PREVALENCE, ANTIBIOTIC AND PULSED-FIELD GEL ELECTROPHORESIS PATTERNS OF *STAPHYLOCOCCUS AUREUS* SMALL-COLONY VARIANTS IN CYSTIC FIBROSIS PATIENTS

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Abstract. *Staphylococcus aureus* is the most common pathogen isolated from respiratory tract samples in cystic fibrosis (CF) cases. Rate of infection with *S. aureus* small-colony variants (SCVs) also is increasing in CF patients. In this study, we aimed to determine the prevalence, antibiotic susceptibility and genotypic property of *S. aureus* SCVs in respiratory tract samples of CF patients admitted to Istanbul Faculty of Medicine Hospital, Turkey. Among 305 respiratory tract samples from 84 CF patients, normal *S. aureus* isolates were present in 71% of the CF patients and *S. aureus* SCVs in 21%. The highest antibiotic resistance was against penicillin (82%) followed by clarithromycin (21%) in *S. aureus* SCVs, while resistance to levofloxacin was low (2%) in normal *S. aureus* isolates but was 16% in *S. aureus* SCVs. No *mecA* and *mecC* were detected. The *S. aureus* strains constituted 24 different genotypes based on pulsed field gel-electrophoresis assay. The possible existence of *S. aureus* SCVs that are more resistant to antibiotis than normal *S. aureus* should be taken into considerstion when treating CF patients for this pernicious bacterial infection.

Keywords: *Staphylococcus aureus*, small-colony variant, genotypic propperty, cystic fibrosis patient

INTRODUCTION

Staphylococcus aureus and *S. aureus* small-colony variants (SCVs) constitute a major portion of pathogens isolated from respiratory tracts of patients with cystic fibrosis (CF)(Govan and Deretic, 1996; Lyczak *et al*, 2002). *S. aureus* SCVs are slow-growing subpopulations of *S. aureus* that form smaller, non-pigmented and non-hemolytic colonies (Lowy, 1998). Due to differences in phenotypic features of the colonies, they can easily be overlooked as part of the normal flora in laboratory assessments (Proctor *et al*, 2006) and cause treatment failures as these pathogens can be much more resistant to antibiotics normally prescribed.

In addition, SCVs can survive intracellularly, enabling their evasion from the

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host defense system and thereby causing chronic recurrent infections. The persistence of *S. aureus* infection in CF patients is attributed to the presence of SCV variants (Kahl *et al*, 2003) and characterization of *S. aureus* SCVs have provided new insights concerning chronic *S. aureus* lung infections in CF patients (Proctor *et al*, 2014).

In this study, prevalence and antibiotic resistance patterns of *S. aureus* SCVs isolated from respiratory tract samples of CF patients and genetic relationship among these isolates were investigated.

MATERIALS AND METHODS

Sample collection

A total of 305 clinical samples, including sputum, deep throat secretion and bronchoalveoler lavage, obtained routinely from CF patients attending Istanbul University Faculty of Medicine Hospital, Turkey between April 15, 2013 and February 20, 2014 were included in the study. Deep throat secretion refers to cough throat specimen from a child who is unable to produce sputum is obtained from a swab placed at the back of the throat to induce coughing (Garcia, 2010).

Specimen cultivation

Specimens were cultured on Columbia sheep blood agar (CSBA) (Oxoid, Hampshire, UK) and mannitol salt agar medium (MSA) (Becton Dickinson, Franklin Lakes, NJ) at 35ºC aerobically for 24 and 48 hours respectively. Colonies on MSA that changed from orange to yellow due to fermentation of mannitol and hemolytic, pigmented large colonies on CSBA were considered as putative *S. aureus*. The non-hemolytic, non-pigmented, pinpoint or "fried egg" colonies on CSBA and small colonies on MSA that did not change color were considered putative *S. aureus* SCVs (Proctor and Peters, 1998; Kahl *et al*, 2003). Putative *S. aureus* SCV colonies were subcultured on to Columbia blood agar (CBA) and Schaedler agar (SA) (Becton Dickinson) under an atmosphere of 5-10% CO_2 at 35ºC. Normal size, hemolytic and pigmented colonies were considered *S. aureus* SCV (Kahl *et al*, 1998). For confirmation, Gram staining and catalase, coagulase and *S. aureus*-specific latex agglutination tests (Plasmatec, Bridport, UK) were performed, as well as determination for the presence of *nucA* by PCR (Brakstad *et al*, 1992).

Determination of antibiotics susceptibility

Susceptibility of normal *S. aureus* isolates (on Mueller Hinton agar) to cefoxitin (30 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin, levofloxacin (5 μg), linezolid (30 μg), penicillin G (10 IU), teicoplanin (30 μg), telithromycin (15 μg), tetracycline (30 μg), and trimethoprim-sulphamethoxazole (SXT) (1.25-23.75 μg) (Becton Dickinson) were determined using a disc diffusion method (CLSI, 2013). *S. aureus* ATCC 25923 was the bacterial control. Susceptibility of *S. aureus* SCV isolates (on Mueller Hinton agar supplemented with 5% sheep blood) to cefoxitin, penicillin G, clarithromycin, clindamycin, tetracycline, tigecycline and vancomycin (BioMérieux, France), were conducted using E-test method (CLSI 2013); and to gentamicin, levofloxacin, SXT, and rifampicin (5 μg) (Oxoid) using the disc diffusion method. Breakpoint values provided for *S. aureus* by CLSI (2013) were used for assessment of antibiotic susceptibility results. Tigecycline susceptibility was evaluated according to breakpoint of EUCAST (2013).

Methicillin resistance of *S. aureus* and *S. aureus* SCV were analyzed using cefoxi-

tin disc diffusion method and confirmed by PCR amplification of *mecA* and *mecC* (Paterson *et al*, 2012).

Determination of clonal relationship

Clonal relationship among *S. aureus* SCV isolates was determined using pulsed field gel-electrophoresis (PFGE) of *Sma*I (New England, Ipswich, MA) digested bacterial DNA and PFGE bands were categorized in accordance with criteria of Tenover *et al* (1995).

Statistical analysis

Clinical data of the patients were compared using chi-square test (SPSS version 15.0; SPSS, Chigago, IL) and a p-value <0.05 is accepted as statistically significant.

RESULTS

A total of 305 clinical samples, comprising 207 (68%) deep throat secretion, 97 (32%) sputum and one (0.3%) bronchoalveolar lavage, from 84 CF patients were assessed. There were 51 males and 33 females, with median age of 11.3 years. Sixty (71%) harbored normal *S. aureus* isolates in their respiratory samples and 18 (21%) harbored SCVs as well, of whom 7 were females and 11 were males. The median age of patients with normal *S. aureus* and *S. aureus* SCVs was 11 and 15 years, respectively. The median age of patients with SCVs was higher than CF patients.

Ninety-one normal *S. aureus* isolates were obtained from 88 samples and 38 SCV isolates from 34 samples.The prevalence of SCVs together with normal *S. aureus*-positive patients was 30% (18/60). Isolates numbers 1, 5 and 17 that were detected with different phenotypes, including pinpoint and "fried egg" colonies, from the same sample were included as different isolates. It is worth noting that isolates numbers 1 and 17 showed the same phenotypes 8 months later.

S. aureus SCVs were present in all samples (n = 34) from which methicillin-sensitive *S. aureus* (MSSA) isolates were found, but only in 2 (6%) samples with methicillin-resistant *S. aureus* (MRSA). In addition, *S. aureus* SCVs were present in 14 (41%) and 11 (32%) samples with mucoid and non-mucoid *Pseudomonas aeruginosa*, respectively. *S. aureus* SCV independent of normal *S. aureus* growth was never detected.

Among 34 samples with *S. aureus* SCVs, 16 were deep throat secretion samples and 18 were sputum. No hemolysis was found in 24/38(63%) such isolates and no pigment in 26 (68%). While narrow zones of hemolysis were observed in 11 (29%) isolates, larger hemolysis zone were evident in hemolytic colonies on SA plates. Similarly, while 9 (24%) isolates had a light yellow color, pigmented colonies were dark yellow on SA. Nine (24%) SCV isolates formed "fried egg" colonies (Fig 1) and 18 (47%) pinpoin type (Fig 2).

All *S. aureus* SCV isolates formed pleomorphic gram-positive cocci clusters of various sizes, were positive for catalase and latex agglutination test but 36/38 were coagulase positive. All of *S. aureus* SCV isolates carried *nucA* but not *mecA* or *mecC*, and all normal *S. aureus* and SCV isolates were susceptible to cefoxitin.

Antibiotics susceptibility tests revealed that among normal *S. aureus* isolates 83% were resistant to penicillin G, followed by erythromycin (22%), tetracycline (14%), and clindamycin (13%); but no isolates were resistant to linezolid, teicoplanin or telithromycin. As for *S. aureus* SCV isolates, 82% were resistant to penicillin G, followed by clarithromycin (21%), levofloxacin16%, clindamycin

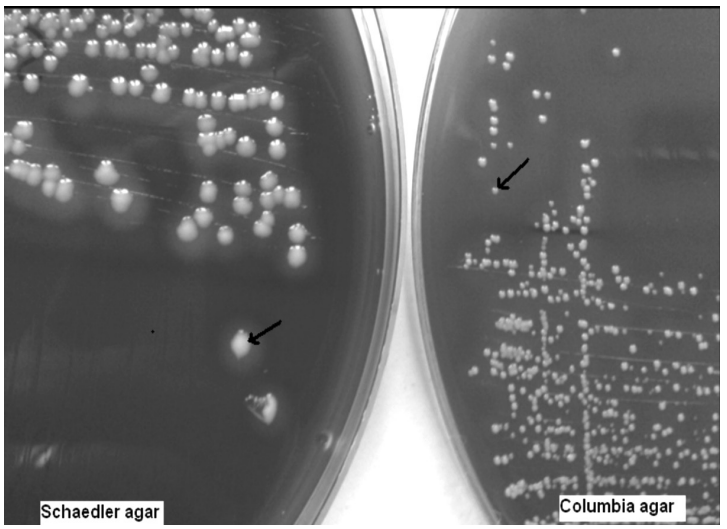

Fig 1–Hemolysis feature and colony sizes of *S. aureus* SCVs on Schaedler and Columbia agar plates. Fried egg colonies on Schaedler agar (left). Pinpoint and non-hemolytic colonies on Columbia agar (right).

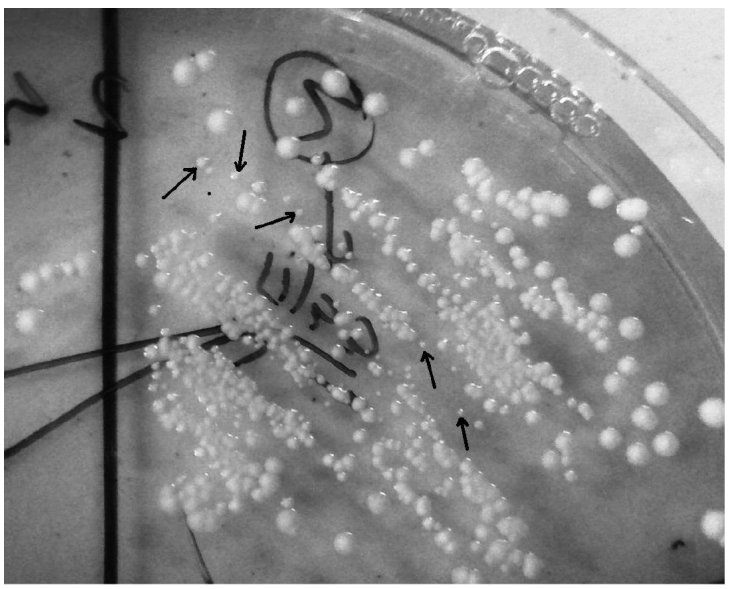

Fig 2–Normal *S. aureus* and *S. aureus* SCV colonies (marked with arrows) in mannitol salt agar medium.

and tetracycline (each 10%), and gentamicin and rifampicin (each 8%); but no resistance to vancomycin and tigecycline (Fig 3). MIC ranges and MIC_{50} and MIC_{90} values for *S. aureus* SCV isolates are presented in Table 1.

S. aureus SCV isolates from 18 patients could be divided into 22 different genotypes by PFGE (Table 2). The most predominant genotypes were 1 and 3, followed by genotype 4. Genotypes 4, 11 and 13 also were detected in the repetitive cultures of same patients. Genotypes 1 and 3 were identified not only the repetitive cultures of same patients but also from samples of other patients. Some representative PFGE patterns are shown in Fig 4.

DISCUSSION

S. aureus prevalence of varying rates has been reported in different studies carried out with samples from CF patients. Kolak *et al* (2003) reported the presence of normal *S. aureus* and/or SCV in respiratory specimens of more than 70% of CF patients. Paixoa *et al* (2010) found 35% *S. aureus* among the most frequent pathogens isolated from CF patients (Paixao *et al*, 2010). A comparable rate (50%) was obtained by Yağcı *et al* (2013).

As regards *S. aureus* SCV prevalence studies, Kahl *et al* (1998) reported a prevalence of SCVs in *S. aureus* carriers of 49.1%. Sadowska *et al* (2002) reported that in Po-

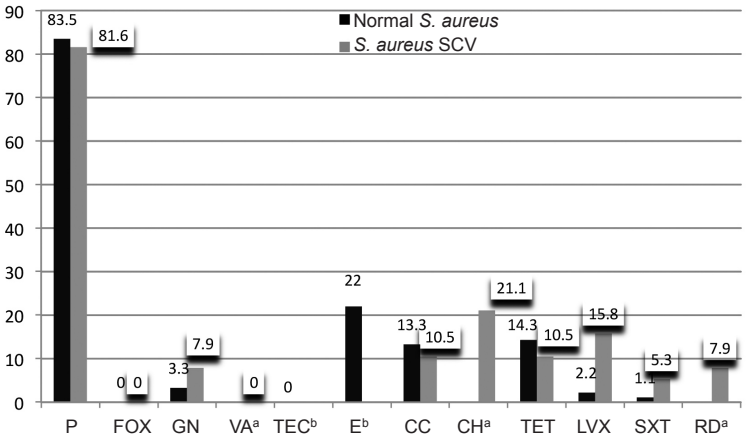

Fig 3–Resistance rates of normal *S. aureus* and *S. aureus* SCVs.

P, penicillin; FOX, cefoxitin; GN, gentamicin; VA[a], vancomycin; TEC[b], teicoplanin; E[b], erythromycin; CC, clindamycin; CH[a], clarithromycin; TET, tetracycline; LVX, levofloxacin; SXT, trimethoprim-sulphamethoxazole; RD, rifampicin. [a]Only for SCVs, [b]only for normal *S. aureus*.

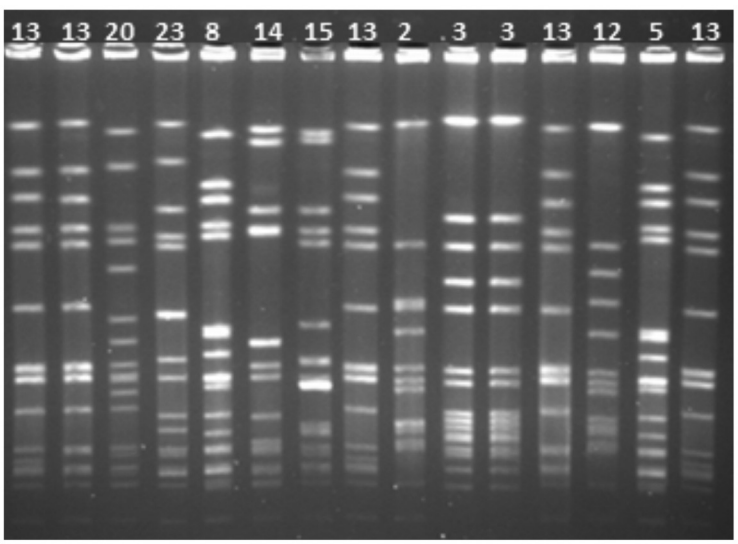

Fig 4–Pulsed-field gel electrophoresis patterns of *S. aureus* SCVs (13, control strain).

land *S. aureus* SCVs were isolated in 32% of respiratory samples containing normal *S. aureus* from CF patients between 1.5-19 years of age. In a study of Besier *et al* (2007) analyzing sputum and deep throat swab samples of 252 CF patients, 48% of the patients were colonized/infected with *S. aureus* and that SCVs were detected in 17% of such samples. More recently, Wolter *et al* (2013) detected *S. aureus* SCVs were in 24% of CF patients while normal *S. aureus* was isolated from 88% of the patients, and Yağcı *et al* (2013) reported a prevalence of *S. aureus* SCVs of 6.4%. The high prevalence rates in the aforementioned studies could be due to the high usage of SXT in the populations where the studies were carried out.

In our study, *S. aureus* SCVs were isolated in 21% of CF patients and in 11% of 305 clinical samples, much higher prevalence rates compared to other studies in the literature. This might be an indicator of increasing rate of *S. aureus* SCV infection and/or colonization in CF patients, and this issue in Turkey needs to be addressed. In addition,the presence of isolates with different phenotypes from the same sample and their persistence in some patients showed that *S. aureus* SCVs can have different phenotypes and persistence as observed with *P. aeruginosa* and *S. aureus* co-infection (Hogardt and Heesemann, 2010; Abdul-Wahab *et al*, 2014).

Table 1
MIC values of *S.aureus* SCV strains (*N*=38).

Antibiotics	MIC limit values	MIC 50	MIC90	Resistant: *n* (%)
P	<0.016-96	1.5	16	30 (81.6)
FOX	1-3	2	2	0
CC	<0.016-256	0.047	1	4 (10.5)
CH	0.047-256	0.094	256	8 (21.1)
TET	0.064-64	0.25	24	4 (10.5)
TGC	<0.016-0.19	0.047	0.094	0
VA	0.38-1	0.75	1	0

P, penicillin G; FOX, cefoxitin; CC, clindamycin; CH, clarithromycin; TET, tetracycline; TGC, tigecycline; VA, vancomycin.

The median age (11.3 years) of the patients in our study was lower than those (15 years) infected with *S. aureus* SCVs. Schneider *et al* (2008) reported that patients positive for *S. aureus* SCVs are significantly of more advanced age. Similarly, Vergison *et al* (2007) noted the median age (21 years) of CF patients positive for *S. aureus* SCV is higher compared with that (16 years) of patients with normal phenotype *S. aureus*. The detection of *S. aureus* SCVs in older CF patients with *S. aureus* infection more advanced years is thought to be caused from the increasing rate of antibiotics prescribed to CF patients over the years (Besier *et al*, 2007). Kahl *et al* (1998) reported that usage of antifolates or aminoglycosides causes an increase in the rate of *S. aureus* SCV isolation.

Various media, such as MSA, CSBA and chromogenic *S. aureus* agar, can be used for *S. aureus* SCV isolation. Studies conducted in recent years reported satisfactory results with MSA and CSBA (Vergison *et al*, 2007; Yağcı *et al*, 2013). Cultivation of clinical samples on CSBA or chromogenic *S. aureus* agar provides a rapid and accurate method (Kipp *et al*, 2005). As *S. aureus* SCVs can form pink colonies in MSA as they do not always ferment mannitol, this must be taken into consideration in their identification when using this culture medium (Proctor and Peters, 1998). The presence of pinpoint colonies, characteristic feature of *S. aureus* SCVs, along with the presence of normal size *S. aureus* colonies should be interpreted with caution. In addition to this type of colony, colonies resembling "fried egg" with tiny pigmented field surrounding the edges can be observed especially in CSBA (Kahl *et al*, 2003).

With regards to the coagulase test, it has been reported that tube coagulase test is positive after >18 hours of incubation for the majority most *S. aureus* SCVs (Proctor and Peters, 1998). On the other hand, it was reported that *S. aureus* SCV isolates can produce false negative coagulase test results and that the presence of *nuc* and *coa* should be confirmed in these types of isolates (Becker *et al*, 2004). In our hands, the coagulase test (using rabbit plasma) was 95% positive. Yağcı *et al* (2013) reported 87% positive result in detecting *S. aureus* SCVs using coagulase test with human plasma.

There is no standardized antibiotic susceptibility test for *S. aureus* SCVs. A number of methods, including disc

Table 2
Genetic diversity, co-isolation and antibiotic resistance patterns of *S. aureus* SCVs isolated from 18 CF patients.

Patient no	Date of isolation	Co-isolation	PFGE genotype	P	FOX	GN	VA	CC	CH	TET	LVX	SXT	TGC	RD
1	30.04.2013	MSSA/MPA/NMPA	13	R	S	S	S	S	S	S	S	S	S	S
	14.01.2014	MSSA/NMPA	13	R	S	S	S	S	S	S	S	S	S	S
2	12.02.2014	MSSA	20	S	S	S	S	S	S	S	S	S	S	S
3	09.09.2013	MSSA	23	R	S	S	S	S	S	S	S	S	S	S
	30.12.2013	MSSA	8	R	S	S	S	S	S	S	S	S	S	S
4	19.06.2013	MSSA	14	R	S	S	S	S	S	S	S	S	S	S
	28.01.2014	MSSA	15	R	S	S	S	S	S	S	S	S	S	S
5	14.05.2013	MSSA	2	R	S	S	S	S	S	S	S	S	S	S
	17.06.2013	MSSA	3	R	S	S	S	S	S	S	S	S	S	S
	17.06.2013	MSSA	3	R	S	S	S	S	S	S	S	S	S	S
	17.02.2014	MSSA	3	R	S	S	S	S	S	S	S	S	S	S
6	03.09.2013	MSSA	2	R	S	S	S	S	S	S	R	S	S	S
	01.10.2013	MSSA	1	R	S	S	S	I	S	S	R	S	S	S
7	16.05.2013	MSSA	12	R	S	S	S	S	R	S	S	S	S	S
8	26.12.2013	MSSA/NMPA	5	R	S	S	S	S	S	S	S	S	S	S
9	17.06.2013	MSSA/MPA/NMPA	1	R	S	S	S	S	S	S	S	S	S	S
	17.06.2013	MSSA/MPA/NMPA	6	S	S	S	S	S	S	S	S	S	S	S
	16.09.2013	MSSA	7	S	S	S	S	S	S	S	S	S	S	S
	23.12.2013	MSSA/MPA/NMPA	1	R	S	S	S	S	S	S	S	S	S	S
	18.02.2014	MSSA/MPA/NMPA	1	S	S	S	S	S	S	S	S	S	S	S
10	10.07.2013	MSSA	2	R	S	S	S	S	S	S	S	S	S	S
	13.11.2013	MSSA	17	R	S	S	S	S	S	S	S	S	S	S
	13.11.2013	MSSA	9	R	S	S	S	S	S	S	S	S	S	S
	25.11.2013	MSSA	19	R	S	S	S	S	S	S	S	S	S	S
11	25.11.2013	MSSA	3	R	S	S	S	S	S	S	S	S	S	S
12	01.11.2013	MSSA/NMPA	18	R	S	S	S	S	S	S	S	S	S	S
13	06.01.2014	MSSA/MRSA/MPA	10	R	S	S	S	S	S	R	S	S	S	S
14	23.09.2013	MSSA/MPA	2	S	S	S	S	S	S	S	S	S	S	S
15	17.09.2013	MSSA/MPA	4	R	S	R	S	R	R	R	R	S	S	R
	21.01.2014	MSSA/MPA	24	R	S	S	S	S	S	S	R	S	S	S
	06.01.2014	MSSA/MRSA/MPA	4	R	S	R	S	R	R	R	R	S	S	R
	12.11.2013	MSSA/MPA	4	R	S	R	S	R	R	R	R	S	S	R
16	14.01.2014	MSSA/NMPA	16	R	S	S	S	S	S	S	S	R	S	S
	14.01.2014	MSSA/NMPA	5	R	S	S	S	S	S	S	S	R	S	S
17	03.06.2013	MSSA/MPA/NMPA	11	S	S	S	S	S	R	S	S	S	S	S
	16.01.2014	MSSA/MPA/NMPA	11	S	S	S	S	S	R	S	S	S	S	S
18	10.09.2013	MSSA/MPA	1	R	S	S	S	S	R	S	S	S	S	S
	17.02.2014	MSSA/MPA/NMPA	1	R	S	S	S	S	R	S	S	S	S	S

PFGE, pulsed field gel electrophoresis; P, penicillin; FOX, cefoxitin; GN, gentamicin; VA, vancomycin; CC, clindamycin; CH, clarithromycin; TET, tetracycline; LVX, levofloxacin; SXT, trimethoprim-sulphamethoxazole; TGC, tigecycline; RD, rifampicin; R, resistant; S, sensitive; I, intermediate; MSSA, methicillin sensitive *S. aureus*; MRSA, methicillin resistant *S. aureus*; MPA, mucoid *P. aeruginosa*; NMPA, non-mucoid *P. aeruginosa*.

diffusion, have been used (Besier *et al*, 2007). In addition, it was reported that detection of MIC may be more accurate via such methods as broth dilution or E-test (Besier *et al*, 2007, 2008). The E-test method was utilized in our study but disc diffusion method also was performed for some antibiotics. Comparing the resistance rates of *S. aureus* SCVs with normal *S.aureus* isolates, it was noticeable that normal *S. aureus* isolates were more susceptible to gentamicin, levofloxacin and SXT, in agreement with an earlier study (Besier *et al*, 2007).

MIC values detected for tigecycline was between < 0.016 and 0.19 μg/ml for all *S. aureus* SCVs. Yağcı *et al* (2013) reported MIC values for tigecycline of <0.5μg/ml in all normal *S. aureus* isolates, but 12% of *S. aureus* SCV isolates have MIC values >1 μg/ml. This is an important finding impacting on the prescription of tigecycline for treatment of *S. aureus* infection in CF patients.

It has been reported that certain *S. aureus* SCVs are susceptible to aminoglycoside antibiotics (Von Eiff*et al*, 2006). Aminoglycoside resistance rates of *S. aureus* SCVs vary between 6.3% and 9% (Vergison *et al*, 2007; Yagci *et al*, 2013). Gentamicin resistance was 8% in our study.

Genotyping by PFGE of the 38 *S. aureus* SCV isolates revealed that certain genotypes could be detected in isolates taken from the same patient and analyzed on different dates. In addition, certain genotypes could be identified in repeat samples from the same patient and in different patients as well. Thus, patients can be persistently or temporarily infected with *S. aureus* SCVs.

In conclusion, *S. aureus* SCVs can easily be failed to be noticed and considered as normal flora in laboratory assessments due to their distinct differences in comparison with normal *S. aureus* isolates. Due to their various features, accurate diagnosis of *S.aureus* SCV infection in CF patients is of great importance for effective treatment and improved outcome.

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