Besier *et al.*, 2007a; Yagci *et al.*, 2013; Kahl *et al.*, 1998) reported an *S. aureus* SCV prevalence between 8% (Yagci *et al.*, 201) and 33% (Kahl *et al.*, 1998).

The median age of SCV colonized patients in our study was 24 years, whereas in other studies the median age was 21 years (Besier *et al.*, 2007a), 14.4 years (Yagci *et al.*, 2013) or 13 years (Kahl *et al.*, 1998). Although *S. aureus* is commonly found in CF patients during infancy, the association between *S. aureus* SCV and non-paediatric age in our patients could be a consequence of a longer exposure to antimicrobial agents, such as antifolates and aminoglycosides (Kahl *et al.*, 1998; Massey *et al.*, 2001). In fact, when we analysed the data collected on antimicrobial treatment we found that patients becoming colonized by *S. aureus* SCV were widely treated with trimethoprim/sulfamethoxazole, aminoglycosides and linezolid. On the other hand, patients colonized with *S. aureus* normal received less antibiotic treatment with trimethoprim/sulfamethoxazole and linezolid.

Moreover, 75.0% of patients with SCV strains were co-colonized with *P. aeruginosa,* which is known to produce 4-hydroxy-2-heptylquinoline-N-oxide, a substance that in the CF lung protects *S. aureus* from commonly used aminoglycoside antibiotics such as tobramycin (Hoffman *et al.*, 2006). Prolonged growth of *S. aureus* in the presence of *P. aeruginosa* causes the emergence of *S. aureus* SCV (Hoffman *et al.*, 2006).

We found that 27 out of 28 strains were thymidine-dependent and this prevalence has already been described in the literature (Besier *et al.*, 2007b; Kahl *et al.*, 1998). The thymidine-dependence is expected since continuous expo-

sure to antibiotics in CF lung favours resistance to antifolates. Indeed, the bacteria acquire the ability to use the exogenous nucleotide sources available in huge quantities in the pus of the CF pulmonary environment. Thymidine-dependent *S. aureus* SCV are also described as hypermutators (Besier *et al.*, 2008) and our results describing the presence of mutators with significantly (P<0.01) high mutation frequency rates among *S.aureus* SCV are in agreement with these data. In addition, the number of SCV hypermutator strains is higher in comparison to strains with normal phenotype (39.3% and 20.7%, respectively).

A recent publication by Oliver *et al.* showed that mutators may additionally have important effects on the evolution of virulence, genetic adaptation to the airways of CF patients, persistence of colonization, transmissibility, and perhaps lung infection decline (Oliver *et al.*, 2010). Moreover the mutators were found to be much more frequently resistant to antibiotics than non-mutators, representing a major problem for antimicrobial therapy. However, in our study we did not find a statistically significant difference in resistance rate between mutators and non-mutators.

Biofilm formation has been linked to persistence of *S. aureus* and other pathogens in pulmonary CF infection. In agreement with previous data reporting a significantly increased biofilm-formation ability of *S. aureus* SCV (Maduka-Ezeh *et al.*, 2012), our results showed that, unlike the normal isolates (58.6% form biofilm), all SCV tested were able to form biofilm to different degrees (P<0.01), in particular: moderate (53.6%), weak (25.0%) and strong (21.4%) producers.

Metabolic defects combined with intracellular survival and biofilm formation alter *S. aureus* SCV susceptibility to antibiotics, contributing to therapeutic failures. No large epidemiological survey is yet available in the literature, and the present results expand our knowledge of SCV antibiotic susceptibility.

This study found 8/28 (28.6%) strains with high-level resistance (MIC=32mg/L) to rifampicin, while a previous study described one clinical isolate, recovered from a chronic infection, with an SCV phenotype resistant to rifampicin (Gao *et al.*, 2010).

In agreement with a study on a collection of 48 SCVs isolated from CF patients that reported increased MICs to tigecycline (Garcia *et al.*, 2013), our results show high MICs (4-8-12-32 and 256 µg/mL).

In comparative studies examining several antibiotics against SCV with different auxotrophisms, moxifloxacin appeared consistently highly effective against thymidine-dependent SCVs (Garcia *et al.*, 2013), while in this study the SCV strains tested show a high rate of resistance to this fluoroquinolone.

Finally, the analysis of *S.aureus* SCV MIC showed a tendency toward increased vancomycin MICs (MIC_{50}=1.5 µg/mL - MIC_{90}=2 µg/mL). Recent guidelines suggested considering alternative antibiotics in complicated MRSA infection when vancomycin MIC is >=2 µg/mL (Liu *et al.*, 2011).

Daptomycin, linezolid and trimetoprim/sulfmetaxozol are the only alternative treatments to vancomycin. In the SCV strains analyzed in this study, the antimicrobial treatment options are further reduced, since these strains have become intrinsically resistant to trimetoprim/sulfmetoxazol due to the auxotrophism that characterizes them. Furthermore for the treatment with linezolid our analysis showed that the MIC values, even if included in the susceptibility range, are high (MIC50=1,5 µg/mL e MIC90=µg/mL).

Moreover, daptomycin is not recommended for treatment of MRSA-related lung infections, including pneumonia, because it is inactivated by lung surfactant. Therefore it is evident that the therapeutic options for the treatment of MRSA SCV strains are drastically reduced.

Finally, the relevant data found by molecular typing disclosed same SCV strain, belonging to Clone A, and the same MR strain, belonging to type C, in different patients, suggesting patient-to-patient transmission.

PVL is an important virulence factor associated with typical community-acquired (CA) MRSA strains (Zetola *et al.*, 2005). The absence of genes coding PVL in the CF MRSA tested may suggest a probable initial hospital acquisition of these strains.

Therefore the spread of the biofilm producing MRSA-SCV clone C stresses the importance of accurate and prompt isolation, identification

and characterization of SCV strains in CF in order to allow an effective treatment and prevent cross-infections.

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