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Article type : Letter to the Editor

LETTER TO THE EDITOR

***Staphylococcus aureus* small colony variants: prevalence in chronic rhinosinusitis and induction by antibiotics**

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/all.13580](https://doi.org/10.1111/all.13580)

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Short title: *Staphylococcus aureus* SCVs in chronic rhinosinusitis

Keywords: *staphylococcus aureus*, small colony variants, chronic rhinosinusitis, gentamicin, rifampicin, clindamycin

Author Contributions: ZG and ZY performed all experiments and drafted first manuscript; AJH, AJP and PJW critically reviewed manuscript; SV designed research questions, critically reviewed manuscript

Conflicts of interest: none relevant to this study

To the Editor,

Staphylococcus aureus (*S. aureus*) small colony variants (SCVs) are an altered bacterial phenotype characterized by slow growth rate and small colony size. Due to reduced antimicrobial sensitivity and a capacity to invade host cells, *S. aureus* SCVs can escape immune defense mechanisms and contribute to therapy-refractory Staphylococcal infections (reviewed in (1)). *S. aureus* SCVs have been isolated from sites of several persistent and relapsing chronic infections such as osteomyelitis (2) and from within the nasal polyps of patients with Chronic Rhinosinusitis (CRS) (3), however the triggers and mechanisms of SCV formation are largely unknown. Previous work has demonstrated *S. aureus* can be induced to the SCV phenotype by exposure to different stressors *in vitro* (2, 4, 5) and SCVs are also isolated from patients with ongoing antibiotic treatment (1, 6-8). However, the incidence and characteristics of SCVs amongst *S. aureus* clinical isolates (CIs) from CRS patients and whether these isolates are induced into a SCV phenotype by clinically relevant antibiotics is not known. As antibiotics form the mainstay of treatment for CRS patients this information is important to advise treatment options.

The purpose of this study was to determine the prevalence of *S. aureus* SCVs in CRS CIs, analyse the capacity of clinically relevant antibiotics to induce SCVs and compare the characteristics of induced SCVs with their Normal Colony Phenotype (NCP) parents. To achieve this, the SCV status of 66 CRS *S. aureus* CIs was determined and 11 types of antibiotics tested for their capacity to induce SCVs in representative CIs and the reference strain ATCC51650. SCVs were defined by decreased colony size and reduced pigmentation compared to parent NCP. Biochemical activity as well as infection ability, antibiotic sensitivity and stability were also tested. Details of materials and methods are found in this article's Online Repository. Of the 66 *S. aureus* CIs from CRS patients assessed for SCV phenotype half (33/66) grew large sized white or yellow colonies, typical of *Staphylococci* and 26/66 of *S. aureus* CIs grew small (≤ 1 mm) colonies with some of these (5/26) appearing white in color and lacking hemolytic activity (SCVs). Mixed populations of small and large colonies were present in the remaining 7/66 CIs with 3 of these containing colonies with SCV phenotype (Supplementary Table 1) Representative images of *S. aureus* CIs are shown in Supplementary Figure 1.

After SCV status was determined we then tested 11 antibiotics for their capacity to induce SCVs of ATCC51650 and three representative CIs. The MIC of these antibiotics was determined and is shown in Supplementary Table 2. Incubation with gentamicin was particularly effective at inducing SCVs. This was consistent with previous reports that have shown gentamicin exposure induces reversible rapid switching of gentamicin-sensitive strains into gentamicin-resistant SCVs (5). Isolates CI1 and ATCC51650 showed switching to SCV phenotype after as little as 1 hour at $\frac{1}{2}$ MIC whilst all isolates showed a gradual increase in SCVs over the 7-day period in continuous culture with 2 MIC gentamicin (Figure 1A and Supplementary Figures 2 and 3). The other antibiotics tested did not induce SCVs at short exposure times (< 24 hours). However, SCVs were induced by 24-hour incubation with clindamycin from 3 MIC to 10 MIC and by rifampicin from 8 MIC to 10 MIC (Figure 1B-D). Using different protocols, Tuchscher et al similarly showed gentamicin and clindamycin induced *S. aureus* SCV formation and reported a high resistance rate of bacteria when exposed to rifampicin for 24 hours (2). Our results add to these findings, demonstrating that bacteria exposed to high concentrations of rifampicin were rifampicin-resistant and of a SCV phenotype. Stability testing of the isolates indicated SCVs induced by short exposure times to gentamicin (< 2 hours) reverted back to NCP when cultured overnight. However, incubation with 2 MIC gentamicin for 3 days or more induced SCVs that were stable for up to 5 days

incubation. The phenotypic instability of SCVs is a known phenomenon, with phenotype reversion occurring frequently after incubation in stress-free conditions making laboratory-based investigation of clinically isolated SCVs difficult (1).

The catalase, coagulase and hemolytic activity as well as infection ability and antibiotic sensitivity of one representative SCV clinical isolate (CI1-SCV) was also assessed. CI-SCVs were induced by gentamicin (G-C1SCV), clindamycin (C-C1SCV) and rifampicin (R-C1SCV) for 4 days and were stable for at least 5 days continuous culture in antibiotic free broth. All antibiotic-induced C1-SCVs lost their coagulase activity and kept catalase activity, with loss of haemolytic activity only observed in gentamicin and clindamycin induced SCVs. Induced SCVs were also resistant to the antibiotics used for induction but more sensitive to other antibiotics (Table 1). G-CI1SCVs also showed lower infection rate compared to the NCP parent strain (32.7 % vs 76.1 % for the CI1- SCVs and CI1 respectively) when applied to Human Bronchial Epithelial (HBE) cells for 6 hours. However, the percentage of infected cells decreased rapidly for the CI1-NCP after extracellular bacteria were removed and after 24 hours the CI1-SCVs infection rate was similar to the CI1-NCP parents (18.3% vs 22.2%) (Supplementary Figure 4). Both phenotypes could survive in the cells for more than 4 days. This suggests that the CI1-SCVs, whilst not as capable of initiating intracellular infection, are better able to persist in the intracellular environment. Previous research has indeed shown intracellular infection with *S. aureus* SCVs induced a less widespread up regulation of pro-inflammatory factors in comparison to intracellular infection with NCP parent strains (9) which may in part explain these results.

In conclusion, 12% of *S. aureus* CIs from the sinonasal cavities of CRS patients had colonies with characteristics reminiscent of SCVs when cultured *in vitro*. Moreover, *S. aureus* CIs could be induced into antibiotic-tolerant SCVs by prolonged exposure to different clinically used antibiotics. In our experiments, antibiotic-induced *S. aureus* SCVs were tolerant to the antibiotics used for induction, but were more sensitive to alternative antibiotics tested. These findings may have important implications for the design of optimal antibiotic treatment strategies for relapsing infections in the context of CRS.

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Table 1 Characteristics of CI1-SCVs induced by Gentamicin, Clindamycin and Rifampicin for 4 days.

G-SCVs	C-SCVs	R-SCVs	NCP-Parents
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Catalase	positive	positive	positive	positive
Coagulase	negative	negative	negative	positive
Hemolysis	negative	negative	positive	positive
MIC-Gent	8 ~ 16*	1	1	2
MIC-Clin	0.04 ^Δ	>10*	0.04 ^Δ	0.2
MIC-Rif	<0.001	<0.001	0.625 ~ 2.5*	0.001 ~ 0.004
MIC-Met	0.39 ^Δ	0.78	0.39 ^Δ	0.39 ~ 0.78
MIC-Ery	0.39 ^Δ	0.39 ^Δ	0.2 ~ 0.39 ^Δ	0.78

G-SCVs: SCVs induced by gentamicin; C-SCVs: SCVs induced by clindamycin; R-SCVs: SCVs induced by rifampicin; (MIC-Gent), (MIC-Clin), (MIC-Rif), (MIC-Met), (MIC-Ery): MIC (μg/ml) to gentamicin, clindamycin, rifampicin, methicillin and erythromycin respectively; *: MIC of SCVs significantly higher than parents (P<0.05); Δ MIC of SCVs significantly lower than parents (P<0.05)

Figure 1: Induction of SCVs by antibiotics after 24hrs culture in TSB: A) representative plate images of gentamicin-induced ATCC51650 and CI1 *S. aureus* SCVs. ATCC 51650 and CI1 parental strains were observed as large colonies (a, e); Continuous incubation with 2MIC gentamicin induced an increasing number of SCVs over time; shown at 1 hour (b, f), day 3 (c, g) and day 6 (d, h) as small colonies with reduced pigmentation **B-E) induction of SCVs in representative isolates ATCC51650 (B), CI1 (C), CI2 (D) and CI3 (E) by 11 different clinically used antibiotics.** Clindamycin, Gentamicin and Rifampicin were the only antibiotics observed to induce SCV phenotype.

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Title:

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Date:

2018-12-01

Citation:

Zhang, G., Zhao, Y., Hayes, A. J., Psaltis, A. J., Wormald, P. -J. & Vreugde, S. (2018). Staphylococcus aureus small colony variants: Prevalence in chronic rhinosinusitis and induction by antibiotics. ALLERGY, 73 (12), pp.2403-2405. <https://doi.org/10.1111/all.13580>.

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