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Review

Staphylococcus aureus small colony variants: a challenge to microbiologists and clinicians

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Abstract

The pathogen *Staphylococcus aureus* may use various strategies to resist antibiotic therapy. One of these strategies is the formation of small colony variants (SCVs), a naturally occurring, slow-growing subpopulation with distinctive phenotypic characteristics and pathogenic traits. SCVs are defined by mostly non-pigmented and non-haemolytic colonies ca. 10 times smaller than the parent strain. In the past decade, many reports and prospective studies have supported a pathogenic role for these variants in patients with persistent and/or recurrent infections. The tiny size of clinical and experimentally derived SCVs on solid agar is often due to auxotrophy for hemin and/or menadione, two compounds involved in the biosynthesis of electron transport chain components. The morphological and physiological features of SCVs present a challenge to clinical microbiologists in terms of recovery of organisms, their identification and susceptibility testing. Based on the knowledge that SCVs may persist intracellularly, treatment including antimicrobial agents with intracellular antistaphylococcal activity appears appropriate. SCVs potentially use the upregulated arginine deiminase pathway to produce ATP or, through ammonia production, to counteract the acidic environment that prevails intracellularly, as shown using a site-directed mutant with SCV phenotype in transcriptomic studies.

Keywords: Small colony variants; *Staphylococcus aureus*; Persistent infections; Recurrent infections; Electron transport chain; Intracellular persistence; Arginine deiminase pathway; Oxidative phosphorylation; *hemB* mutant; *menD* mutant

1. Small colony variants as a cause of persistent and recurrent infections

Small colony variants (SCVs), formerly designated as 'G' (gonidial) variants or dwarf colonies, constitute a naturally occurring, slow-growing subpopulation of bacteria with distinctive phenotypic characteristics and pathogenic traits. The recovery of SCVs of *Staphylococcus aureus* from clinical specimens was first described around 100 years ago. However, the connection of this phenotype to persistent and recurrent infections has only been appreciated in recent years [1]. Five patients were described with unusually persistent and/or antibiotic-resistant infections due to *S. aureus* SCVs. Since then, many reports and prospective studies have supported a pathogenic role for SCVs in patients with chronic and/or persistent infections such as chronic osteomyelitis and persistent skin and soft-tissue infection [2–4]. The results of a 6-year prospective study analysing the prevalence and persistence of *S. aureus* in patients with cystic fibrosis demonstrated that the airways of >70% patients were persistently colonised/infected by normal and/or SCV *S. aureus*, with a median persistence of 37 months (range 6–70 months) [5]. Some patients who initially harboured both normal and *S. aureus* SCVs subsequently lost the normal strain, whilst SCVs persisted for extended periods. The longer persistence of the SCV phenotype indicated a survival advantage of SCVs compared with the normal phenotype in the hostile milieu of the airways, possibly due to optimised adaptation of the SCVs.

2. Intracellular persistence of SCVs

S. aureus has various strategies for resisting therapy that extend beyond classic mechanisms. Such strategies include the potential for evading the effect of a given antibiotic even though it tested susceptible by production of diffusion

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barriers, e.g. biofilm production, or by withdrawal into the intracellular milieu. The latter mechanism has been documented for SCVs. Indeed, in several assays using various non-professional phagocytes such as endothelial or epithelial cells, these variants were able to persist intracellularly [4,6–8].

To identify the intracellular location of SCVs, primary human endothelial cells were infected with various strain pairs displaying either the normal or the SCV phenotype [9]. Subsequently, maturation of phagosomes using live cell imaging was visualised. Within 1 h, all internalised staphylococci accumulated in lysosomal organelles and remained there for up to 5 days. Whilst an effective bactericidal activity of human endothelial cell lysosomes towards staphylococci was observed, these studies provided evidence that SCVs of selected strains are able to withstand this activity.

3. SCVs: a challenge in terms of recovery, identification and susceptibility testing

SCVs are defined by mostly non-pigmented, non-haemolytic colonies ca. 10 times smaller than the parent strain (hence their name). This tiny size of clinical and experimentally derived SCVs on solid agar is often due to auxotrophy for menadione, hemin or thymidine [1–4,6]. When the medium is supplemented with these compounds, SCVs grow as rapidly as the parent strains. Menadione and hemin are required for the biosynthesis of electron transport chain components, as menadione is isoprenylated to form menaquinone, the acceptor of electrons from nicotinamide adenine dinucleotide (NADH)/flavin adenine dinucleotide (FADH2) in the electron transport chain, and hemin is required for the biosynthesis of cytochromes, which accept electrons from menaquinone.

In addition to the atypical colonial morphology, absent or reduced biochemical reactions (e.g. mannitol salt agar-negative using the Api Staph system) are typical features [10]. Most *S. aureus* SCVs are coagulase-positive by the tube test only after incubation for >18 h. Thus, these uncommon morphological and physiological features of SCVs present a challenge to clinical microbiologists in terms of recovery and identification [2].

A prerequisite for the isolation of this subpopulation is the application of extended conventional culture and identification techniques [10]. Recently, we showed that the most accurate method to detect both the species *S. aureus* and the SCV phenotype is to inoculate specimens both on Columbia blood agar and on a new chromogenic agar (*S. aureus* ID agar) [11]. However, SCVs are easily overgrown and missed when the normal *S. aureus* is present because SCVs divide approximately nine times slower than *S. aureus* with normal phenotype [2]. *S. aureus* isolates suspected of being SCVs, which may give a false-negative coagulase test, should be confirmed as *S. aureus* by testing the species-specific *nuc* and *coa* genes. Another diagnostic approach was shown in a patient with a brain abscess in which *S. aureus* SCVs were detected in the brain tissue using a 16S rRNA-directed in situ hybridisation technique [12].

Since clinical SCVs often exhibit an unstable phenotype, Fourier-transform infrared spectroscopy has been used to investigate the phase variation from SCV phenotype into the normal phenotype and vice versa [13]. Indeed, this non-invasive technique offered a rapid, reliable and non-destructive approach to trace directly the process of reversion from the normal phenotype into the SCV phenotype and vice versa. Based on spectral information in three different spectral ranges, clustering resulted in dendrograms showing a clear discrimination between the normal and SCV phenotype.

For several reasons, SCVs also present a challenge with regard to susceptibility testing. First, SCVs are often present in mixed populations with the normal phenotype (see above), thus even a small percentage of normally growing organisms will rapidly replace the SCVs in liquid medium in an overnight culture, thereby making susceptibility testing of the SCVs difficult [2]. Second, delayed growth makes standardisation of testing difficult because slow growth alters diffusion tests and the times for measuring susceptibility [14]. Third, errors may occur when these variants are resistant to oxacillin when tested by disk diffusion test, Etest, microdilution test and automated susceptibility testing systems as well as anti-PBP2a slide latex agglutination tests [15]. As a consequence, detection of the *mecA* gene by molecular methods or the use of an anti-PBP2a slide latex agglutination test using a drastically increased inoculum (approximately a loopful with 100–200 SCV colonies) should be used for the reliable diagnosis or validation of methicillin-resistant *S. aureus* (MRSA) SCVs.

4. Treatment of patients infected with *S. aureus* SCVs

Interruption of electron transport reduces the electrochemical gradient across the bacterial membrane, resulting in a decreased uptake of antimicrobial agents that require a charge differential to be active. Therefore, substances such as gentamicin or other aminoglycosides should not be used for the therapy of infections caused by *S. aureus* SCVs, although single strains with SCV phenotype might be susceptible to aminoglycosides [16].

So far, prospective studies on the treatment of patients infected with *S. aureus* SCVs are not available, thus optimal therapy has not yet been defined. However, based on the knowledge that SCVs may persist intracellularly, treatment including antimicrobial agents with intracellular antistaphylococcal activity such as rifampicin appears appropriate. As monotherapy with rifampicin is not recommended owing to rapid development of resistance, a combination regimen with either β-lactam antibiotics such as oxacillin or a second-generation cephalosporin for methicillin-susceptible *S. aureus* SCVs, or vancomycin for MRSA SCVs, is necessary [17]. In the past it was found that trimethoprim/sulfamethoxazole combined with rifampicin was the most active therapeutic regimen in a tissue culture system