Prevalence and genetic diversity of *Staphylococcus aureus* small-colony variants in cystic fibrosis patients

S. Yagci[1], G. Hascelik[2], D. Dogru[3], U. Ozcelik[3] and B. Sener[2]

1) *Infectious Diseases and Clinical Microbiology Clinic, Ankara Training and Research Hospital,* 2) *Department of Microbiology and Clinical Microbiology, Hacettepe University Medical Faculty and* 3) *Chest Diseases Unit, Hacettepe University Medical Faculty, Ihsan Dogramaci Children's Hospital, Ankara, Turkey*

Abstract

Staphylococcus aureus small-colony variants (SCVs) are being isolated more frequently in cystic fibrosis (CF) patients. We aimed to determine the prevalence of *S. aureus* SCVs and their phenotypic and genotypic properties in CF patients admitted to a university hospital. Specimens of 248 patients were examined during a period of 11 months. Colonies supposed to be SCVs were evaluated on Columbia blood agar, mannitol salt agar, and brain–heart infusion agar with 5% NaCl (BHIA 5% NaCl). Strains were confirmed by *S. aureus nucA* PCR. Antibiotic susceptibilities of SCVs and simultaneously isolated *S. aureus* strains were determined for oxacillin, gentamicin, trimethoprim–sulphamethoxazole, vancomycin, ciprofloxacin, linezolid, and tigecycline. Genetic relatedness between SCVs and normal *S. aureus* strains was determined with a pulsed-field gel electrophoresis (PFGE) method. *S. aureus* SCVs were detected in 20 of 248 patients (8.1%). The highest SCV isolation rate was obtained with MSA, followed by BHIA 5% NaCl. Auxotrophism for thymidine was demonstrated in six SCVs. The tigecycline susceptibilities of 48 SCV strains isolated in this study showed higher MIC values than those of 33 simultaneously isolated normal *S. aureus* strains. Whereas SCVs and normal *S. aureus* strains showed identical genotypes in 14 of the patients, five patients showed different genotypes. This first study from Turkey evaluating *S. aureus* SCVs in CF patients has indicated the importance of considering and reporting SCVs in chronic infections such as CF. The presence of SCVs will probably indicate persistent infection, and this might impact on antibiotic treatment decisions, as they are more resistant to antibiotics.

Keywords: Cystic fibrosis, genotypic diversion, phenotypic characteristics, small-colony variant, *Staphylococcus aureus*
Original Submission: 18 July 2011; **Revised Submission:** 1 November 2011; **Accepted:** 23 November 2011
Editor: J.-M. Rolain
Article published online: 28 November 2011
Clin Microbiol Infect 2013; **19:** 77–84
10.1111/j.1469-0691.2011.03742.x

Corresponding author: S. Yagci, Fakulteler M. Keskin S. 9/3 Cebeci, 06590 Cankaya/Ankara, Turkey
E-mail: serveryagci@yahoo.com.tr

Introduction

Cystic fibrosis (CF) patients are often colonized with *Staphylococcus aureus*. The spectrum of microorganisms isolated from the respiratory tract specimens of CF patients has changed in recent years, and, as well as the frequent pathogens *S. aureus* and *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *S. aureus* small-colony variants (SCVs) are being isolated more frequently [1–3]. *S. aureus* SCVs are known as slow-growing subpopulations of *S. aureus* that grow as small, non-pigmented and non-haemolytic colonies on agar plates, and their isolation and identification is a challenge for clinical microbiology laboratories [4]. This difficulty may lead to diagnostic underestimation and therefore therapeutic failures in the clinical setting. *S. aureus* SCVs have been associated with chronic and recurrent infections; however, this relationship has only been appreciated in recent years [5,6].

The discovery and characterization of *S. aureus* SCVs have provided new insights into our understanding of the pathogenesis of chronic *S. aureus* lung infections in CF patients. As SCVs can survive intracellularly, the isolation of an SCV phenotype is usually associated with the persistence of *S. aureus* in CF patients. This study was aimed at determining the prevalence and antibiotic susceptibility profiles of *S. aureus*

SCVs in CF patients, identifying the best method for detection of SCVs in clinical specimens, and evaluating the genetic relatedness between normal *S. aureus* strains isolated from CF patients and their paired SCV strains.

Materials and Methods

This prospective study was conducted at Hacettepe University Children's Hospital, Chest Diseases Unit and Clinical Microbiology Laboratory, Ankara, Turkey between February 2007 and January 2008. Respiratory specimens of 248 CF patients were screened for the presence of *S. aureus* SCVs. The specimens comprised sputum and deep throat swab samples.

Specimens were cultured on Columbia sheep blood agar (BD, Franklin Lakes, NJ, USA) and chocolate agar (Fluka, Sigma-Aldrich, St. Louis, MO, USA) with 300 U/mL bacitracin (Sigma-Aldrich, St. Louis, MO, USA), Eosin Methylene Blue agar (Becton Dickinson), mannitol salt agar (MSA) (Becton Dickinson), oxidation/fermentation polymyxin–bacitracin–lactose agar, and brain–heart infusion agar (Oxoid, Basingstoke, UK) with 5% NaCl (Merck, Darmstadt, Germany) (BHIA 5% - NaCl). All agar plates were incubated at 35°C for at least 48 h aerobically, except for BHIA 5% NaCl plates, which were incubated anaerobically.

Colonies suspected of being *S. aureus* on sheep blood agar, MSA and BHIA 5% NaCl were further isolated at 35°C for 24–48 h on Columbia sheep blood agar. Non-haemolytic, non-pigmented, pinpoint or fried-egg colonies on sheep blood agar and small colonies on MSA and BHIA 5% NaCl were considered to be *S. aureus* SCVs. These suspected colonies were inoculated onto Columbia blood agar and Schaedler agar (Becton Dickinson). Columbia blood agar was incubated in a normal atmosphere and Schaedler agar was incubated in 5–10% CO_2, both at 35°C. If the colonies were observed to be normal-sized, haemolytic and pigmented on Schaedler agar, they were considered to be *S. aureus* SCVs. Strains were subjected to species identification by Gram staining, catalase reaction, tube coagulase result, and latex agglutination test (Slidex Staph Plus; bioMérieux, Marcy l'Etoile, France). Identification of *S. aureus* was confirmed by *nucA* PCR [7].

Auxotrophy for haemin (5.4 μg) was tested by using standard disks (Sigma), and auxotrophy for thymidine and menadione was tested by impregnating disks with 1.5 μg of thymidine (Sigma) or 1.5 μg of menadione (Sigma), respectively. A strain was positive for auxotrophy if a zone of growth surrounding the impregnated disks on Mueller–Hinton agar was detected after 24 h of incubation at 35°C [8,9].

Antimicrobial susceptibilities of the strains were determined by the broth microdilution method for oxacillin, gentamicin, vancomycin, ciprofloxacin, linezolid, trimethoprim–sulphamethoxazole and tigecycline according to CLSI guidelines [10]. Strains with a normal phenotype were tested on Mueller–Hinton broth, and SCVs were tested on brain–heart infusion broth [11]. The MIC values of SCVs were evaluated according to CLSI breakpoints for staphylococci [12]. As there are no CLSI criteria for tigecycline, EUCAST susceptibility criteria were applied [13].

Clonal identity and genetic relatedness between normal *S. aureus* strains and their paired SCVs were analysed with a pulsed-field gel electrophoresis (PFGE) method after *Sma*I (Sigma) restriction of bacterial DNA, as described previously [14]. The 33 normal *S. aureus* strains selected for PFGE were the pairs of related SCVs isolated from each patient. The PFGE bands produced were evaluated according to the Tenover criteria [15]. PFGE genotypes were numbered separately within each patient. No interpatient genetic relatedness analysis was performed.

Clinical data of patients with SCV isolation were collected and evaluated from patients' medical records by the physicians of the paediatric chest diseases unit. Statistical analysis was performed with SPSS software, version 15.0. Definitive statistics were represented as means, medians, and percentages. Differences between means were evaluated with the Mann–Whitney test for the numerical variables and the chi-square, Mantel–Haenzel or Fisher exact test for the categorical variables. A p-value cut-off of ≤ 0.05 was considered to be statistically significant for all analyses.

Results

A total of 519 respiratory specimens from 248 CF patients were evaluated for the presence of *S. aureus* SCVs. Of these 519 specimens, 209 (40.3%) were sputum and 310 (59.7%) were deep throat swab samples. The number of male patients (129, 52%) was similar to the number of female patients (119, 48%). The median age of patients was 9.9 years (range: 1–58 years), and was similar in the two genders (Table 1).

Of the 248 patients, 123 (49.6%) harboured normal *S. aureus* strains in their respiratory specimens, and 20 (8.1%) harboured SCVs as well. The prevalence of SCVs in *S. aureus*-positive patients was 16.2% (20/123). Of these 20 SCV-positive patients, 13 (65%) were females and seven were males. The median age of patients with normal *S. aureus* strains was 10.4 years (range: 1–58 years), and that of patients with SCVs was 14.4 years (range: 2–31 years); the isolation rate was highest in those between 11 and 15 years of age, who constituted a total of 25% of the

TABLE 1. Comparison of the ages of patients with normal *Staphylococcus aureus* and the ages of patients with *S. aureus* small-colony variants (SCVs)

	Patients with normal *S. aureus*	Patients with *S. aureus* SCVs
Age (years)	*n* (%)	*n* (%)
1.0–5.0	42 (34.1)	3 (15.0)
6.0–10.0	31 (25.2)	4 (20.0)
11.0–15.0	23 (18.7)	5 (25.0)
16.0–20.0	17 (13.8)	3 (15.0)
21.0–25.0	5 (4.1)	4 (20.0)
≥26.0	5 (4.1)	1 (5.0)
Total	123 (100.0)	20 (100.0)

patients ($p < 0.05$). Patients with SCVs were older than patients with normal *S. aureus* strains only (p 0.015, Mann–Whitney *U*-test) (Table 1). Among the 20 SCV carriers, 13 had SCVs once, two had SCVs twice, and five had SCVs three times. Persistent colonization was defined as three or more positive cultures during the study period, and was demonstrated in 25% ($n = 5$) of SCV carriers. From these 20 patients, 48 SCVs and 30 normal *S. aureus* strains were isolated in a total of 32 samples. Fifteen (46.9%) of these 32 samples also revealed the simultaneous presence of *P. aeruginosa* and *S. aureus* SCVs.

The review of patients' medical reports revealed that no antistaphylococcal prophylaxis had been given to the patients. The most frequently used antibiotics for the staphylococcal infections in CF patients were amoxycillin–clavulanate, ampicillin–sulbactam, and fluoroquinolones. Trimethoprim–sulphamethoxazole was used in only five patients and gentamicin in four patients. All of the five patients with SCVs with trimethoprim–sulphamethoxazole resistance had been treated with trimethoprim–sulphamethoxazole. The length of the treatment varied from 1 month to 2 years for different patients. The trimethoprim–sulphamethoxazole-resistant SCVs from these five patients were thymidine-dependent SCVs (Table 2).

Of the 48 SCVs, 28 were isolated after an incubation period of 24 h and 20 after an incubation period of 48 h. The highest SCV yield was from MSA (66.7%), followed by BHIA 5% NaCl (50%). For patients infected or colonized with SCVs, a reliable bacterial count could not be obtained, as they grow as tiny colonies and are usually covered by normal *S. aureus* colonies. Typical small colonies were observed for 36 (75%) of strains (Fig. 1). Six strains (12.5%) formed pinpoint colonies and six (12.5%) formed fried-egg colonies.

Among the 48 SCVs, six were thymidine-dependent (Fig. 2). Of these six, one was also haemin-dependent. Five of the thymidine-dependent strains formed fried-egg colonies. No menadione-dependent strains were detected.

The resistance rates of SCVs were as follows: oxacillin, 10.4%; trimethoprim–sulphamethoxazole, 16.7%; ciprofloxacin, 14.6%; and gentamicin, 6.3%. When compared with those for simultaneously isolated normal *S. aureus* strains, these resistance rates showed no significant difference. No resistance was detected for vancomycin and linezolid. MICs for tigecycline were significantly higher for SCVs than for simultaneously isolated normal *S. aureus* strains (p 0.041, Fisher) (Table 3).

A total of 81 strains (33 normal *S. aureus* strains and 48 paired SCVs) were genotyped by PFGE (Fig. 3). PFGE bands of *S. aureus* and SCV strains with the same profile were considered to indicate clonality [15]. Whereas SCVs and their normal counterparts showed clonality in 14 of the patients, five patients exhibited different genotypes for SCV and normal strains. One patient exhibited different SCV genotypes and also different normal *S. aureus* genotypes in a single sample. Persistence of the same SCV was seen in five patients.

Discussion

This is the first study to demonstrate the prevalence and phenotypic and genotypic characteristics of *S. aureus* SCVs in CF patients in Turkey. The prevalence of *S. aureus* colonization in our CF centre, which is the reference CF centre in Turkey, was 57.7% during the study period. The US Patient Registry Annual Data Report 2009 indicated that the *S. aureus* prevalence in CF patients was 51.3% [16]. The European CF *S. aureus* prevalence varies between 60.6% and 72% in different CF centres (UK, Germany, Belgium, and France) [1,17,18]. SCVs were isolated from 8.1% of CF patients and 6.4% of specimens evaluated. Among *S. aureus*-positive patients, the prevalence was 16.2%. In the study of Besier *et al.*, respiratory specimens of 252 CF patients in Germany were evaluated, and the SCV prevalence was found to be 17% among *S. aureus* carriers, which is similar to our findings. The median age of our patient population was 9.9 years, and that of the SCV-positive patients was 14.4 years. The median age of CF patients in Besier's study was 16 years, and of the SCV-positive patients was 21 years [9]. Kahl *et al.* [11] reported a high prevalence (49.1%) of SCVs in their *S. aureus*-positive CF population, in which the median age of SCV-positive patients was 13 years, and all SCV-positive patients received long-term trimethoprim–sulphamethoxazole prophylaxis. In one recent study, SCV prevalence was reported to be 8.2%, similar to our findings. However, the authors focused on only one respiratory specimen per patient in a short period [10]. The emergence of *S. aureus* SCVs has been reported to be associated with antibiotic use, but isolation after extended antibiotic-free intervals may also be possible [6,19]. The median ages of our patient population

TABLE 2. **Genetic diversity of *Staphylococcus aureus* small-colony variants (SCVs) and paired *S. aureus* strains from 20 patients harbouring *S. aureus* SCVs**

Patient no.	Date of admission	Phenotype	PFGE genotype	OX	GM	VA	CIP	LZ	SXT	TG
1	6 March 2007	SCV	II	R	R	S	R	S	R	R
	16 March 2007	SCV	II	R	R	S	R	S	S	S
		S. aureus	I	S	S	S	S	S	S	S
2	17 April 2007	SCV	I	S	S	S	I	S	S	S
		SCV	I	S	S	S	S	S	R	S
		S. aureus	III	S	S	S	S	S	S	S
	3 August 2007	SCV	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	I	S	S	S
		SCV	I	S	S	S	I	S	S	S
	19 November 2007	*S. aureus*	IV	S	S	S	S	S	S	S
		SCV	II	R	S	S	S	S	S	S
3	18 April 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	I	S	S	S
4	26 April 2007	*S. aureus*	I	R	S	S	I	S	S	S
		SCV	I	S	S	S	S	S	S	S
	7 June 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
	17 December 2007	*S. aureus*	I	S	S	S	S	S	S	S
		S. aureus	I	R	S	S	S	S	S	S
		SCV	I	S	I	S	S	S	S	R
		SCV	I	S	S	S	S	S	S	R
5	30 March 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
	24 October 2007	*S. aureus*	I	R	S	S	S	S	S	S
		SCV	I	R	S	S	S	S	S	S
	11 December 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	R	S	S	S	S	S	S
		SCV	I	R	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	R
6	5 June 2007	SCV	I	S	S	S	I	S	S	S
		SCV	I	S	S	S	S	S	S	S
		SCV	I	R	S	S	I	S	S	S
		S. aureus	I	S	S	S	I	S	S	S
	26 September 2007	SCV	I	S	S	S	I	S	S	S
	17 January 2008	*S. aureus*	I	S	S	S	I	S	S	S
		SCV	I	S	S	S	I	S	S	S
7	5 June 2007	*S. aureus*	I	S	S	S	R	S	S	S
		SCV	I	S	S	S	I	S	S	S
8	5 June 2007	*S. aureus*	I	S	S	S	R	S	S	S
		SCV	I	S	S	S	S	S	S	S
9	27 June 2007	*S. aureus*	II	S	S	S	I	S	S	S
		SCV	I	S	S	S	I	S	R	S
	17 August 2007	*S. aureus*	II	S	S	S	S	S	R	S
		SCV	I	S	S	S	R	S	S	S
	8 October 2007	*S. aureus*	II	R	S	S	S	S	S	S
		S. aureus	I	S	S	S	I	S	S	S
		SCV	I	R	S	S	S	S	S	S
10	4 July 2007	*S. aureus*	II	R	S	S	S	S	S	S
		S. aureus	II	R	S	S	S	S	S	S
		S. aureus	I	S	S	S	S	S	S	S
		SCV	–	R	S	S	S	S	R	S
		SCV	I	R	S	S	S	S	R	S
		SCV	II	R	S	S	S	S	R	S
		SCV	I	S	S	S	S	S	R	S
11	27 June 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	R	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
12	3 August 2007	*S. aureus*	I	S	S	S	R	S	S	S
		SCV	I	R	S	S	I	S	S	S
13	22 August 2007	SCV	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
		S. aureus	I	S	S	S	I	S	S	S
14	1 October 2007	*S. aureus*	I	R	S	S	R	S	S	S
		SCV	I	R	S	S	R	S	S	S
	7 January 2008	*S. aureus*	I	R	S	S	R	S	S	S
		SCV	I	R	S	S	R	S	S	R
15	16 October 2007	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
16	15 November 2007	*S. aureus*	I	R	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
17	11 December 2007	*S. aureus*	I	R	S	S	S	S	S	S
		S. aureus	I	S	S	S	S	S	S	S
		SCV	I	S	S	S	S	S	S	S
18	10 December 2007	SCV	I	R	S	S	S	S	S	R
		SCV	I	S	S	S	S	S	S	S
19	29 November 2007	*S. aureus*	I	S	S	S	R	S	S	S
		SCV		S	S	S	S	S	S	S

TABLE 2. Continued

Patient no.	Date of admission	Phenotype	PFGE genotype	OX	GM	VA	CIP	LZ	SXT	TG
20	31 January 2008	*S. aureus*	I	S	S	S	S	S	S	S
		SCV	II	R	S	S	R	S	R	S
		SCV	II	R	R	S	R	S	S	S

PFGE, pulsed-field gel electrophoresis; OX, oxacillin; GM, gentamicin; VA, vancomycin; CIP, ciprofloxacin; LZ, linezolid; SXT, trimethoprim–sulphamethoxazole; TG, tigecycline; R, resistant; I, intermediate; S, sensitive.
PFGE genotypes are numbered separately for each patient. No interpatient genetic relatedness analysis was performed.

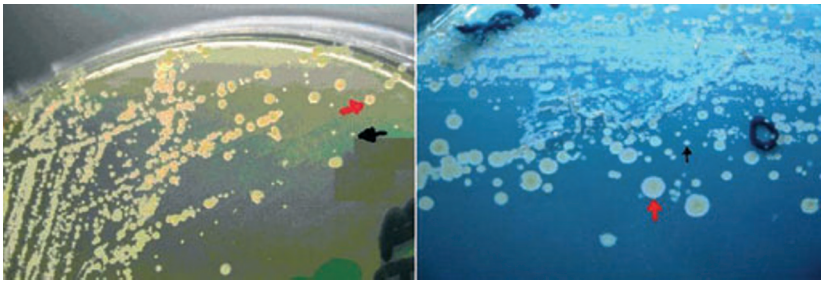

FIG. 1. *Staphylococcus aureus* small-colony variants (black arrow) and normal *S. aureus* colonies (red arrow) on mannitol salt agar (left) and brain–heart infusion agar with 5% NaCl (right).

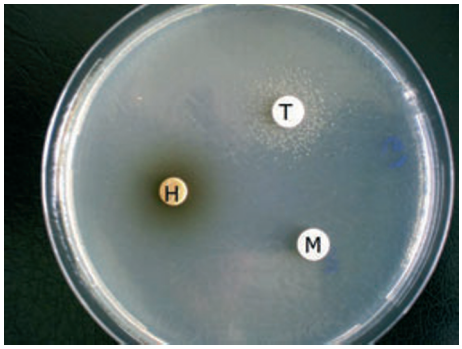

FIG. 2. Thymidine-dependent *Staphylococcus aureus* small-colony variants on Mueller–Hinton agar. H, haemin; M, menadione; T, thymidine.

seemed to be lower than that mentioned in the studies of Besier and Kahl, and it was also determined that no trimethoprim–sulphamethoxazole prophylaxis had been applied in our CF population. The lower SCV prevalence in our study group might be attributable to these two factors.

S. aureus colonization is highest in infants and young children with CF [20]. However, the emergence of SCVs was found to be associated with advanced age, and the reason might be the increased consumption of antibiotics with increasing age [9,17]. This relationship was also observed in our study.

It is well documented that the use of MSA for the recovery of *S. aureus* is a prerequisite for CF microbiology [21]. In a multicentre survey from Belgium, SCV prevalence was found to be lower (4%) than in other studies; however, the authors indicated that almost all SCVs were recovered from patients in a single laboratory, which processed specimens from two centres. It was also indicated that appropriate techniques were not used in most centres, so they failed to detect any SCVs [17]. In this study, all SCVs were initially detected in MSA plates, allowing proper isolation and further identification. According to our study, the best method for detecting SCVs was to inoculate specimens onto both MSA and BHIA 5% NaCl as well as Columbia blood agar. When MSA and BHIA 5% NaCl were used together, detection and identification of 86.6% of SCVs were achieved. These selective media suppress the growth of *P. aeruginosa* and support

TABLE 3. Antibiotic susceptibilities of *Staphylococcus aureus* small-colony variants (SCVs) and paired normal *S. aureus* strains

	S. aureus (*n* = 33)				*S. aureus* SCV (*n* = 48)			
Antibiotic	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	R (%)[a]	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	R (%)[a]
Oxacillin	≤0.06–2	0.25	1	–	≤0.06 to >64	0.25	4	5 (10.4)
Gentamicin	≤0.125–4	1	4	–	≤0.125 to >128	1	4	4 (8.4)
Vancomycin	0.25–2	1	2	–	0.125–2	1	2	–
Ciprofloxacin	0.25 to >32	0.5	4	27 (81.8)	0.03 to >32	1	16	37 (77.1)
Linezolid	0.25–4	2	4	–	0.25–4	2	2	–
TMP-SXT	0.016–32	0.125	0.25	1 (3.0)	0.023 to >32	0.25	32	8 (16.7)
Tigecycline[b]	0.015–0.5	0.125	0.25	–	0.015–2	0.06	1	6 (12.5)

R, resistant; TMP-SXT, trimethoprim–sulphamethoxazole.
[a]Intermediate strains were included in the resistant category.
[b]According to EUCAST breakpoints.

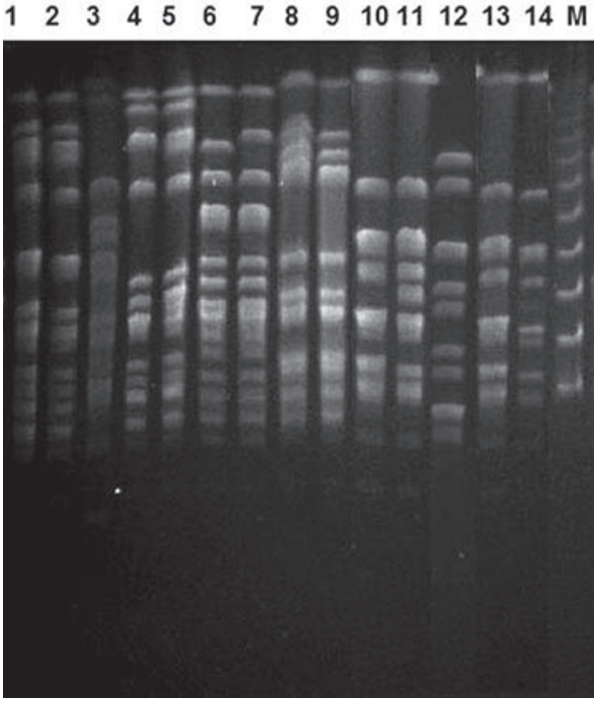

FIG. 3. Pulsed-field gel electrophoresis patterns (*Sma*I digest). Lanes 1 and 2: patient 14 (small-colony variants (SCVs)). Lane 3: patient 20 (normal *Staphylococcus aureus*). Lanes 4 and 5: patient 20 (SCVs). Lane 6: patient 8 (normal *S. aureus*). Lane 7: patient 8 (SCVs). Lanes 8 and 9: patient 10 (normal *S. aureus*). Lanes 10, 11, 13, and 14: patient 2 (SCVs). Lane 12: patient 2 (normal *S. aureus*). M: molecular marker.

the growth of normal *S. aureus* and SCVs [11]. Thus, MSA together with BHIA 5% NaCl could be an appropriate choice for SCV isolation, especially in laboratories with fewer facilities.

P. aeruginosa strains in CF patients produce 4-hydroxy-2-heptylquinoline-*N*-oxide, which suppresses the growth of *S. aureus*, and prolonged exposure causes the emergence of *S. aureus* SCVs [22–24]. Besier *et al.* reported that co-colonization with *P. aeruginosa* was significantly more frequent in patients with *S. aureus* SCVs than in patients with normal *S. aureus* only [9]. We found that 50% patients with *S. aureus* SCVs (10 of 20 patients) were co-colonized with *P. aeruginosa*, whereas only 28.56% (30 of 105) of patients with normal *S. aureus* were simultaneously positive for *P. aeruginosa*. However, the difference between the groups was not significant in our study.

Lack of biosynthesis of menadione or haemin occurs in electron transport-deficient SCVs, and this phenotype can be reversed by supplementation of media with menadione or haemin. Nonetheless, SCVs without auxotrophism for thymidine, menadione or haemin were reported to be CO_2-dependent [4,25]. Auxotrophism for thymidine was detected in a small number of SCVs in our study as compared with the results of some previous studies. Auxotrophism was reported for haemin in 10 strains, for menadione in two strains and for thymidine in 41 strains among 78 *S. aureus* SCVs by Kahl *et al.* However, the selection of these auxotrophic strains was attributed to long-term trimethoprim–sulphamethoxazole prophylaxis or interventional aminoglycoside treatment [11]. In another study, thymidine auxotrophism was detected in 63% of the strains [9]. The lower number of auxotrophic strains detected in our study might be attributable to the fact that there is no antistaphylococcal prophylaxis and no predominant use of aminoglycoside treatment in cases of staphylococcal lung disease in our centre. Also, the SCV phenotype of our strains might also be attributable to CO_2 auxotrophism, which we could not detect. Another possible explanation could be the reversion of some of the strains during auxotrophism testing on Mueller–Hinton agar. Besides MHA, several other media, such as chemically defined medium (CDM) or tryptic soy agar, were used in previous studies [5,11]. Further studies are needed to determine which media are best for testing auxotrophism in *S. aureus* SCVs.

As *S. aureus* SCVs grow slowly, they have decreased susceptibility to cell wall-active antibiotics [26,27]. *In vitro* and *in vivo* selection of mutants that are more resistant to ciprofloxacin among clinical *S. aureus* SCVs has been reported before [9,28,29]. Thus, it is not surprising that the number of strains that were resistant/intermediately resistant to ciprofloxacin was higher than the number that were resistant/intermediately resistant to the other antibiotics among normal *S. aureus* and SCV strains, as fluoroquinolones have been used predominantly in our study population. All of the thymidine-dependent strains that formed fried-egg colonies were resistant to trimethoprim–sulphamethoxazole in our study, in agreement with previous studies [1]. Although *S. aureus* SCVs were found to be more antibiotic-resistant than their simultaneously isolated counterparts, no significant difference was detected, probably because of the small number of strains tested.

The intracellular activity of tigecycline was evaluated in an infected cell model, and no higher activity was observed against SCV strains than against normal or revertant phenotypes [23]. To the best of our knowledge, our study is the first to show that MIC values for tigecyline were significantly higher in SCVs than in the simultaneously isolated normal *S. aureus* strains. Tigecycline resistance among *S. aureus* strains is very rare [13]. Consistent with the literature, we did not detect any resistance among normal *S. aureus* strains. However, the noteworthy finding of our study is that resistant strains are SCVs.

Genotyping by PFGE revealed clonality between *S. aureus* SCVs and simultaneously isolated normal *S. aureus* strains in a group of patients. However, some of the patients harboured different clones. When consecutive strains of patients were analysed longitudinally, it was observed that *S. aureus* strains in some patients could transform into different genotypes. The persistence of a predominant clone in a patient might indicate prevention of colonization by other lineages in the airways of patients, probably because of bacterial interference. Although SCVs and normal *S. aureus* strains have a tendency to persist in CF airways, transient/persistent colonization by another clone could be possible. Similar observations have been made for persistent *P. aeruginosa* colonization in CF patients [30,31].

This first study to evaluate *S. aureus* SCVs in CF patients in Turkey indicated the need for proper isolation, identification and reporting of *S. aureus* SCVs in the CF population, as the presence of *S. aureus* SVCs in airways will probably indicate adaptation of bacteria in airways and persistent infection. This information might have an impact on antibiotic treatment decision-making, as SCVs tend to resist intracellular killing and are more resistant to antibiotics.

Acknowledgements

We are deeply grateful to M. Hayran for his help with the statistical analysis.

Transparency Declaration

This research was supported by grants from Hacettepe University Scientific Research Foundation, Ankara, Turkey (Project no. 06 D03 101 007). We declare that we have no conflicts of interest.

References

1. Kahl BC, Duebbers A, Lubritz G *et al.* Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *J Clin Microbiol* 2003; 41: 4424–4427.
2. Steinkamp G, Wiedemann B, Rietschel E *et al.* Prospective evaluation of emerging bacteria in cystic fibrosis. *J Cyst Fibros* 2005; 4: 41–48.
3. Goss CH, Mayer-Hamblett N, Aitken ML *et al.* Association between *Stenotrophomonas maltophilia* and lung function in cystic fibrosis. *Thorax* 2004; 59: 955–959.
4. Proctor RA, von Eiff C, Kahl BC *et al.* Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* 2006; 4: 295–305.
5. Proctor RA, van Langevelde P, Kristjansson M *et al.* Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis* 1995; 20: 95–102.
6. von Eiff C, Bettin D, Proctor RA *et al.* Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clin Infect Dis* 1997; 25: 1250–1251.
7. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphlococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J Clin Microbiol* 1992; 30: 1654–1660.
8. Lannergard J, von Eiff C, Sander G *et al.* Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2008; 52: 4017–4022.
9. Besier S, Smaczny C, von Mallinckrodt C *et al.* Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease. *J Clin Microbiol* 2007; 45: 168–172.
10. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*. CLSI Document M100-S18. Wayne, PA: CLSI, 2008.
11. Kahl B, Herrmann M, Everding AS *et al.* Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J Infect Dis* 1998; 177: 1023–1029.
12. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; twentieth informational supplement*. CLSI document M100-S20. Wayne, PA: CLSI, 2010.
13. European Committee on Antimicrobial Susceptibility Testing: Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3, 5 January, 2011.
14. Goering RV, Winters MA. Rapid method for epidemiological evaluation of gram-positive cocci by field inversion gel electrophoresis. *J Clin Microbiol* 1992; 30: 577–580.
15. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
16. Cystic Fibrosis Foundation Patient Registry. Annual Data Report. Bethesda, MD: Cystic Fibrosis Foundation, 2009.
17. Vergison A, Denis O, Deplano A *et al.* National survey of molecular epidemiology of *Staphylococcus aureus* colonization in Belgian cystic fibrosis patients. *J Antimicrob Chemother* 2007; 59: 893–899.
18. Goss CH, Muhlebach MS. *Staphylococcus aureus* and MRSA in cystic fibrosis. *J Cyst Fibros* 2011; 10: 298–306.
19. Proctor RA, Balwit JM, Vesga O. Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. *Infect Agents Dis* 1994; 3: 302–312.
20. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 2003; 168: 918–951.
21. Schneider M, Muhlemann K, Droz S *et al.* Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol* 2008; 46: 1832–1834.
22. Hoffman LR, Deziel E, D'Argenio DA *et al.* Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2006; 103: 19890–19895.
23. Nguyen HA, Denis O, Vergison A *et al.* Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: study of antibiotic combinations. *Antimicrob Agents Chemother* 2009; 53: 1443–1449.
24. Nguyen HA, Denis O, Vergison A *et al.* Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis

patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. *Antimicrob Agents Chemother* 2009; 53: 1434–1442.
25. Gomez-Gonzalez C, Acosta J, Villa J *et al.* Clinical and molecular characteristics of infections with CO_2-dependent small-colony variants of *Staphylococcus aureus*. *J Clin Microbiol* 2010; 48: 2878–2884.
26. Proctor R. Bacterial energetics and antimicrobial resistance. *Drug Resist Updates* 1998; 1: 227–235.
27. von Eiff C, Peters G, Becker K. The small colony variant (SCV) concept—the role of staphylococcal SCVs in persistent infections. *Injury* 2006; 37 (suppl 2): 26–33.
28. Pan XS, Hamlyn PJ, Talens-Visconti R *et al.* Small-colony mutants of *Staphylococcus aureus* allow selection of gyrase-mediated resistance to dual-target fluoroquinolones. *Antimicrob Agents Chemother* 2002; 46: 2498–2506.
29. Besier S, Zander J, Kahl BC *et al.* The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 2008; 52: 2183–2189.
30. Sener B, Koseoglu O, Ozcelik U *et al.* Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. *Int J Med Microbiol* 2001; 291: 387–393.
31. Cramer N, Wiehlmann L, Tummler B. Clonal epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis. *Int J Med Microbiol* 2010; 300: 526–533.