Insights into the Emergence, Clinical Prevalence, and Significance of *Staphylococcus aureus* Small Colony Variants

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Abstract Small colony variants (SCVs) of *Staphylococcus aureus* (*S. aureus*), one of the most commonly observed pathogens, have been observed in clinical patients for more than half a century in a variety of infectious diseases (e.g., osteomyelitis, cystic fibrosis, endocarditis, skin infections, and abscess). The presence of *S. aureus* SCVs in patients has been rising and recent clinical studies have raised concerns about their potential roles in chronic and persistent infections. In this chapter, the emergency and clinical prevalence of *S. aureus* SCVs are examined; their characteristics and types of samples and techniques studied are discussed; and perspectives and recommendations for their diagnosis, pathogenesis, and treatment are proposed. Clinical cases involving *S. aureus* infections lasting for weeks or longer, or when pinpoint colonies are noted on routine cultures, should be screened for *S. aureus* SCVs and, if present, antibiotics that are effective in eliminating *S. aureus* SCVs must be considered.

Keywords Small colony variant · *Staphylococcus aureus* · Chronic infection · Persistent infection · Intracellular disease

Introduction

Staphylococcus aureus (*S. aureus*) is found to be most prevalent in inpatient specimens and second most prevalent in outpatient specimens [1]. Small colony variants (SCVs) of *S. aureus* have been reported in persistent and chronic infections as well

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as in secondary infections in genetic and acquired diseases. They have been observed in infections of implants (prosthetic joint, pacemaker), skin (cutaneous abscesses), tissue (muscular abscesses), bone (osteomyelitis), blood (bacterial sepsis), sinuses (sinusitis), and airways (cystic fibrosis or CF) [2–14].

The clinical presence of *S. aureus* SCVs has been documented since the 1950s when a dwarf colony was isolated from a skin abscess [15]. This subpopulation of *S. aureus* produced colonies that were noted to be nonpigmented and nonhemolytic [16]. In 1978, using eight clinical samples, *S. aureus* SCVs were acquired from blood, osteomyelitis, subcutaneous abscess, and cerebrospinal fluid [17]. These clinical isolates displayed delayed growth and reverted to the parent strain (normal-type or wild-type) when supplemented with certain nutritional needs [16]. Quite a few recent clinical reports have raised concerns about the roles of *S. aureus* SCVs in the occurrence and persistence of infections. In 2013, Yagci et al. examined 123 CF patients (out of a total of 248 patients) with persistent airway infections and, of these patients, 16% presented with *S. aureus* SCVs [18]. In 2014, Tande et al. retrospectively examined 35 patients (among a total of 134 infected patients) with periprosthetic joint infections caused by *S. aureus*; 28.6% of these patients were found to possess *S. aureus* SCVs [19].

Infections associated with *S. aureus* SCVs seem to be chronic and usually persist even after long antimicrobial therapies. SCVs, compared to their normal-type, are more resistant to traditional antibiotics and, because of their location within the cells and their reduced uptake of antimicrobial agents, they persist [20, 21]. Certain subtypes are also associated with different treatment types, such as menadione; hemin-dependent subtypes are probably associated with the use of aminoglycoside antibiotics (e.g., kanamycin, tobramycin, gentamicin, streptomycin, and others) [22].

To deal with established infections, delivering an effective and early antimicrobial treatment works the best. In the early stages of infection, localized antimicrobial applications are preferred because they act in the location they are needed, and do not cause as many side effects as the systemic circulation is bypassed. However, the use of antibiotics likely has contributed to the appearance of *S. aureus* SCVs [4, 23, 24]. Strategies for reducing or eliminating *S. aureus* SCVs in infections have yet to be developed, but the clinical implications of SCVs are apparent. Understanding the roles and mechanisms of *S. aureus* SCVs in infection seems to be important to chronic and recurrent infections, and putting an effective therapeutic strategy in place to decrease the chance of chronic and recurrent infections will save patients' frustration, time, and money.

What Are *S. aureus* SCVs and Their Characteristics?

S. aureus may be phagocytized by professional phagocytes (e.g., macrophages) and a fraction of the phagocytized bacteria may survive and reside intracellularly. Alarmingly, bacteria like *S. aureus* have also been found to enter human cells (nonprofessional phagocytes) that do not typically phagocytize foreign materials [25].

Adherence of *S. aureus* onto such host cell surfaces is a prerequisite for such invasions. *S. aureus* is known to express an array of adhesins on their surfaces including the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). *S. aureus* utilizes these MSCRAMMs to adhere directly and efficiently to host cells or via bridging ligands with host proteins (e.g., fibronectin-binding proteins). Next, the attachment of *S. aureus* to the host cell surfaces can induce changes in the host cells' cytoskeleton which leads to the phagocytosis of *S. aureus* into the host cells [26]. Upon phagocytosis, in general, it requires several characteristics of *S. aureus* in order for them to survive intracellularly; these characteristics include resistance to the intracellular host defense mechanisms and no killing of the host cells (either by lysis or by inducing apoptosis). Once it has invaded host cells, *S. aureus* may be destroyed by the intracellular defense mechanisms, maintained as the normal-type in a relatively short time, or, for certain strains of *S. aureus*, switched to SCVs. For instance, upon infection with *S. aureus*, epithelial cells were found to contain mainly normal-type *S. aureus* in the beginning; however, the number of normal-type *S. aureus* decreased and the number of *S. aureus* SCVs increased with increasing postinfection time. *S. aureus* SCVs reached approximately 90% after 28 days [27].

S. aureus SCVs are phenotypically quite different from the normal-type strain and, clinically, their variations are mostly limited to types deficient in electron transport substrates (menadione and hemin) and those deficient in thiamine biosynthesis. As shown in Fig. 1, *S. aureus* SCVs were observed in at least one of the airway cultures from 24 pediatric patients with CF; 6 of these patients had menadione- and/or hemin-deficient SCVs [24]. The deficiencies of menadione, hemin, and thiamine caused the colonies to grow much more slowly when compared to the normal-type and kept them from being targeted by the host's intracellular defense mechanisms.

S. aureus SCVs are much smaller (10× difference in size), nonpigmented, and nonhemolytic colonies compared to the parent strain (normal-type or wild-type) (Fig. 2); their small size makes them frequently missed in hospital laboratories. Table 1 lists the characteristics of *S. aureus* SCVs. Their prominent features include decreased pigmentation and hemolysis, increased resistance to aminoglycosides, and an unstable colony phenotype. Unlike their normal-type, *S. aureus* SCVs are mostly non-virulent which allows them to be overlooked by host cell defenses. In the normal-type, *S. aureus* produces alpha-toxin which causes an intracellular signaling cascade that results in the lysis of host cells [28]. In *S. aureus* SCVs, the synthesis of alpha-toxin is downregulated and the host cells stay intact which provides these facultative bacteria a reservoir in which to persist [26]. Some SCV phenotypes could even survive the bacteriostatic environment of the lysosome; for instance, a menadione auxotroph SCV strain, obtained from an osteomyelitis patient, survived within the lysosome of endothelial cells for 48 h [29]. However, upon supplementing the deficient substrates (e.g., menadione, hemin, and thiamine), *S. aureus* SCVs may rapidly reverse to the normal-type and lyse the host cells [22]. It is believed that the ability of *S. aureus* SCVs to persist intracellularly is due in part to the ability of *S. aureus* to phenotypically switch from the parent strain to the SCVs.

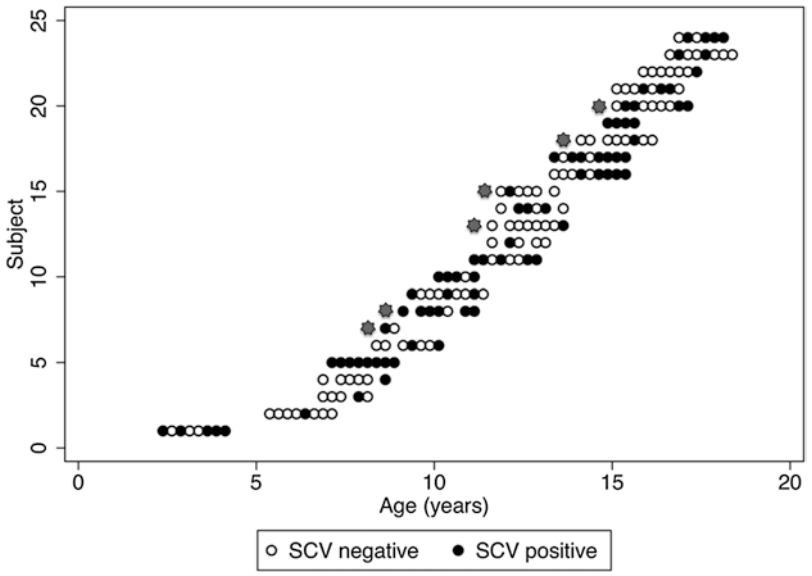

Fig. 1 Schematic of culture positivity for *S. aureus* SCVs among the 24 subjects from whom *S. aureus* SCV was isolated on one or more occasions. Each horizontal line of circles represents a series of culture results from one SCV-positive subject (each indicated by subject number on the *y*-axis) by age quarter during the study, with each culture plotted by subject age at the midpoint of each age quarter. Closed circles indicate cultures positive for *S. aureus* SCVs; open circles are negative. Asterisks indicate subjects who were culture positive for either menadione- or hemin-deficient *S. aureus* SCVs. (Reprinted with permission from Clinical Infectious Diseases 57:384–391 (2013) [24])

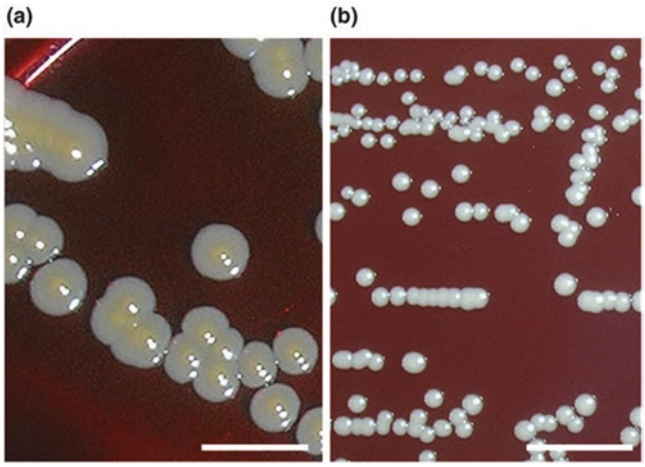

Fig. 2 Pictures of *S. aureus* of same clonal origin on sheep blood agar plates after 48 h incubation. Scale bar = 1 cm. (**a**) Normal-sized colonies, of 2–3 mm in size, show a typical golden pigmentation. (**b**) SCVs. (Reprinted with permission from Trends in Microbiology 17:54–58 (2009) [26])

Table 1 Characteristics of SCVs of *S. aureus*, as compared to parent strains

Phenotypic characteristics	
Colony size	10× smaller than normal-type colonies
Pigmentation	Weak
Hemolytic activity	Weak
Coagulase production	Weak
Resistance toward aminoglycosides	Increased
Auxotrophism	Present
Growth	Slow
Cell wall	Thick
Electrical potential across membrane	Low
Metabolism	
Tricarboxylic acid cycle	Reduced
Acetate catabolism	Reduced
Arginine deiminase pathway	Increased
Virulence determinants	
Toxin production	Weak or absent
Clumping factor	Increased levels
Fibronectin binding proteins	Increased levels
Polysaccharide intercellular adhesion	Increased
RNAIII	Very low levels
sigB	Upregulated
agr	Downregulated
hla	Downregulated

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Screening and Identification of *S. aureus* SCVs from Clinical Patient Samples

S. aureus SCVs have been isolated from a variety of clinical patient samples such as blood, bone, bronchial secretion, bronchoalveolar lavage, cerebrospinal fluid, joint aspiration, tissue aspirate, nares sample, oropharyngeal swab, pus, skin tissue, and sputum (Table 2). They have been characterized mainly by culturing for relatively long time periods and auxotrophy tests (Table 3). *S. aureus* SCVs are typically cultured, isolated, and identified as pinpoint, nonpigmented, nonhemolytic colonies after 24–72 h incubation on blood agar or Columbia agar. These characteristics set them apart from normal-type *S. aureus* which is ten times larger and golden. Normal-type *S. aureus* also grows much faster than SCVs; therefore, SCVs are overgrown by the normal-type and frequently overlooked. *S. aureus* SCVs can be further classified by testing them for thymidine, hemin, and menadione auxotrophy using agar disk diffusion tests; testing auxotrophy for hemin uses standard disks and testing auxotrophy for thymidine and menadione uses disks with thymidine and menadione, respectively. In addition, the samples must be confirmed as *S. aureus* by

Table 2 Types of clinical samples used for studies of *S. aureus* SCVs from human specimens

Disease		Sample studied	Reference
Cystic fibrosis		Bronchial secretion, sputum, bronchoalveolar lavage, or oropharyngeal swab	[6, 7, 18, 23, 24, 30, 31, 41–43]
Osteomyelitis		Bone, deep tissue aspirate	[4, 9, 17, 35]
Skin infection	Darier's disease	Skin tissue, anterior nare sample	[8]
	Hip abscess	Infected tissue	[44]
	Brain abscess	Tissue, pus	[30]
Implant/ device-related infections	Pacemaker	Blood	[9]
	Ventriculoperitoneal shunt infection	Cerebrospinal fluid	[36]
	Joint infection	Joint aspiration, tissue	[2, 47]

Table 3 Techniques applied to examine *S. aureus* SCVs in clinical samples

Technique	Procedure	Finding	Reference
Culture	Culture specimens on Columbia blood agar, brain-heart infusion agar, mannitol salt agar, and Schaedler agar. May subculture bacterial isolates representing each visible morphotype on blood agar plates	SCVs appeared as small, nonpigmented, and nonhemolytic colonies on Columbia agar while they grew normally on Schaedler agar; these SCVs were confirmed as *S. aureus* by coagulase tube test, or PCR for nuc and coa genes	[6, 7, 18, 23, 24, 30, 42]
Auxotrophy	Test auxotrophy for hemin using standard disks, and auxotrophy for thymidine and menadione using disks with thymidine and menadione, respectively	SCV demonstrated auxotrophy for hemin, menadione, or thymidine	[6, 18, 30, 31]

testing for the species specific genes *nuc* and *coa* via polymerase chain reaction (PCR) [18], 16S-rRNA-directed in situ hybridization [30], or genotypic analysis "Spa typing" [31].

Emergence and Prevalence of Clinical Cases of *S. aureus* SCVs

Due to their slow growth rate, atypical colony morphology, unusual biochemical reactions, and reduced coagulase activity, the isolation and identification of *S. aureus* SCVs have been challenging (especially in the early years). The roles of *S. aureus* SCVs in infections have been underestimated and likely have contributed to therapeutic failures in clinical settings. The first clinical observations of *S. aureus* SCVs were reported in the 1950s where pure growth of "dwarf-colony variants"

(now known as SCVs) of *S. aureus* was obtained in cultures from patients with abscesses, septic lesions, and whitlows [15, 32, 33]. Since the mid-1990s, *S. aureus* SCVs have been increasingly appreciated clinically after they were isolated from a variety of infected patients. *S. aureus* SCVs have been reported in clinical cases including CF, abscesses, bacteremia, pneumonia, septic arthritis, implant/device-related infection, and skin, soft tissue, and bone infections (Table 4). These clinical cases have shown the versatility of *S. aureus* SCVs and their presence in diverse clinical scenarios. *S. aureus* SCVs have been identified as the sole or predominant isolate in some cases [17] and have been increasingly seen in patients with recurrent, persistent infections, especially those that have been treated with antimicrobial therapy such as aminoglycosides and cell-wall-active antibiotics (Table 4).

Table 4 History of SCVs identified in diseases and their prevalence

Year	Disease	Finding	Refs.
1951	Abscess	Pure growth of "dwarf-colony variant" of *S. aureus* was obtained in primary culture of pus from an abscess and presented normal large-colony type of growth in the presence of 10% CO_2	[15]
1952	Closed septic lesion	*S. aureus* SCVs were isolated in pure culture from two independent closed septic lesions	[32]
1955	Recurrent whitlow	Pure growth of *S. aureus* SCVs were repeatedly obtained from a patient who had multiple whitlows treated with antibiotics in primary cultures of pus from two whitlows and a boil, and also from the patient's nose. There was strong evidence that these SCVs were the primary pathogens at least in the last two lesions	[33]
1969	Bacteremia, lymphatic leukemia, cadaver kidney recipient, etc.	*S. aureus* SCVs ("microcolonies") were recovered from the anterior nares of an asymptomatic adult, the blood of a patient being treated with Nafcillin for *Staphylococcal* bacteremia secondary to an infected aortic valve prosthesis, the blood of an anephric patient receiving Methicillin and chloromycetin, the blood of a patient with lymphatic leukemia receiving cortisone, methotrexate, and penicillin, and the blood of a cadaver kidney recipient receiving cortisone, penicillin, and polymyxin B treatment	[12]
1978	Pneumonia	*S. aureus* SCVs were revealed from a 29-year-old patient who developed right lower lobe pneumonia following chest trauma	[11]
1978	Infection (e.g., osteomyelitis)	*S. aureus* SCVs were identified as the sole or predominant isolate in eight human infection cases from osteomyelitis specimens, blood, subcutaneous abscess, and cerebrospinal fluid. These organisms were shown to be menadione- or thiamine-dependent	[17]
1987	Cystic fibrosis	Sputum, bronchial washings, pharyngeal swabs, tracheal aspirates, and Bartlett bronchial brushings were collected from 200 patients (0.5–37 years old), 95 patients harbored *S. aureus*, and 20/95 (21%) patients had thymidine-dependent *S. aureus* SCVs	[41]

(continued)

Table 4 (continued)

Year	Disease	Finding	Refs.
1995	Chronic osteomyelitis, septic arthritis	*S. aureus* SCVs were cultured from five patients with persistent and recurrent infections	[5]
1996	Sternoclavicular joint septic arthritis	Blood samples from an 11-year-old boy with shoulder discomfort were found to have *S. aureus* SCVs	[47]
1997	Chronic osteomyelitis	Bone specimens or deep tissue aspirates were acquired from 14 patients treated with gentamicin beads and 4/14 (29%) patients had *S. aureus* SCVs. One SCV was a menadione and three were hemin auxotrophs. There was infection recurrence in all four patients with SCVs but not in patients with normal-type *S. aureus* only	[4]
1998	Cystic fibrosis	Bronchial secretion samples were collected from 78 patients (6 months to 43 years). 53 patients harbored *S. aureus*, and among them, 26/53 (49%) patients had *S. aureus* SCVs	[7]
1998	Chronic osteomyelitis	Menadione auxotrophic *S. aureus* SCVs were recovered in multiple bone specimens from one patient with chronic osteomyelitis who had previously been treated with gentamicin beads	[34]
1999	Hip abscess	The first case of a fatal infection with *S. aureus* SCVs in an AIDS patient was reported and *S. aureus* methicillin-resistant SCVs were recovered from hip abscess	[9]
2000	Hip abscess	*S. aureus* SCVs were recovered from persistent wound infection in the right inguinal crural region after herniotomy	[44]
2001	Skin infection	*S. aureus* SCVs were detected from skin tissue and anterior nares in a 39-year-old male with Darier's disease	[8]
2002	Cystic fibrosis	63 *S. aureus* isolates were collected from sputum samples of children (1.5–19 years old); 20/63 (32%) contained *S. aureus* SCVs	[43]
2002	Chronic osteomyelitis	*S. aureus* SCVs were recovered from a patient treated with gentamicin beads	[35]
2003	Brain abscess	*S. aureus* SCVs were identified in a patient with brain abscess; the patient was treated with a combination of vancomycin and rifampin followed by prolonged treatment with teicoplanin, with no sign of infection at 9-month follow-up	[30]
2003	Cystic fibrosis	Sputum samples from 52 patients (21–72 years old) were found to present *S. aureus* and 24/52 (46%) patients had *S. aureus* SCVs	[23]
2003	Pacemaker-related infection	Blood samples from a recurrent pacemaker-related bloodstream infection contained *S. aureus* SCVs	[48]
2005	Ventriculoperitoneal shunt infection	*S. aureus* SCVs were recovered from cerebrospinal fluid in a 69-year-old woman with recurrent ventriculoperitoneal shunt-related meningitis	[36]
2006	Periprosthetic joint infection	Joint aspirations or intraoperative tissues from 5 (6%) out of 83 (66 total hip and 17 total knee arthroplasty) patients contained *S. aureus* SCVs. Intracellular cocci in fibroblasts were observed in periprosthetic tissue samples	[45]

(continued)

Table 4 (continued)

Year	Disease	Finding	Refs.
2007	Cystic fibrosis	Sputum samples and deep throat swabs were obtained from 252 patients (maximum of 61 years), and 17% (95% confidence interval, 10 to 25%) among *S. aureus* carriers had *S. aureus* SCVs	[6]
2008	Cystic fibrosis	*S. aureus* SCVs were identified in respiratory secretion samples from 8/40 (20%) patients harboring *S. aureus*, particularly those with advanced pulmonary disease and prolonged antibiotic exposures	[42]
2013	Cystic fibrosis	*S. aureus* SCVs were detected in 20 (16%) of 123 patients harboring *S. aureus*. SCVs and normal-type *S. aureus* strains showed identical genotypes in 14 patients, while 5 patients showed different genotypes	[18]
2013	Cystic fibrosis	Sputum, bronchoalveolar lavage, or oropharyngeal swabs were obtained from 100 pediatric patients (maximum of 16 years), and 24/100 (24%) patients had *S. aureus* SCVs	[24]
2015	Skin, soft tissue, and bone infections	Clinical samples were collected from skin, bone, and soft tissues, and 10 (15%) out of 66 samples with positive growth of *S. aureus* contained thymidine independent *S. aureus* SCVs	[46]
2016	Cystic fibrosis	9% of *S. aureus* positive patients were positive for *S. aureus* SCVs. 17 different SCV isolates and 12 corresponding normal-type isolates were obtained from 147 patients. 13 isolates were determined thymidine auxotroph, 2 isolates were auxotroph for hemin, and none of the tested isolates were auxotroph for both, respectively	[31]
2018	Cystic fibrosis	37 MRSA isolates from 28 patients were found to be SCVs, which presented higher rates of antibiotic resistance to moxifloxacin, erythromycin, and trimethoprim/sulfamethoxazole, compared to normal colony variant MRSA isolates. Moreover, patients with such SCVs had lower lung function, higher rates of persistent infection, compared to individuals with normal colony variant MRSA	[13]

Osteomyelitis

Osteomyelitis is an infection of the bone that occurs after trauma, surgery, presence of a foreign body such as a prosthesis or after hematogenous seeding. One of the major infecting organisms is *S. aureus* making *S. aureus* SCVs a suspect in patients that have persistent or recurrent infections. von Eiff et al. examined bone specimens and deep tissue aspirates from 14 patients with osteomyelitis [4]. Four (29%) out of the 14 patients had *S. aureus* SCVs. After antimicrobial (gentamicin) treatment for more than 4 weeks, strikingly, infection recurred in the four patients who had *S. aureus* SCVs whereas those without SCVs did not have recurrence [4].

Along with recurrent infection, patients were more likely to have *S. aureus* SCVs when they were treated with gentamicin beads; these beads release gentamicin over a period of weeks to months to provide a sustained local level of antibiotics and are commonly used to treat osteomyelitis. All four patients treated with gentamicin beads had *S. aureus* SCVs while the other patients who were not treated with gentamicin beads did not have *S. aureus* SCVs [4]. *S. aureus* SCVs were also recovered in two cases of chronic osteomyelitis where the patients were previously treated with gentamicin beads [34, 35]. These data may suggest that gentamicin beads might have selected for *S. aureus* SCVs (Table 5), which should alert physicians that gentamicin may select for *S. aureus* SCVs that could contribute to recurrent and persistent infections.

In 2006, Sendi et al. identified *S. aureus* SCVs in five periprosthetic joint infection patients [2]. These five patients had a mean age of 62, all experienced treatment failure prior to isolation of *S. aureus* SCVs despite as many as three surgical revisions and up to 22 months of antibiotics (e.g., intravenous flucloxacillin followed by a combination of rifampin and levofloxacin after a few days for methicillin-susceptible Staphylococcal infections). Even with antimicrobial therapies during early treatment consisting of various combinations of flucloxacillin,

Table 5 SCVs and normal-type of *S. aureus* in patients with or without previous local gentamicin treatment

Case no.	Colony type	Previous local gentamicin therapy	Other previous systemic antibiotics	Auxotroph	Cause of osteomyelitis	Recurrence of osteomyelitis[a]
1	n	No	PenG, Ctax, Cm, Cpfx	No	Postoperative	No
2	n	No	None	No	Hematogenous	No
3	n	No	Cm	No	Contiguous	No
4	n	No	None	No	Posttraumatic	No
5	n	No	None	No	Hematogenous	No
6	n	No	None	No	Hematogenous	No
7	n	No	Oxa	No	Posttraumatic	No
8	n	No	None	No	Hematogenous	No
9	n	No	Ctax	No	Postoperative	No
10	n	No	Amox/CA	No	Contiguous	No
11	SCV	Yes	Vm, Cfur	Hemin	Postoperative	Yes
12	SCV, n	Yes	Cm	Hemin	Postoperative	Yes
13	SCV, n	Yes	Vm	Hemin	Posttraumatic	Yes
14	SCV, n	Yes	Oxa, Ctax, Cm	Menadione	Postvaccination	Yes

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Amox/CA amoxicillin/clavulanic acid, *Cfur* cefuroxime, *Cm* clindamycin, *Cpfx* ciprofloxacin, *Ctax* cefotaxime, *n* normal, *Oxa* oxacillin, *PenG* penicillin G, *SCV* small colony variant, *Vm* vancomycin
[a]Relapse of osteomyelitis occurring more than 1 year after primary diagnosis and treatment

vacomycin plus cefepime, ciprofloxacin, and rifampin, all patients had recurrent infections. The spacers were removed after detection of *S. aureus* SCVs and antimicrobial treatment was chosen based on susceptibility testing. A combination of levofloxacin and rifampin was administered for a course ranging between 5.5 and 7 weeks. Four patients proceeded to receive reimplantations, while one refused in fear of reinfection. Follow-up for these patients ranged between 12 and 48 months; three patients were cured while two were likely cured. Therefore, it seems that, in cases that involve persistent or recurrent infections, *S. aureus* SCVs should be examined and antibiotics that can eliminate SCVs may need to be considered in order to advance toward proper treatments and possibly avoid surgical revisions. Due to the stubborn nature of SCVs, removal of all implants and extensive debridement are recommended.

Implant/Device-Related Infection

S. aureus is one of the most common causes of infections associated with biomedical implants or devices. *S. aureus* SCVs have been isolated in cases of pacemaker and ventriculoperitoneal-shunt infections in 2003 and 2005, respectively [2, 36]. In these *S. aureus* SCV cases, the patients had poor clinical and microbiologic responses to prolonged antimicrobial therapies. Patients were treated unsuccessfully with antibiotics (e.g., cefuroxime) which led to multiple instances of recurrent fever, infection, hospitalization, and surgeries. These cases further emphasize the versatility and infectious nature of *S. aureus* SCVs. With the increasing use of invasive implants/devices, *S. aureus* SCVs are expected to become more common; implant/device-related infections that are persistent and resistant should be tested for *S. aureus* SCVs. Similar to the periprosthetic joint infection cases discussed above, the best way to treat *S. aureus* SCVs was to completely remove the foreign implant or device and administer appropriate antimicrobial treatment that is effective against *S. aureus* SCVs.

Cystic Fibrosis (CF)

CF is a progressive, genetic disease that causes persistent lung infections and may also affect the pancreas, liver, kidneys, and intestines. *S. aureus* SCVs have been frequently isolated in studies involving patients affected by CF [13]. *S. aureus* is one of the most common bacteria found in the respiratory tracts of children with CF [37] and continues to be one of the major pathogens along with *Pseudomonas aeruginosa* and *Haemophilus influenza* [38]. In order to combat these pathogens, long-term prophylactic oral antimicrobial agents such as tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole (SXT), and certain cephalosporins and penicillins [39, 40] are administered. Although normal-type *S. aureus* can be eliminated

from the airways, unfortunately, *S. aureus* SCVs can form and adapt to the hostile environment leading to chronic and recurrent infections. When looking at 9 studies involving a total of 1266 patients, *S. aureus* SCVs were identified in 9%, 16%, 17%, 20%, 21%, 24%, 32%, 46%, and 49%, respectively, among patients harboring *S. aureus* (Table 4) [6, 7, 18, 23, 24, 31, 41–43]. Alarmingly, carriers of *S. aureus* SCVs had been infected with *S. aureus* longer than those with the normal phenotype [42], showed significantly higher antimicrobial resistance rates than those with the normal phenotype [6], and the presence of SCVs was directly related to poor clinical outcomes [31]. Moreover, patients positive for SCVs were significantly older [6, 18, 42], more commonly co-colonized with *Pseudomonas aeruginosa* [6], and showed signs of more advanced disease, such as lower forced expiratory volume than patients who had only normal-type *S. aureus* [6, 42]. Lower weight, advanced age, and prior use of SXT were found to be independent risk factors for *S. aureus* SCV positivity [6].

Wolter et al. illustrated a unique pattern of culture positivity for *S. aureus* SCVs in 24 pediatric patients with CF [24]. The patients tended to have "alternating positive and negative culture positivity suggesting repeated selection and enrichment for *S. aureus* SCVs, incomplete detection, or both" (Fig. 1). Infection with *S. aureus* SCVs led to a greater drop in lung function and was independently associated with worse CF respiratory outcomes (Table 6). Patients treated with SXT for longer than 18 months or those receiving interventional aminoglycoside treatment were more likely to have SCVs [23, 24]. In fact, it was indicated that SXT was the strongest predictor of *S. aureus* SCV detection suggesting that SXT strongly selected for *S. aureus* SCVs. *S. aureus* SCVs should be a concern for all CF patients especially in those with reduced lung function and those treated with antibiotics for a long period of time. Screening and identification of these SCVs can help guide proper therapeutic treatments.

Abscess

S. aureus SCVs have been isolated from abscesses. One of the first clinical cases of *S. aureus* SCVs from an abscess patient showed pure tiny colonies (i.e., SCVs) in the cultures of pus samples. The smears of these tiny colonies presented typical Staphylococcal morphology and these tiny colonies reverted to typical large Staphylococcal colonies when cultured in the presence of carbon dioxide [15]. *S. aureus* SCVs were also identified from a patient with a persistent wound infection (abscess and fistula). A combination treatment of flucloxacillin and rifampicin for 4 weeks led to healing of the chronic wound infection [44].

In another report, methicillin-resistant *S. aureus* SCVs were identified in a patient with a brain abscess [30]. In this study, computed tomography (Fig. 3) showed a left temporal mass where, 10 years earlier, a neurosurgical intervention had been performed to treat a subarachnoid hemorrhage. The patient was treated with cefamandole for 2 weeks due to a febrile episode and *S. aureus* was confirmed via 16S

Table 6 SCV status as an independent predictor of change in lung function over the study period[a]

Predictor	Coefficient estimate (mean difference in change in FEV_1% predicted over study period[b])	95% Confidence interval	*P* value
Model 1			
Ever SCV positive on study	−11.00	−18.81, −3.18	0.007
Age at enrollment	−1.31	−2.43, −0.18	0.023
FEV_1% predicted at enrollment	−0.28	−0.48, −0.08	0.007
Model 2			
Ever SCV positive on study	−11.32	−19.10, −3.55	0.005
Age at enrollment	−1.51	−2.75, −0.26	0.019
FEV_1% predicted at enrollment	−0.28	−0.48, −0.08	0.007
Ever *Pseudomonas aeruginosa* positive on study	2.53	−4.75, 9.80	0.490
Model 3			
Ever SCV positive on study	−11.29	−19.61, −2.97	0.009
Age at enrollment	−1.29	−2.44, −0.14	0.028
FEV_1% predicted at enrollment	−0.28	−.48, −0.08	0.008
Ever MRSA positive on study	0.59	−5.92, 7.10	0.857
Model 4			
Ever SCV positive on study	−12.98	−21.55, −4.41	0.004
Age at enrollment	−1.34	−2.51, −0.17	0.026
FEV_1% predicted at enrollment	−0.26	−0.47, −0.06	0.013
Ever *Stenotrophomonas maltophilia* positive on study	4.41	−2.67, 11.49	0.218
Model 5			
Ever SCV positive on study	−10.91	−18.79, −3.03	0.008
Age at enrollment	−1.28	−2.41, −0.16	0.026
FEV_1% predicted at enrollment	−0.29	−0.49, −0.08	0.007
Any exacerbations on study	−1.04	−7.55, 5.46	0.749
Model 6[c]			
Ever SCV positive on study	−11.92	−20.18, −3.66	0.006
Age at enrollment	−1.29	−2.45, −0.12	0.031
FEV_1% predicted at enrollment	−0.28	−0.49, −0.08	0.007
Use of TMP–SMX during the study	2.19	−4.54, 8.92	0.517

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FEV_1% percent predicted forced expiratory volume in 1 s, *MRSA* methicillin-resistant *S. aureus*, *SCV* small-colony variant, *TMP–SMX* trimethoprim–sulfamethoxazole

[a]Adjusting for sex did not alter the results (not shown). Each potential confounding variable was evaluated by adding it to the base model (model 1). Due to sample size constraints, we did not evaluate all potential confounding variables simultaneously

[b]For covariates coded as yes/no (such as culture positivity), this is the mean difference in change in FEV_1% predicted over the study period between subjects coded as yes versus those coded as no. For continuous covariates, this is the mean difference per 1 unit increase in covariate (e.g., for age, the mean difference per 1 year increase in age)

[c]Includes data for 59 participants who had antibiotic data reported. Similar results were obtained when adjusting for total quarters of TMP–SMX use

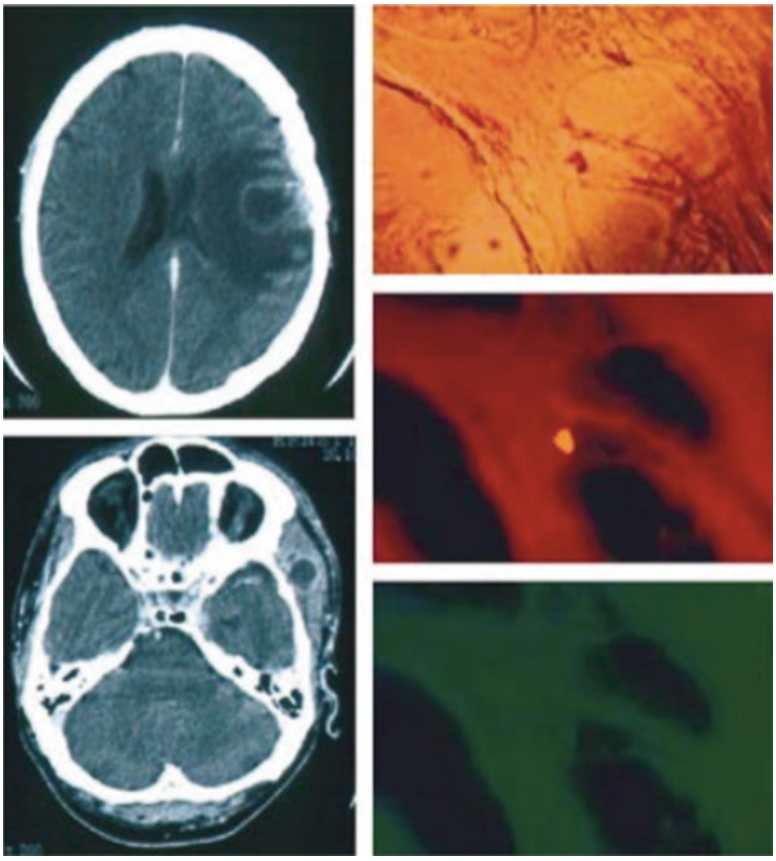

Fig. 3 Brain abscess caused by *S. aureus* SCVs. Left: cerebral computed tomography with contrast medium; (top) intracerebral abscess and (bottom) left temporal intramuscular abscess. Right: Detection of *S. aureus* cells by in situ hybridization of a tissue section obtained from brain abscess; (top) phase contrast microscopy, (middle) in situ hybridization using a Cy3-labeled *S. aureus* specific SA-P1 probe, and (bottom) control hybridization with a FLUOS-labeled *S. epidermidis* probe SEP1. (Reprinted with permission from Journal of Neurology, Neurosurgery, and Psychiatry 74:1000–1002 (2003) [30])

rRNA-directed in situ hybridization. *S. aureus* SCVs were subsequently identified after culturing tissue and pus samples from the abscess for a long time period (i.e., 72 h). This patient was treated with vancomycin, rifampin, and teicoplanin and no infection was observed at the 9-month follow-up. The medical history indicated that this patient had no signs of acute or recurrent infection between the two surgeries. The authors claimed that *S. aureus* SCVs were the causative microorganisms for the infection; to our understanding, this diagnosis was not conclusive, although it is possible that the surgery performed 10 years prior might be linked to the formation and later proliferation of *S. aureus* SCVs.

In 1999, the first case of a fatal infection with *S. aureus* SCVs was reported in an acquired immune deficiency syndrome (AIDS) patient (36-year-old man) who was under long-term treatment with trimethoprim/sulfamethoxazole for prophylaxis of *Pneumocystis carinii* pneumonia [9]. *S. aureus* methicillin-resistant SCVs were recovered from a hip abscess in the patient. Vancomycin treatment was administered but the patient's status deteriorated rapidly; the patient died of refractory septic shock 6 days after admission with fever and progressive pain (of 6 weeks duration) in the right hip [9].

Skin Infection

The first recorded case of a persistent and antimicrobial resistant skin infection due to *S. aureus* SCVs was reported in 2001 in a 39-year-old patient with Darier's disease [8]. The patient was hospitalized several times in previous years due to recurrent herpes virus infections and recurrent purulent infections. In 1999, the patient was hospitalized again. Methicillin-resistant *S. aureus* was isolated from skin and anterior nares leading to a 4-week intravenous course of antimicrobial therapy consisting of vancomycin, rifampicin, and clindamycin. A topical mupirocin ointment was also given for 2 weeks for the nasal mucosa. The skin condition did not significantly improve and topical treatments with steroids and antiseptics (povidone-iodine, chlorhexidine, and chlorquinaldol) were administered. Over a course of 28 months, 119 isolates were derived from 53 clinical specimens obtained from different areas of the affected skin and anterior nares. Hemin-auxotrophic SCVs along with various *S. aureus* strains were found in the skin infections. With this being said, *S. aureus* SCVs may be related to skin infections that persist for a long period of time and may be resistant to various therapeutic treatments.

Clinical Significance of *S. aureus* SCVs

From the clinical cases reported (Table 4), we can see that *S. aureus* SCVs have significant clinical implications because:

- *S. aureus* SCVs are found in a broad range of percentages among *S. aureus* positive patients such as 6% [45], 9% [31], 15% [46], 16% [18], 17% [6], 20% [42], 21% [41], 24% [24], 29% [4], 32% [43], 46% [23], 49% [7], and 100% [15, 32, 33]. *S. aureus* SCVs are responsible for [15, 32, 33], most likely responsible for [4, 17], or may contribute to [5–9, 11, 12, 18, 23, 24, 30, 31, 34–36, 41–48] the infections observed clinically.
- A variety of factors including lower weight, advanced age, and prior use of antibiotics may contribute to the development of *S. aureus* SCVs [6]. For instance, the history of antimicrobial treatments seems to contribute to increased occurrences

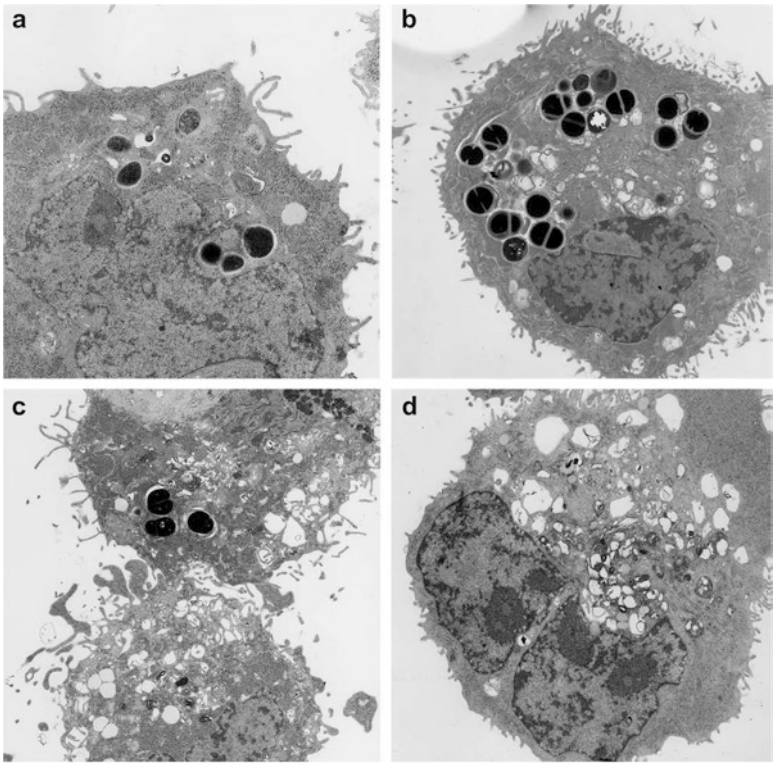

Fig. 4 Electron micrographs of keratinocyte HaCaT cells that were infected with isolates of clinical isogenic normal-type *S. aureus* and *S. aureus* SCVs. After incubation of infected HaCaT cells in the presence of lysostaphin for 30 min or 48 h (analogous to an intracellular persistence assay), cells were washed, dehydrated, and embedded in Epon. Ultrathin sections were counterstained and examined by electron microscopy. (**a**, **b**), Intracellular persistence of SCVs (SCV1) within viable HaCaT cells after (**a**) 30 min or (**b**) 48 h of incubation. Epithelial cells appear viable and show no signs of degeneration (original magnification, ×3400). (**c**, **d**) *S. aureus* of the normal phenotype (NP1) is incorporated (**c**) after 30 min by intact HaCaT cells; however, (**d**) after 48 h of incubation, most epithelial cells show severe lytic degeneration and release of bacteria (original magnification, ×3400). (Reprinted with permission from Clinical Infectious Diseases 32:1643–1647 (2001) [8])

of *S. aureus* SCVs, since *S. aureus* SCVs were more often obtained from patients who were treated with antibiotics (e.g., gentamicin) [4, 6, 9, 12, 33–35, 42, 45].

- *S. aureus* SCVs have the ability to persist longer within host cells compared to their wild-type strains (Fig. 4) [8], which may explain why *S. aureus* seems to be eliminated but infection may recur weeks or months later [33]. Because they reside intracellularly and have relatively low virulence, *S. aureus* SCVs can remain inside other cells and be protected from conventional antibiotic treatments as well as from the intracellular host defense mechanisms.
- *S. aureus* SCVs are often more resistant to antibiotics compared to their normal-type strain (Fig. 5) [6] and are difficult to eliminate. However, they can be treated but the optimal treatments still need to be identified and consequences of failure

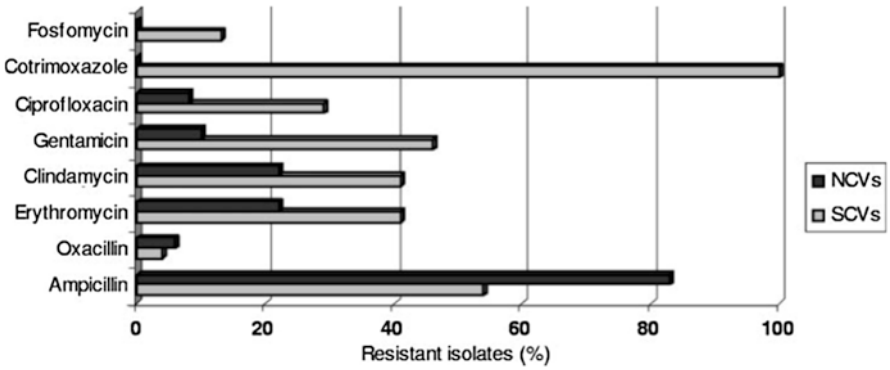

Fig. 5 Percent antimicrobial resistance of *S. aureus* isolates. Isolates with the SCV (n = 24) and normal colony variant or NCV (n = 110) phenotypes are compared. (Reprinted with permission from Journal of Clinical Microbiology 45:168–72 (2007) [6])

could be unexpected. In 2003, a patient with a brain abscess had *S. aureus* SCVs and was effectively treated with a combination of vancomycin and rifampin followed by prolonged treatment with teicoplanin; no signs of infection were observed at the subsequent 9-month follow-up [30]. However, various antibiotics have failed to treat *S. aureus* SCVs and led to recurrent infections [4] and might even have contributed to death [9].

- The presence of *S. aureus* SCVs most likely contributed to a poorer clinical outcome, since patients who had *S. aureus* SCVs were significantly more commonly co-colonized with other bacteria (e.g., *Pseudomonas aeruginosa*), infected with *S. aureus* longer, had chronic or persistent infections or infection recurrence, and presented signs of more advanced disease (e.g., lower forced expiratory volume) compared to patients who had only wild-type *S. aureus* [6, 18, 31, 42]. For instance, it was reported that four patients among 14 infected patients who had *S. aureus* SCVs all had infection recurrence while the remaining 10 patients with normal-type *S. aureus* showed no recurrence [4]. *S. aureus* SCVs were also more often observed among patients with chronic, persistent, or recurrent infections [5, 34, 36, 44, 48].

Summary and Perspectives

S. aureus SCVs have been observed in patients for more than half a century in a variety of infectious diseases including CF, sepsis, bacteremia, endocarditis, skin infections, rhinosinusitis, osteomyelitis, brain abscess, implant/device-related infections, etc. So far, clinical cases of *S. aureus* SCVs in CF patients have been documented relatively better compared to the other diseases, but this does not necessarily mean that *S. aureus* SCVs are less commonly found in the other infections. More clinical cases involving *S. aureus* infections should be examined for presence or absence of *S. aureus* SCVs. Similarly, SCVs of other bacteria should be carefully

Table 7 SCVs of non-*Staphylococcal* bacteria recovered from human specimens

Small colony variants of non-*Staphylococcal* bacteria recovered from human specimens	
Microorganism	Type or site of infection and/or specimen
Brucella melitensis	Subacute bacterial endocarditis (blood culture)
Burkholderia cepacia	Lung and other airway specimens from patients with cystic fibrosis
Burkholderia pseudomallei	Experimental melioidosis
Escherichia coli	Chronic prosthetic hip infection; urinary tract infection; human feces
Lactobacillus acidophilus	Human feces
Neisseria gonorrhoeae	Gonorrhoea (urethra, cervix, and vagina)
Pseudomonas aeruginosa	Lung and other airway specimens from patients with cystic fibrosis
Salmonella serovars	Typhoid fever

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examined and treated; SCVs are not limited to *S. aureus* and have been reported for non-*Staphylococcus* bacteria that have been recovered from human specimens (Table 7) [28]. More details on diagnosis, pathogenesis, and treatment of *S. aureus* SCVs are discussed below.

Diagnosis *S. aureus* SCVs have been identified from various specimens including bronchial secretion, sputum, bronchoalveolar lavage, oropharyngeal swab, bone, tissue/joint aspirate, skin tissue, anterior nare sample, pus, cerebrospinal fluid, and blood. Currently, most hospital laboratories have the ability to isolate, characterize, and identify normal-type *S. aureus*, via targeting genes such as *nuc*, *clfA*, *eap*, and *coa*. In contrast to the normal-type, *S. aureus* SCVs are much smaller, nonhemolytic, and nonpigmented; these characteristics have made them difficult to recover and classify. Because of their slow growth and atypical morphology, *S. aureus* SCVs are often missed, misidentified, or misinterpreted by the automated systems routinely used in many clinical laboratories. For instance, *S. aureus* SCVs were misidentified as coagulase-negative *Staphylococcus* [9, 44, 48]. Therefore, special efforts should be taken to identify *S. aureus* SCVs when an infection is particularly resistant to treatment, persists for a long period, or fails to respond to adequate antimicrobial therapy. We recommend that *S. aureus* SCVs should be suspected whenever pinpoint colonies are noted on routine cultures (even with a small number), and such samples should be run for *S. aureus* SCVs using appropriate selective media and growth conditions.

Pathogenesis The development of *S. aureus* SCVs is likely when the presence of normal-type *S. aureus* lasts for hours, weeks, or longer; we have confirmed the formation of *S. aureus* SCVs in osteoblasts and macrophages [49], and that they may contribute to bone infections in vivo [50]. The presence and contribution of *S. aureus* SCVs in clinical infections most likely have been underestimated and underreported. It is likely that *S. aureus* SCVs have played an important role in infection persistence; however, complete pathogenesis for *S. aureus* SCVs has yet to be discovered. The ability of *S. aureus* SCVs to phenotypically switch back and forth between the nor-

mal and variant forms help the organism to evade both host defense and antimicrobial treatments thereby contributing to the persistence of their associated infections. Moreover, the menadione deficient strain of *S. aureus* SCVs can form more diverse and highly structured biofilms compared to the normal-type [51] and may contribute to their persistence as well.

Treatment *S. aureus* SCVs persist intracellularly within their host cells and they are often more resistant to antibiotics than the normal-type *S. aureus*. Their ability to "hide" intracellularly may protect them from intracellular host defense mechanisms and decrease their exposure to antibiotics. As a result, *S. aureus* SCVs are difficult to treat. Two approaches may be applied. One is to prevent the development of *S. aureus* SCVs by striking early in the acute infection stage before SCVs can develop by using effective local and systemic antimicrobial treatments; screening for *S. aureus* SCVs must be done if such treatments fail since certain antimicrobial treatments may be selective (Table 5) for the development of *S. aureus* SCVs. The other approach is to identify effective antimicrobial approaches to treat *S. aureus* SCV-associated infections. Currently, the optimal treatments for infections caused by *S. aureus* SCVs have not been identified. However, some treatments seem to be promising. A combination of levofloxacin and rifampin cured three patients with *S. aureus* SCVs [45], a combination of vancomycin and rifampin followed by prolonged treatment with teicoplanin presented no sign of infection at 9-month follow-up in a patient with an *S. aureus* SCV-associated brain abscess [30]. A combination of flucloxacillin and rifampicin led to healing of a persistent wound infection associated with *S. aureus* SCVs [44]. In preclinical studies, we have shown that antimicrobial peptides may be effective in eliminating *S. aureus* that are "hiding" in other cells [52], and tuning immune responses may be promising as well [53–55]. Nanomedicine, due to the unique characteristics of nanomaterials, is also emerging as improved or alternative therapies for intracellular pathogens like *S. aureus* SCVs [56]. Therefore, there are promising treatments and we recommend that antimicrobial agents (e.g., rifampin) which have potent intracellular activity should be used in treating infections caused by *S. aureus* SCVs.

Overall, *S. aureus* SCVs should be aggressively and accurately identified whenever infections induced by *S. aureus* fail apparently "adequate" antimicrobial therapy. The identification will help physicians end ineffective antimicrobial therapeutic treatments, which may inadvertently induce the development of *S. aureus* SCVs, and promptly initiate proper antimicrobial treatments. Failure to identity and treat *S. aureus* SCVs may lead to chronic, persistent, and recurrent infections, wound complications, and even death.

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