The persistence of intracellular *Staphylococcus aureus* in the sinuses: a longitudinal study*

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Abstract

Background: *Staphylococcus aureus* (*S. aureus*) can reside within the sinonasal mucosa in chronic rhinosinusitis patients and causes recurrent infections. Within the host cell, *S. aureus* can evade host immune detection enabling its own survival. We hypothesise that *S. aureus* can persist within the sinonasal epithelium for a prolonged period without immune activation.

Methodology: Patients with chronic rhinosinusitis with nasal polyps (CRSwNP) undergoing two sinus surgeries were included. Immunohistochemistry and Haematoxylin and Eosin stains were used to determine intracellular *S. aureus* (ICSA) status and inflammatory cell count, respectively. One-way ANOVA and paired t-tests were performed for comparison between ICSA subgroups and within each subgroup, respectively.

Results: Histopathological specimens from 34 patients (68 procedures) were included. ICSA positivity ($ICSA^+$) was seen in 43 specimens (63.2%) from 26 (76%) patients. 18 (52.9%) of those were $ICSA^+$ in both operations while 8 (23.5%) patients were $ICSA^+$ in only one of the operations. 8 (23.5%) patients were ICSA negative in both operations. There was no difference in the number of eosinophils, lymphocyte and neutrophils between ICSA subgroups.

Conclusions: This study demonstrated that *S. aureus* is found intracellularly within CRSwNP tissue at multiple time points without an increase in the number of eosinophils, lymphocytes and neutrophils. This finding supports our hypothesis that ICSA is able to escape from host detection and resides within the sinonasal mucosa despite intense treatment.

Key words: chronic rhinosinusitis, *Staphylococcus aureus*, transnasal endoscopic surgery, inflammation, fibrosis

Introduction

Intracellular *Staphylococcus aureus* (ICSA) has been demonstrated within the sinonasal epithelium in patients with chronic rhinosinusitis (CRS) as well as in healthy individuals [1-5]. It is known that upon entrance into the cells, *Staphylococcus aureus* (*S. aureus*) undergoes significant phenotypic changes by modifying its metabolism and down regulating the expression of virulence factors [6]. A study conducted by Wood et al. [4] showed a lack of local immune response to intramucosal microcolonies in patients with CRS, supporting the concept that these adaptation mechanisms allow its survival within the cells and facilitate its evasion from host immune detection. Further compounding this problem is the fact that antibiotics commonly used to treat methicillin sensitive *S. aureus* infection in the setting of CRS, such as β-lactams, have low intracellular activity and therefore are ineffective in eliminating ICSA [7]. The combination of these factors allows ICSA to remain undetected as well as survive and persist within the cells.

In vivo persistence of ICSA in CRS patients was firstly demonstrated by Clement et al. [1] and Plouin-Gaudon et al. [2]. Both groups showed that the same patient specific genotypes of *S. aureus* were repeatedly cultured in patients that had ICSA. These findings indicate that one specific *S. aureus* strain dominates the niche and can cause recurrent infections. Further support of this finding was a study that showed identical *S. aureus* genotypes

in CRS patients isolated from their sinonasal cavities at multiple time points [8]. Together, these findings support the notion that ICSA might act as a reservoir causing recurrent infections and may play a role in CRS pathogenesis. The long-term consequence of ICSA in CRS remains unclear although ICSA has been found to be more prevalent in CRS patients with surface biofilm [3] and in those requiring revision surgery suggesting a role in recalcitrant disease [5, 9].

It is unknown whether the presence of ICSA is affected by current standard medical management of CRS. This study was designed with an aim to investigate the persistence of ICSA in patients with recalcitrant CRS who had been treated with intense medical and surgical therapies. The second aim of this study was to gain further understanding of the longitudinal changes in histopathological features, inflammatory cell load and fibrosis in association with ICSA.

Materials and methods

Ethics approval for this study was granted by the Human Research Ethics Committee of The Queen Elizabeth Hospital (South Australia, Australia).

Patient recruitment

Patients diagnosed with CRS with nasal polyps (CRSwNP) according to the 2012 European Position Paper on Rhinosinusitis and Nasal Polyps [10] diagnostic criteria and had undergone two endoscopic sinus surgeries (ESS) between 2003 and 2015 were recruited into the study. Histopathological specimens of polyps taken at each surgical procedure were examined in the study. Specimens from patients who had a history of immunosuppression or were less than 18 years of age were excluded from the study. Clinical data including pre- and post-operative management was obtained from patient medical records.

Histology staining - haematoxylin and eosin (H&E) and immunohistochemistry (IHC)

Archived histology blocks of sinonasal mucosa from each ESS were retrieved. Two consecutive sections of 4μm thickness were cut from each block and placed on separate slides. Specimens underwent both routine H&E staining to assess inflammatory cell counts and basement membrane thickness and immunohistochemistry (IHC) staining to determine ICSA status.

IHC was performed using an automated system Leica BOND-III (Leica Biosystems, Germany). The autostainer was programmed as follows: 15 minutes heat fix at 60°C followed by rehydration by changes of decreasing concentrations of ethanol. Dual endogenous peroxidase block (Dako EnVision + Dual Link System-HRP, K4065) for 10 minutes at room temperature followed by 20 minutes of pH 6 citrate buffer incubation at 98°C to antigen retrieve. Primary mouse anti *S. aureus* antibody (Millipore MAB930) was used at 1 in 100 concentration for 1 hour at room temperature. Unimmunised mouse IgM (Sigma M2521) and normal saline were used as negative controls. 3,3'-Diaminobenzidine (DAB) stain was developed using Dako EnVision kit (K4065) as per manufacturer's instructions.

Image analysis

All stained slides were scanned into digital files using a NanoZoomer 2.0-HT (Hamamatsu, Japan) to allow whole image analysis. The images were reviewed using NDP.view2 software (Hamamatsu, Japan).

ICSA status

To determine the presence of ICSA, two blinded independent investigators (JO & AD) assessed all IHC stained slides. The slides were coded with specimen identifier number only and evaluated in a computer generated randomized order to avoid assessing the histology of the same patient consecutively. The whole mucosa of each section was assessed for the presence of ICSA. Positive ICSA was defined as discrete and round dark brown staining within cells in the epithelium (Figure 1a) and/or subepithelium (Figure 1b). A specimen was only categorised ICSA positive when it was scored positive by both assessors.

Inflammatory cell load and basement membrane (BM) thickness
For each H&E stained section, areas immediately below the epithelium were used for inflammatory cell counting. A total of 10 areas (0.035mm^2 each) were randomly selected at 5X magnification per slide. Areas containing submucous glands and major vessels were avoided [11]. Each area was then examined under 40X magnification for eosinophil, lymphocyte and neutrophil counting using ImageJ software (National Institute of Health, USA). The investigator (AB) that assessed the H&E slides was blinded from the ICSA status of each patient.
Basement membrane was measured at two different points on each of the area selected in H&E stain using a ruler tool provided in NDP.view2 software (Hamamatsu, Japan).

Statistical analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software Inc, La Jolla, CA, USA). One-way ANOVA was used to compare between the ICSA subgroups and a paired parametric t-test was performed to compare the difference between the 1st and 2nd operations within each of the subgroups. Statistical significance was defined as p-value <0.05.

Results

Patients' characteristics

A total of 34 patients (68 operations) were included in the study. Details of the demographical information is summarised in

Table 1. Patient demographics and pre-operative characteristics.

Total number of patients	34
Total number of operations	68
Age	46.1 (27- 70)
Male: Female	22: 12
Asthma	30 (67.6%)
Aspirin sensitivity	9 (26.5%)
Smoking	1 (2.9%)
Number of primary operations included in the study	8
Mean number of operations prior to the study in revision patients (range)	3.1 (1- 10)
Mean time between operations in months (range)	35.0 (6- 84)

Table 1. The majority of patients in this cohort were male (65%). Asthma was common in this recalcitrant group (67.6%) and 26.5% of patients suffered aspirin sensitivity. The mean number of previous surgical procedures of patients included in this study was 3.1 (1-10), with the mean duration between operations 35 (6- 84) months.

ICSA prevalence and persistence

Within the 68 specimens assessed, 43 (63.2%) from 26 patients (76%) were found to be positive with ICSA ($ICSA^+$). 18 of these patients (52.9%) had ICSA in specimens taken at both operations and this group was designated as $ICSA^{+/+}$. 8 patients (23.5%) were ICSA negative at both operations ($ICSA^{-/-}$). There were 8 patients (23.5%) who were only ICSA positive in one of the two operations during the study period (ICSAΔ). Out of these 8 patients, 5 patients were only positive at the first operations ($ICSA^{+/-}$) and 3 were only positive in the second operations ($ICSA^{-/+}$). Among the 23 patients who were $ICSA^+$ at the first operation, 78.2% of them remained $ICSA^+$ despite medical and surgical therapies. The mean duration between the operations in $ICSA^{+/+}$ group was 33.9 (12.0- 75.0) months. The demographic characteristics between the ICSA subgroups were summarised in Table 2. There were no differences found between the use of antibiotics and corticosteroid in pre- and post-operative management between the subgroups (Table 2).

Inflammatory cell load

There was no statistical difference found in numbers of eosinophils, lymphocytes and neutrophils between the 3 ICSA subgroups. When comparing between the 2 operations within the same patients in each subgroup, the number of eosinophils was reduced from the first to the second operation in both $ICSA^{-/-}$ and ICSASΔ groups but this did not reach statistical significance. The total number of eosinophils remained the same at both time points in the $ICSA^{+/+}$ group (Figure 2).

BM thickness

There was no statistical difference found in BM thickness between the 3 ICSA subgroups and no significant temporal change in BM thickness noted in any subgroup.

Both clinical and histological data were compared between all 4 ICSA subgroups initially, however there were no differences

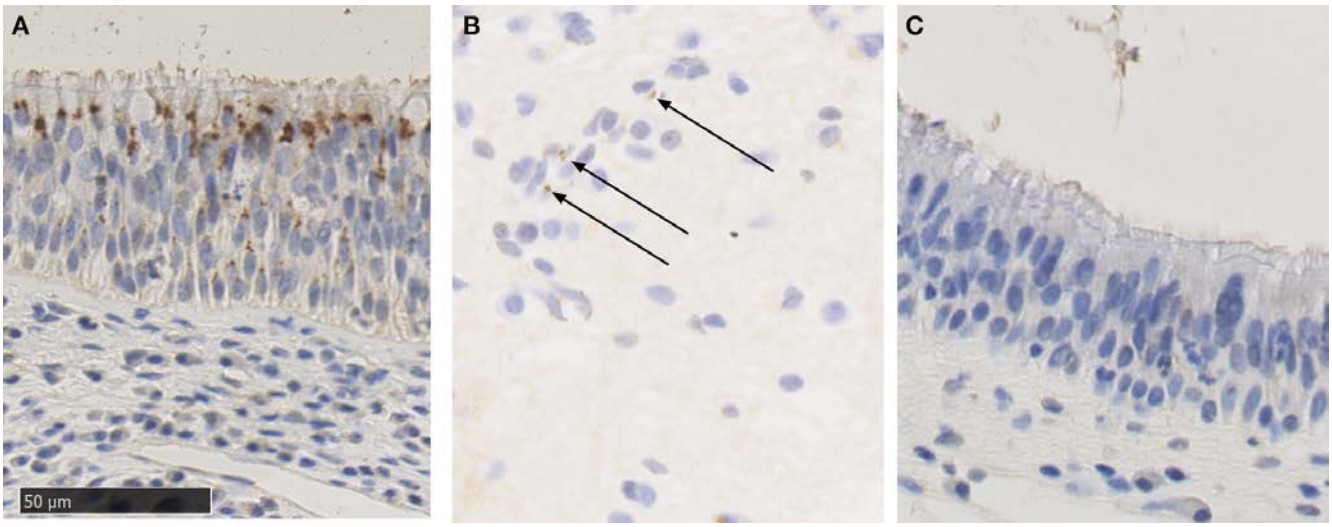

Figure 1. Immunohistochemistry stain of intracellular S. aureus (ICSA). A. An image of an $ICSA^+$ specimen at 40X magnification. ICSA is most abundant within epithelium in the sinonasal mucosa. Positive ICSA stain is dark brown, discrete and round shaped. B. Positive ICSA staining within inflammatory cells in subepithelium digitally zoomed into 80X magnification. C. An image of an $ICSA^-$ specimen at 40X magnification.

Table 2. Demographics and clinical characteristics of each intracellular *S. aureus* (ICSA) subgroup.

	ICSA$^{+/+}$	ICSA$^{-/-}$	ICSAΔ	p-value
N	18 (52.9%)	8 (23.5%)	8 (23.5%)	-
Age (range)	45. 8 (29- 63)	46.9 (27- 70)	46.1 (33- 54)	0.9
Male: Female	14:4	4:4	4:4	0.06
Asthma	11 (61.1%)	5 (62.5%)	7 (87.5%)	0.2
Aspirin sensitivity	5 (27.7%)	1 (12.5%)	3 (37.5%)	0.3
Smoking	1 (5.5%)	0	0	0.4
Number of primary operations included in the study	5 (28%)	0	3 (37.5%)	0.2
Mean number of operations prior to the study in revision patients (range)	2.2 (1- 4)	3.6 (2- 8)	4.6 (1-10)	0.1
Mean time between operations in months (range)	33.9 (12- 75)	28.8 (11- 59)	42.8 (6- 84)	0.7
Pre-operative management (within 6 months)				
Number of operations with complete pre-operative data	26/36 (72%)	12/16 (75%)	14/16 (88%)	-
Topical antibiotics†	2/26 (8%)	1/12 (7%)	0/14 (0%)	0.6
Oral antibiotics†,‡	3/26 (12%)	0/12 (0%)	0/14 (0%)	0.2
Oral corticosteroid†,§	9/26 (35%)	1/12 (8%)	2/14 (14%)	0.1
Post-operative management (18 months)				
Number of operations with complete follow up data	29/36 (81%)	13/16 (81%)	13/16 (81%)	-
Topical antibiotics†	12/29 (41%)	3/13 (23%)	2/13 (15%)	0.2
Short term oral antibiotics¬‖	1.8	2.5	1.8	0.5
Long term (>2 weeks) oral antibiotics†	11/29 (38%)	5/13 (38%)	5/13 (38%)	0.9
Oral corticosteroid‖,§	2	1.8	1.5	0.6

† A maximum of one treatment course prescribed per operation. ‡ Only 1 patient (in ICSA$^{+/+}$ group) in the whole cohort received oral antibiotics within a month prior to the operation. § Each corticosteroid course was defined as a 3-weeks tapering regime. ‖ Mean number of treatment courses per operation. Note. Topical corticosteroid was prescribed to all patients post-operatively.

found between the 4 groups. Given the low number of patients in the ICSA$^{+/-}$ and ICSA$^{-/+}$ groups, these data were combined and presented as one group (ICSA$^{\Delta}$).

Discussion

This study was conducted to evaluate the prevalence of ICSA in patients with recalcitrant CRSwNP as well as the changes in ICSA status over time. In this cohort, 76% of patients were found to have ICSA at some point during the study period. This is consistent with previous studies that report a prevalence of ICSA in patients with CRSwNP ranging between 33- 89% [3-5, 12, 13]. This study also showed that in the majority of patients requiring revision surgery, ICSA persisted despite standard post-operative antibiotic treatment. In spite of ICSA persistence, there was no associated increase in eosinophils, lymphocytes, neutrophils and BM thickness found.

Differences in the prevalence rates detected by the different studies may represent differences in the disease process itself but also methodological differences. In our patient cohort, we used whole image analysis technology that reduces random sampling errors. Furthermore, the type and site of tissue harvested could differ between studies. Whereas other studies did not specify the identity of the tissue sampled, this study only examined polyp tissue. A number of different methods currently exist for the detection of ICSA. Early studies relied on Gram staining [4, 14, 15] which is non-specific for *S. aureus*. More recently both Fluorescent in situ Hybridization (FISH)[4, 12] and *S. aureus* immunohistochemistry have been used with similar rates of sensitivity and specificity [16]. This study elected to use immunohistochemistry as it does not require fresh tissue and can be used on paraffin embedded tissue sections. It not only allowed evaluation of ICSA in both the intra- and sub-epithelium location but also permitted assessment of its association with subepithelial inflammatory cells. Disease specific and geographical differences may also explain differences in the prevalence

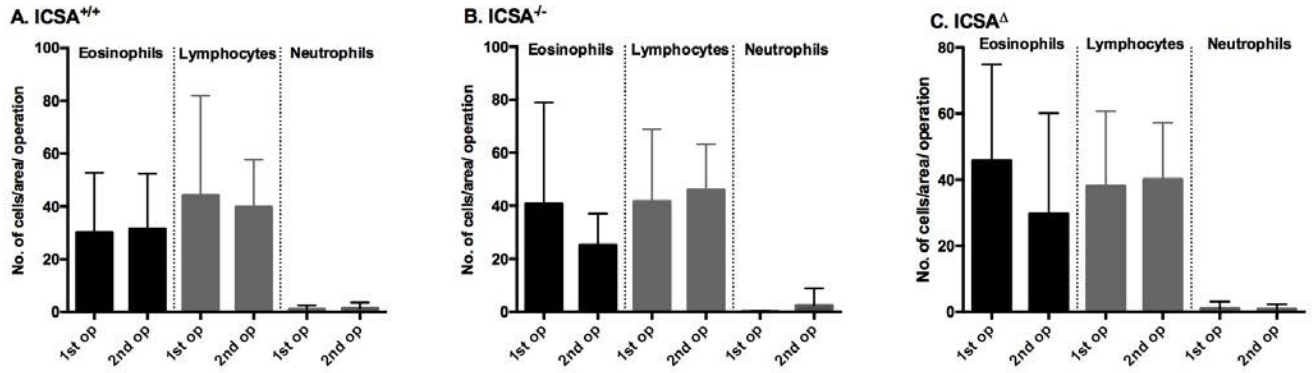

Figure 2. Comparison of the numbers of eosinophils, lymphocytes and neutrophils between 1st and 2nd operations within intracellular *S. aureus* (ICSA) subgroups.

rates of ICSA seen among studies. ICSA has been repetitively found to be more prevalent in patients that have undergone revision surgeries [5, 9] and may explain the high prevalence in this study. Similarly, just as *S. aureus* nasal carriage has been shown to vary between different countries [17-21] so may the prevalence of sinonasal ICSA.

When comparing the presence of ICSA between the sinonasal biopsies taken at different time points within the same patient, we found that the majority of patients who were positive for ICSA in the earlier operation remained ICSA positive at the subsequent procedure. Currently, ICSA is not routinely screened clinically and current management of CRS does not target ICSA directly. The study by Clement et al. [1] showed that ICSA was able to persist for prolonged periods of time despite repeated systemic antibiotic treatments. Their findings together with our results again showed that current standard antibiotics regimes are ineffective for ICSA eradication. This is likely due to poor intracellular activity and penetration of currently used antibiotics [7]. Moreover, it is known that *S. aureus* alters its phenotype and growth rate once inside the host cell, further compromising the antimicrobial effect of antibiotics that are largely dependent on the microbial metabolic activity and proliferation rate [6, 22, 23]. From the data available in this study it was not possible to draw definitive conclusions on whether ICSA was present continuously or intermittently in these patients. It is possible that some of the ICSA$^{+/+}$ patients had eliminated ICSA at some point, however ICSA re-infection occurred subsequently due to a combination of host and pathogen factors. It is also possible that immediately post ESS the bacterial load was reduced by removing diseased sinonasal mucosa and polyps but not sufficient enough to allow complete eradication [8, 24]. Despite the high rate of ICSA persistence found in our cohort, some patients were able to completely eradicate ICSA. Our study did not identify any demographic or clinical characteristics particular to this small cohort, although a larger more powered study is needed to confirm these findings.

It is likely that the virulence of different bacterial strains as well as specific host immune responses may have both contributed to ICSA eradication and molecular studies are needed to further evaluate the immune response to ICSA.

Our study showed no differences in numbers of eosinophils, lymphocytes and neutrophils between the different patient cohorts. Whilst these results indicated that the presence of ICSA did not associated with an influx of these specific cell types, we cannot exclude that ICSA presence might induce a different immune response involving other types of immune cells. There have been a number of studies performed investigating the interaction between *S. aureus* and non-professional phagocytes upon internalisation [6, 22, 23, 25-27]. A down-regulation in the *S. aureus* global virulence factor regulator, accessory gene regulator (*agr*), has been repeatedly demonstrated [23, 25]. A study conducted by Tuchscherr et al. [23] also showed a continuous down-regulation in both *agr* and alpha-haemolysin gene (*hla*) for up to 7 days and 28 days post intracellular *S. aureus* infection in cell and animal models, respectively. Other changes in *S. aureus* gene expression including down-regulation in enterotoxins [6] and metabolic genes as well as up-regulation of cell wall synthesis [22] have also been shown. Matussek et al. [26] and Li et al. [27] examined the in vitro cellular immune response to ICSA using endothelial and epithelial cells respectively. Both of these studies showed an up-regulation of a number of proinflammatory cytokines such as G-CSF, GM-CSF, IL-8 and cox2. The duration of these studies were 8 to 18 hours only and therefore may not be reflective of chronic infections such as CRS. A longer-term animal model by Tuchscherr et al. [23] examined cytokine expression over a 28-day period showing initial upregulation of pro-inflammatory cytokines, CCL5 and CXCL11 (peak at day 7) followed by a down-regulation over the course of 28 days. Overall, these studies provided evidence that once inside the mammalian cells, ICSA is able to adapt to the intracellular environment and switches its phenotype to become less virulent. This could influence the host

immune response such that *S. aureus* is able to remain within the host mucosal tissue.

Basement membrane thickness was used as a measure for fibrosis in this study [11, 28]. Pathological BM thickening was observed in all patients in this cohort and this was consistent with other studies [28, 29]. Pathological BM thickening has been shown to be associated with epithelial damage and eosinophil infiltration [28]. However, there was no association found between ICSA and BM thickness in this study.

The retrospective design and small sample size are limitations of this study. A prospective study with inclusion of detailed clinical data as well as regular sampling of sinonasal biopsies to closely monitor the ICSA status would be ideal to determine whether ICSA persistence was intermittent or continuous. For logistical reasons this would be difficult and result in increased patient discomfort during sampling and possibly also increase the risk of recurrent infections in patients. While harvesting tissue during ESS is the least invasive for patients, the duration between operations is variable and can be many years as observed in this study and intermediate changes in histological parameters could be missed. Overall, this is the first longitudinal study on ICSA in patients with CRSwNP to investigate its natural history under current CRS management.

Conclusion

ICSA was demonstrated within sinonasal mucosa in patients with recalcitrant CRSwNP over prolonged period of time. The presence of ICSA was not associated with an increase in eosinophils, lymphocytes and neutrophils and BM thickening, supporting the current view that ICSA can persist without inducing an inflammatory response involving these cell types. Further studies are needed to gain insight in the interaction between ICSA and host at the cellular and molecular level.

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Authorship contribution

Study conception and design: JO, SV; Acquisition of data: JO, AB, AD; Analysis and interpretation of data: JO, AD; Drafting: JO, AD; Critical revision: SV, AJP, PJW.

Conflict of interest

None relevant to this study.

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