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Prevalence and Clinical Significance of *Staphylococcus aureus* Small-Colony Variants in Cystic Fibrosis Lung Disease▽

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Small-colony variants (SCVs) of *Staphylococcus aureus* can be isolated from the chronically infected airways of patients suffering from cystic fibrosis (CF). These slow-growing morphological variants have been associated with persistent and antibiotic-resistant infections, such as osteomyelitis and device-related infections, but no information is available to date regarding the clinical significance of this special phenotype in CF lung disease. We therefore investigated the prevalence of *S. aureus* SCVs in CF lung disease in a 12-month prospective study and correlated the microbiological culture results with the patients' clinical data. A total of 252 patients were screened for the presence of SCVs. The prevalence rate was determined to be 17% (95% confidence interval, 10 to 25%) among *S. aureus* carriers. *S. aureus* isolates with the SCV phenotype showed significantly higher antibiotic resistance rates than those with the normal phenotype. Patients positive for SCVs were significantly older ($P = 0.0099$), more commonly cocolonized with *Pseudomonas aeruginosa* ($P = 0.0454$), and showed signs of more advanced disease, such as lower forced expiratory volume in 1 s ($P = 0.0148$) than patients harboring *S. aureus* with a solely normal phenotype. The logistic regression model determined lower weight ($P = 0.016$), advanced age ($P = 0.000$), and prior use of trimethoprim-sulfamethoxazole ($P = 0.002$) as independent risk factors for *S. aureus* SCV positivity. The clinical status of CF patients is known to be affected by multiple parameters. Nonetheless, the independent risk factors determined here point to the impact of *S. aureus* SCVs on chronic and persistent infections in advanced CF lung disease.

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disorder in populations of European origin (26). The reported incidences range from 1 in 1,900 to 1 in 4,750 live births (7, 29). Although mutations in the cystic fibrosis transmembrane conductance regulator gene on chromosome 7 result in a myriad of medical problems, the morbidity and mortality of patients with cystic fibrosis are influenced primarily by the degree of bronchopulmonary involvement. Abnormal CF transmembrane conductance regulator function initiates a pathophysiologic cascade of chronic airway inflammation and suppurative infection resulting in progressive pulmonary insufficiency. Ultimately, 95% of patients with CF succumb to respiratory failure (19). *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* are considered to be the major pathogens that chronically infect the airways of these patients (20). With improved medical care and increasing life expectancy, however, the epidemiology of bacterial pathogens in CF patients has become more complex. In the meantime, physicians caring for these patients are increasingly confronted with new emerging bacteria and phenotypes, such as isolates of the *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *S. aureus* small-colony variants (SCVs) (9, 15, 31). The clinical significance of most of these bacteria in CF, especially *S. aureus* SCVs, is currently unclear.

In contrast to the normal *S. aureus* phenotype, SCVs grow as tiny, nonpigmented, and nonhemolytic colonies. Further characteristics are (i) altered expression of virulence genes; (ii) intracellular persistence in in vitro systems; (iii) auxotrophism for distinct growth factors, such as thymidine, hemin, and/or menadione; and (iv) the ability to revert to the normal phenotype (16, 24). *S. aureus* SCVs can be particularly isolated in the context of chronic infections, such as osteomyelitis, persistent skin and wound infection, device-related infections, and CF lung disease (1, 8, 18, 25, 27, 30, 34, 35). The pathogenesis of *S. aureus* SCVs in persistent infections is not fully understood, but one recent study has demonstrated the virulence of a site-directed hemin-auxotrophic *S. aureus* SCV mutant in a murine model of septic arthritis (14). To date, only a few data are available regarding the effects of *S. aureus* SCV on the morbidity of CF patients (18). Hence, our study was aimed at investigating the clinical significance of *S. aureus* SCVs in CF. For this purpose, we (i) determined the prevalence of this phenotype in a large German CF center and (ii) correlated the microbiological culture results with the participating patients' epidemiological and clinical data.

MATERIALS AND METHODS

Patients and specimens. The study was performed at the CF center of the University Hospital of Frankfurt am Main, Frankfurt am Main, Germany. Quarterly routine respiratory specimens of CF patients attending the pediatric or adult ward between January 2004 and December 2004 were screened for the presence of *S. aureus* SCVs. The specimens comprised sputum samples as well as deep throat swabs of children who were not able to produce sputum.

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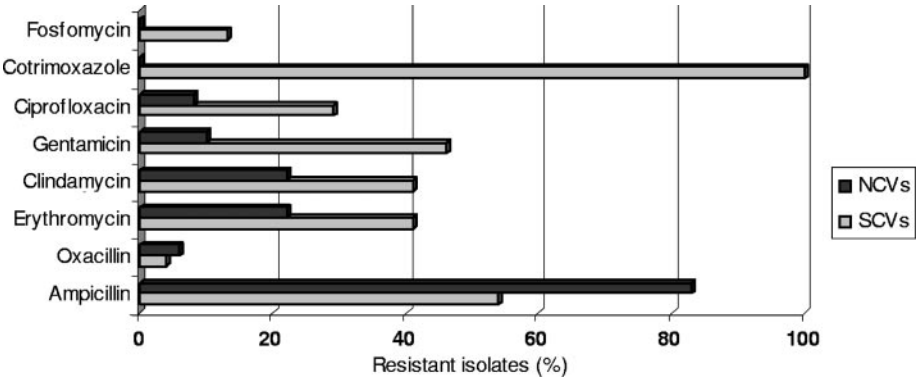

FIG. 1. Percent antimicrobial resistance of *S. aureus* isolates. Isolates with the SCV (n = 24) and NCV (n = 110) phenotypes are compared.

Laboratory methods. Routine specimens were cultured on sheep blood agar, chocolate agar, endo agar, mannitol salt agar (heipha Diagnostika, Eppelheim, Germany), and *B. cepacia* selective agar (bioMérieux, Nürtingen, Germany) at 35°C for at least 48 h. All pathogens from sheep blood agar suspected to be *S. aureus* and all different colony types growing on mannitol salt agar were further isolated at 35°C for 24 to 48 h on sheep blood agar. Isolates morphologically consistent with either SCV or the normal phenotype of *S. aureus* were subjected to species identification, which comprised agglutination with Slidex Staph Plus (bioMérieux), tube coagulase testing (Becton Dickinson, Heidelberg, Germany), Api ID 32 Staph (bioMérieux), and sequence analysis of the 16S rRNA gene (11). Antimicrobial susceptibility was determined by the disk diffusion method according to CLSI (formerly NCCLS) guidelines (6). Isolates with a normal phenotype were tested on Mueller-Hinton (MH) agar, and SCVs were tested on MH agar supplemented with 5% sheep blood (heipha Diagnostika). If susceptibility testing was suggestive of methicillin-resistant *S. aureus*, a *mecA*-specific PCR was employed for confirmation of methicillin-resistant *S. aureus* (37). Auxotrophy for hemin (5.4 μg) was tested by using standard disks (Oxoid, Wesel, Germany), and for thymidine and menadione, it was tested by impregnating disks with 1.5 μg thymidine or 1.5 μg menadione, respectively (Sigma, Hamburg, Germany). An isolate was determined to be positive for auxotrophy if a zone of growth surrounding the impregnated disks on MH agar was detected after 24 h of incubation at 35°C. Clonal identity and relatedness of *S. aureus* isolates were analyzed by pulsed-field gel electrophoresis after SmaI restriction of whole chromosomal DNA. The procedure was performed as described previously (37).

Data collection and statistical analysis. Clinical data were collected by review of medical records. Age, gender, length, weight, body mass index (BMI; patients >16 years of age), percentage of ideal weight for height, age, and gender (weight/height ratio, patients ≤16 years of age), being underweight, forced expiratory volume in 1 s (FEV_1) as percent predicted values (patients ≥6 years of age), cocolonization with *P. aeruginosa* or *B. cepacia*, need for CF-related intravenous (i.v.) antibiotics during the study period, and oral long-term antibiotic prophylaxis (200 or more days per year), especially with trimethoprim-sulfamethoxazole (SXT), during the last 3 years were recorded as hallmarks of epidemiology and morbidity. Weight, height, and FEV_1 were documented quarterly. The best values of BMI and FEV_1 during the study period were used for statistical analysis. Being underweight was defined as a BMI of <19 or a weight/height ratio of <90%, respectively. Three groups of patients were analyzed: group 1 included patients positive for *S. aureus* SCVs, group 2 included patients negative for SCVs but positive for the normal *S. aureus* phenotype, and group 3 included patients entirely negative for *S. aureus*. Two-tailed Fisher's exact test was used to analyze binary variables, whereas continuous scaled variables were evaluated with the nonparametric Kruskal-Wallis test. Continuous data are expressed as median and range. To analyze independent risk factors, a multivariate analysis using the logistic regression model was applied. P values of <0.05 were considered to be statistically significant. Statistical analysis was performed using BiAS software, version 8.1, and SPSS software, version 12.0.

RESULTS

Prevalence of *S. aureus* SCVs. A total of 267 CF patients were screened for the presence of *S. aureus* SCVs in respiratory specimens. Fifteen patients were excluded because of incomplete clinical data. Of the remaining 252 patients, 120 (48%; 95% confidence interval [CI], 41% to 54%) harbored *S. aureus* in their respiratory specimens. Of these 120, 100 (83%; 95% CI, 75% to 90%) had isolates displaying only normal colony variants (NCVs), whereas 20 (17%; 95% CI, 10% to 25%) harbored *S. aureus* SCVs. Among the 20 SCV carriers, 15 patients had SCVs plus normal *S. aureus*, whereas 5 patients carried SCVs alone. One hundred thirty-two CF patients (52%; 95% CI, 46% to 59%) tested negative for *S. aureus*. Persistent colonization defined as three or more positive cultures during the study period could be demonstrated for 50% (n = 10) of SCV carriers.

Characteristics of patients. The median age of patients at study entry was 19 years (range, 0 to 61 years). Nearly half (n = 116 [46%]) were female. Colonization with *P. aeruginosa* was evaluated for 65% (n = 164) of the patients and with isolates of the *B. cepacia* complex for 4% (n = 10) of the patients. The median BMI was 21 (range, 15 to 37), and the median weight/height ratio was 99% (range, 70 to 190%). The percentage of underweight patients was determined as 23% (n = 57). The median FEV_1 predicted value was 76.2% (range, 19 to 141%), and 35% of patients (n = 87) had CF-related i.v. antibiotic therapy during the study period. Prior oral long-term antibiotic prophylaxis was documented for 43% of CF patients (n = 108). SXT (n = 38), cefaclor (n = 22), azithromycin (n = 21), and cefuroxime-axetil (n = 17) were used predominantly as oral long-term prophylactic agents, whereas amoxicillin-clavulanate, cefixime, clarithromycin, clindamycin, doxycycline, ciprofloxacin, levofloxacin, and moxifloxacin were prescribed more infrequently.

Characteristics of *S. aureus* SCVs. Molecular typing by pulsed-field gel electrophoresis identified 17 different genotypes. In detail, 15 clones were individual clones, each isolated from a single patient, while 2 clones were clonal lineages, each cultured from three patients, suggesting patient-to-patient transmission. One patient harbored two different clones. With regard to antibiotic resistance and colony morphology, the 20 SCV carriers had 24 different SCV phenotypes. As shown in Fig. 1, the resistance profile of the SCV isolates differed significantly from the resistance pattern of isolates displaying only the normal *S. aureus* phenotype. Thus, isolates with the SCV

TABLE 1. Epidemiological and clinical characteristics of study patients

Variable	Result for patients:		
	S. aureus positive (n = 120)		*S. aureus* negative (n = 132)
	SCV (n = 20)	NCV (n = 100)	
Median age (range) in yr at study entry	31.5 (2–61)	16.5 (0–45)	20 (1–54)
No. (%) of men	8 (40)	55 (55)	73 (55)
No. (%) positive for *P. aeruginosa*	16 (80)	53 (53)	95 (72)
No. (%) positive for *B. cepacia*	0 (0)	3 (3)	7 (5)
No. (%) underweight[a]	5 (25)	14 (14)	38 (29)
Median (range) % predicted FEV_1[b]	67 (26–104)	85 (26–141)	71 (19–131)
No. (%) who needed i.v. antibiotic[c]	13 (65)	23 (23)	51 (39)
No. (%) with prior long-term oral antibiotics[d]	12 (60)	22 (22)	74 (56)
No. (%) with prior use of SXT[d]	8 (40)	8 (8)	22 (17)

[a] Defined as BMI of <19 or weight/height ratio of <90%.
[b] Patients aged ≥6 years: n = 19 and 90 for *S. aureus*-positive SCV and NCV, respectively, and n = 111 for *S. aureus* negative.
[c] During study period.
[d] During the last 3 years.

phenotype were more frequently and significantly resistant to SXT ($P < 10^{-6}$), gentamicin (P = 0.0001), fosfomycin (P = 0.0052), and ciprofloxacin (P = 0.0096). Furthermore, SCVs showed higher resistance rates for erythromycin and clindamycin than normal isolates (42% versus 23%, respectively), although the observed differences were not significant (P = 0.0673). Resistance to oxacillin was similarly distributed between these two phenotypes (P = 1). The only antibiotic agent with a significant higher resistance rate for NCVs was ampicillin (P = 0.0042). Analysis of the underlying auxotrophism of SCV isolates revealed a predominance of thymidine dependence (63%; n = 15). One combined auxotrophism (thymidine and menadione) could be detected. It was not possible to determine the underlying auxotrophism for eight isolates. Whereas six of these isolates were unstable and reverted before or during auxotrophism testing, two isolates showed no auxotrophy for the growth factors tested. Most of the SCVs (67%; n = 16) exhibited the known "fried-egg" phenotype. Furthermore, 17% (n = 4) showed a pinpoint phenotype, whereas four strains exhibited a mucous phenotype not previously described (17).

Association between microbiological culture results and clinical data. In comparison to CF patients with only NCVs in their respiratory specimens, SCV carriers showed many epidemiological and clinical characteristics (Table 1). Thus, patients positive for SCVs were significantly older (P = 0.0099). Accordingly, SCV-positive patients showed cocolonization with *P. aeruginosa* significantly more often than patients with normal *S. aureus* only (P = 0.0454). Furthermore, patients positive for SCVs showed significant lower FEV_1 values (P = 0.0148). Their median FEV_1 predicted value was 18% less than that of patients with only the normal phenotype. Additionally, SCV-positive patients needed CF-related intravenous antibiotic therapy significantly more often (P = 0.0004) and had received prior oral long-term antibiotic prophylaxis more frequently (P = 0.0012), especially with SXT (P = 0.0008). There were no significant differences between these two groups regarding the variables gender (P = 0.3268), BMI (P = 0.5010), weight/height ratio (P = 0.951), and being underweight (P = 0.3101). Between the group of patients positive for *S. aureus* SCVs and the group of patients entirely negative for *S. aureus*, only two significant differences could be detected. Patients with isolation of *S. aureus* SCVs needed intravenous antibiotics more often (P = 0.0307) and received oral long-term antibiotic prophylaxes with SXT more frequently (P = 0.0209). Some significant differences also could be found between CF patients harboring only normal *S. aureus* in their respiratory specimens and patients entirely negative for *S. aureus*. The former were less often cocolonized with *P. aeruginosa* (P = 0.0037), had a higher BMI (P = 0.014), and showed higher FEV_1 predicted values (P = 0.026). There were no significant differences regarding a cocolonization with *B. cepacia* between all groups of patients.

Evaluation of independent risk factors for being SCV positive. A logistic regression model was applied to determine the independency of epidemiological and clinical variables that were correlated with the presence of *S. aureus* SCVs. As shown in Table 2, the variables weight, age, and prior oral long-term prophylaxes with SXT were significantly and independently associated with the presence of *S. aureus* SCVs in the respiratory specimens of CF patients. Age and antibiotic prophylaxis with SXT showed a positive correlation with the SCV phenotype. The variable weight, however, is negatively correlated with the presence of SCVs. Each increase in weight decreases the probability of being positive for *S. aureus* SCVs.

DISCUSSION

The SCV phenotype is a morphological as well as a physiological variant described for several CF pathogens, including *P. aeruginosa*, *B. cepacia*, and *S. aureus* (12, 13, 18). Although SCVs of *S. aureus* have been described for more than 50 years,

TABLE 2. Epidemiological and clinical characteristics independently associated with the presence of *S. aureus* SCVs in respiratory specimens

Variable	Regression coefficient (β)	SD	P value
Age	−0.145	0.041	0.000
Wt	0.064	0.027	0.016
SXT therapy	−2.284	0.726	0.002

few data are available regarding their pathogenic role in chronic, persistent, and/or relapsing infections (10). In some small, yet well-documented, case series, the clinical features of chronic infection could be directly related to the SCV phenotype (1, 18, 25, 30, 34, 35). In addition, some infection models addressed the question of whether SCVs differ in virulence from isolates that show the normal phenotype. Depending on the model used, however, various authors arrive at dissimilar results. In a murine model of septic arthritis, the SCVs appeared to be more virulent; in the rabbit endocarditis model, virulence was determined to be equal; and in the *Caenorhabditis elegans* model, SCVs were shown to be less virulent than the parental isolates (2, 14, 28). All of these studies, however, examined the virulence of hemin- and/or menadione-auxotrophic mutant clones or clinical isolates, which are frequently encountered in the context of osteomyelitis and device-related infections. The clinical significance and pathogenic role of CF-related *S. aureus* SCVs, however, which are known to be predominantly thymidine auxotrophs, have not been elucidated so far. To the best of our knowledge, the present study is the largest of its kind to investigate the prevalence of *S. aureus* SCVs in the respiratory specimens of CF patients, including an in-depth analysis of the effects of this special phenotype on the clinical status of CF patients (18). We have screened a total of 252 CF patients comprising all age groups. The prevalence of *S. aureus* SCVs was determined to be 17% among *S. aureus* carriers, thereby indicating, in accordance with the pertinent literature, that *S. aureus* SCVs can be isolated often and repeatedly from the chronically infected airways of CF patients (8). One study reported a higher prevalence of *S. aureus* SCVs among CF patients (49.1%), but this phenomenon may be related to differences in the study population and especially the more frequent use of SXT in the study of Kahl et al. (18). Our comprehensive analysis of data suggests that the presence of *S. aureus* SCVs in CF lung disease is associated with more advanced disease. In comparison to patients with merely normal *S. aureus*, carriers of the SCV phenotype are significantly older, are more commonly cocolonized with *P. aeruginosa*, and show a poorer clinical status, as indicated by lower FEV_1 percent predicted values, intense need of i.v. antibiotics, and frequent use of prophylactic long-term antibiotics. After adjusting for potential confounders, the logistic regression model determined three independent variables that are significantly combined with the presence of *S. aureus* SCVs, namely, lower weight, advanced age, and prior long-term prophylaxis with SXT. The significant correlation between the clinical parameter decreased weight and the increased probability of testing positive for *S. aureus* SCVs favors the hypothesis that these patients suffer from a persistent but indolent "smoldering" infection inasmuch as chronic infections commonly induce unwanted weight loss (3, 23).

The association between *S. aureus* SCVs and advanced age of the CF patients studied may be surprising since in CF the highest colonization rates with *S. aureus* are commonly found during infancy. CF has a unique set of bacterial pathogens that are frequently acquired in an age-dependent sequence. Whereas *S. aureus*, at least the normal phenotype, is often the first pathogen cultured from the respiratory tract of young CF children, this organism is often replaced in adulthood by *P. aeruginosa* (7). Interestingly, the SCV carriers studied here showed in comparison to the distinctly younger NCV carriers significantly increased consumption of antimicrobial agents. According to the literature, the emergence of SCVs is enhanced in vitro by prior exposure to diverse antimicrobials and, in vivo, by treatment with antifolates or aminoglycosides (18, 21). Thus, the high consumption of antibiotics may be the reason for the remarkable association between *S. aureus* SCVs and advanced age. The resulting high antibiotic selection pressure, in turn, obviously explains the high resistance rates of SCV isolates against the antimicrobial agents routinely used in CF therapy. Generally, each acquisition of resistance is associated with a distinct fitness cost for the resistant bacteria (4, 5, 36). For example, SCVs gain antifolate resistance at the cost of thymidine auxotrophism. However, due to increased adaptation, this versatile *S. aureus* variant is able to survive in the hostile milieu of the airways. Apparently, the SCVs are able to compensate for the thymidine auxotrophism by the use of exogenous nucleotide sources which are available in quantity in the pus of CF patients.

Antibiotic therapy, however, cannot be the only variable that triggers persistence of SCVs, since SCVs can be isolated even after prolonged antibiotic-free intervals (15). Intracellular survival of *S. aureus* SCVs is a sophisticated mechanism that probably contributes to this phenomenon. In fact, several in vitro models have shown that SCVs are able to persist intracellularly (22, 32, 33). The intracellular location provides a niche for the bacteria, where they are protected against host defense and antibiotic therapy. Here, the SCV carriers were significantly more often cocolonized with *P. aeruginosa* than patients with normal *S. aureus*. Regarding this phenomenon of frequent cocolonization, it is tempting to speculate that, in addition to antibiotic therapy, the extracellular proliferation of *P. aeruginosa* facilitates the intracellular retreat of *S. aureus* as a small-colony variant.

In conclusion, this is the first report to provide evidence that the presence of *S. aureus* SCVs in CF lung disease is associated with more advanced disease. Lower weight, advanced age, and prior prophylaxis with SXT are determined to be independent risk factors for SCV positivity, thus supporting the hypothesis that this particular phenotype is involved in the persistence of chronic infections in CF lung disease.

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