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

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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*
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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