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Small-colony variants and phenotype switching of intracellular Staphylococcus aureus in chronic rhinosinusitis
Allergy, 2014; 69(10):1364-1371

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which has been published in final form at http://dx.doi.org/10.1111/all.12457

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1 December 2015

http://hdl.handle.net/2440/96987
Small colony variants and phenotype switching of intracellular Staphylococcus aureus in Chronic Rhinosinusitis

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Statement of participation: All listed authors have significantly contributed to this work

Conflict of Interest: The authors have no conflicting financial interests.

Short title: Small colony variants in chronic rhinosinusitis

Word count: 2,484
Abstract

Background: Chronic rhinosinusitis (CRS) has been linked to the gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) in its biofilm or intracellular forms. Recent evidence suggests that *S. aureus* also exists in a small colony variant (SCV) form as a mechanism of altering its virulence capabilities. The aim of this study was to investigate the presence of SCVs in sinonasal mucosa of CRS patients and whether the phenomenon of phenotype switching can be applied to intracellular epithelial infections.

Methods: Sinonasal specimens were examined for the presence of intramucosal *S. aureus* and characterised to the strain level. An airway epithelial cell culture infection model was utilised to investigate whether bacteria were capable of alterations in virulence phenotype.

Results: Intramucosal organisms harvested from sinonasal biopsies demonstrate phenotypic growth patterns and lack of coagulase activity consistent with SCVs. Intracellular infection of airway epithelial cell cultures with *S. aureus* led to decreased secretion of enterotoxins and phenotypic growth alterations consistent with SCVs.

Conclusions: Regulation of *S. aureus* virulence factors is a dynamic process and exposure to the intracellular environment appears to provide the necessary conditions to enable these alterations in an attempt for the bacterium to survive and persist within host tissues. Further work is required to ascertain whether SCVs in CRS hold a clinically relevant pathogenic role in recalcitrant disease.

Key Words: Chronic rhinosinusitis, Intracellular infection, Phenotype switching, Small colony variant, *Staphylococcus aureus*
Introduction

Chronic rhinosinusitis (CRS) is characterized by mucosal inflammation affecting the nose and paranasal sinuses with a prevalence of between 10-15% (1) and costs of US$5.8 billion per annum (2). Relapses are common despite ever evolving medical and surgical therapy (3)(4). Bacterial infections, in particular those mediated by *Staphylococcus aureus* (*S. aureus*), are believed to hold an important role in the recalcitrant nature of this condition with frequent relapses attributed to *S. aureus* in planktonic, biofilm and intracellular phenotypes (5, 6). Previous work has demonstrated that *S. aureus* is detectable both intramucosally (7, 8) and intracellularly (9, 10) using different techniques including indirect immunohistochemistry (9, 10) or molecular probes and confocal scanning laser microscopy (CSLM) (7, 11). The association between bacterial biofilms, activation of specific intracellular bacterial pattern recognition receptors (12) and with intracellular status in CRS has been noted (11), with significantly more frequent relapses in disease being recognised when both the biofilm and intracellular organisms were found within *ex vivo* tissue samples (6). Similar relapsing and remitting patterns have been noted in other diseases mediated by *S. aureus* such as chronic osteomyelitis (13), endocarditis (14) and cystic fibrosis (15).

A Small Colony Variant (SCV) is a bacterial phenotype that, per definition, grows in smaller colonies than their wild-type or planktonic counterparts (16) and have been shown to have the ability to survive within Nasal Polyp Epithelial cells *in vitro* (7). They express alterations in pathogenic factors including heightened expression of Fibronectin Binding Proteins (17) and reduction of alpha-toxin production (18), which facilitates access and persistence within the intracellular milieu. The association between chronic diseases and SCVs is one that has been examined in recent years and related to conditions such as osteomyelitis and cystic fibrosis (16). The conversion of bacteria from wild-type to SCV and vice versa is a highly dynamic process that can occur after days or even hours of exposure to an altered environment.
*S. aureus* exhibits its virulence through a number of factors including the secretion of staphylococcal enterotoxins (SE) (19). These superantigenic toxins have the capability of bridging and bypassing the specific antibody-antigen complex between Major Histocompatibility Complex Class II molecules and the receptors on T-helper cells (19), thus stimulating a much larger, non-specific T-cell expansion and related inflammatory response. IgE to *S. aureus* enterotoxins (SE-IgE) is elevated in the serum of patients with asthma and with CRS (20, 21). SE proteins could be identified in the tissue of almost 50% of CRS patients, specifically those with eosinophilic nasal polyposis (22)(19). It is unknown as to whether the production of these toxins is a constant or dynamic process, and thus in this study we examined whether enterotoxin secretion can change through exposure of *S. aureus* to different environments.

There has been little research examining the role of SCVs in CRS, or whether the phenomenon of *S. aureus* phenotype switching is a concept applicable to intracellular and intramucosal infections. In the present study we used a series of *in vitro* and *ex vivo* experiments in order to identify whether SCVs exist within sinonasal mucosa from patients with CRS. Our results indicate that exposure to the intracellular environment causes an alteration from the wild-type phenotype to reduce the production of virulence factors in order to promote their existence as a SCV.
Methods

Bacterial strains

*S. aureus* strains ATCC 13565 and 14458 were obtained from American Type Culture Collection, reference strains for the production of Staphylococcal Enterotoxin A and B (SEA/SEB) respectively. Liquid cultures were prepared as described in the supplementary information.

Harvest of intramucosal bacteria from patient tissue specimens

Ethical approval for tissue collection was granted from the local institution Human Research and Ethics Committee. Informed consent was obtained from eligible patients older than 18 years of age prior to enrolment. Exclusion criteria included active smoking and the use of oral corticosteroids for 4 weeks prior to surgery. Sino-nasal tissue was collected from the ethmoid sinuses from patients (n=6) undergoing endoscopic sinus surgery for CRS (diagnosis being determined according to the guidelines of the European Position Paper on CRS (23)) and placed in Dulbecco’s Modified Eagle’s Medium (Gibco, Invitrogen Corp., Carlsbad, CA). Patient demographic factors and sample processing are specified in the supplementary information.

Superantigen detection

Enterotoxins were detected using the 3M TECRA SIDVIA kit (3M, North Ryde, NSW, Australia) following the manufacturer’s instructions, as detailed in supplementary information.

Clonal typing

Swabs of *S. aureus* were sent to PathWest Laboratory (Perth, Western Australia, Australia) for molecular typing using Pulsed Field Gel Electrophoresis (PFGE) as described (24).
Methods for the isolation of *S. aureus* clinical isolates, cell culture infection, Immunofluorescence, *S. aureus* RNA isolation, qRT-PCR, Lactate Dehydrogenase (LDH) assay and Interleukin 6 (IL-6) ELISA are as described in the supplementary information.

**Results**

Intramucosal *S. aureus* harvested from sinonasal tissue samples demonstrate small colony variant growth patterns

We used immunofluorescent staining to identify intramucosal *S. aureus* cocci in 6 CRS patients. *S. aureus* specific bright green fluorescing dots could be identified within the sinonasal mucosa, both within and beneath the epithelial layer (Figure 1). Clear morphologic differences in terms of colony size and colour could be seen between the bacteria harvested from surface tissue swabs and those harvested from tissue homogenates from these patients. Sinonasal tissue swabs grew numerous bacterial colonies on blood agar plates after 48 hours with a normal phenotype, characterised by yellow pigmentation and weak hemolysis (Fig. 2A). After disinfecting the tissue, we were unable to culture any bacteria from further tissue swabs. However, upon homogenisation of tissue samples we noted the growth of small and very small sized non-pigmented, non-hemolytic colonies of *S. aureus* after 48 hours (Fig. 2B). When these colonies were immediately plated onto chromogenic agar, they demonstrated a lack of coagulase activity, a hallmark of the SCV phenotype (25) (Fig. 2C). Colony sizes were measured using ImageJ and a statistically significant difference in size was noted (p < 0.0001, unpaired t-test) (Fig. 2D). Intramucosal colonies that were subcultured overnight in nutrient broth and then plated onto blood agar demonstrated a return to normal colony growth patterns. Swabs of surface, intramucosal and sub-cultured intramucosal colonies were sent for microbiological analysis that identified the bacterial species and genus
as *S. aureus*. In order to confirm that the differing growth patterns could not be attributed to strain divergence, PFGE was performed, demonstrating that the strains of *S. aureus* cultured held an identical genotype, despite the marked difference in growth patterns (Fig. 2E).

*S. aureus* strains downregulate virulence factors upon internalisation into epithelial cells

Reference strains *S. aureus* ATCC 13565 and ATCC 14458 both expressed the appropriate enterotoxin gene, detected using qRT-PCR and secreted the respective SEA or SEB toxin when cultured into nutrient broth for 24 hours (results not shown). These strains were then used to infect HBE’014 cells. Culture supernatants from flasks of purely intracellular infected cells did not demonstrate any bacterial growth when plated onto agar (Fig. 3A). However, when the same cellular monolayer was lysed and plated onto agar, it grew numerous colonies of bacteria (Fig. 3B). These results indicate that a one hour exposure of the epithelial cells to *S. aureus* enabled the bacteria to enter the cells and that they stayed viable within the intracellular compartments. Also, the lysostaphin/gentamycin treatment could effectively eliminate all extracellular bacteria while leaving the intracellular bacteria unaffected. Control cell cultures that were infected with *S. aureus* and did not get a lysostaphin/gentamycin treatment demonstrated significant bacterial growth in the culture supernatant (Fig. 3C). These were clonally typed by PFGE, confirming that they were the same strain as that used to inoculate the cultures (results not shown).

In the absence of any SEA-positive (ATCC 13565) extracellular bacteria, after 24 hours of intracellular infection we were unable to detect SEA in either the culture supernatant (Fig. 3D) or cell lysate (Fig. 3E) of intracellular infected cells compared to detectable SEA in the supernatant of extracellular *S. aureus* flasks (Fig. 3F). In order to determine if this phenotype switching of intracellular *S. aureus* was reversible, bacteria harvested from the intracellular infected monolayer were cultured in nutrient broth overnight. Analysis of the broth
demonstrated SEA to be present, indicating that SEA secretion was switched back on by exposure to the different environment. Conversely, infection with the SEB reference strain ATCC 14458 demonstrated evidence of SEB within the cell lysate and culture supernatants of both intracellular and extracellular flasks suggesting that the bacteria were unable to downregulate their toxin production. Using the semi-quantitative scale, there was more toxin detected in the cell lysates of intracellular infected cells as opposed to the corresponding culture supernatant (Fig. 3G-I).

Enterotoxin production in clinical isolates

Six *S. aureus* clinical isolates were cultured from 6 CRS patients and analysed for expression of enterotoxin genes using qRT-PCR to identify that one strain expressed the SEA gene and another expressed the SEB gene (results not shown). Analysis using the TECRA kit demonstrated that the respective toxins were produced when the isolates were placed in liquid culture overnight at 37°C. When these strains were used to infect HBE’014 monolayers, the cell lysates or supernatants showed no detectable toxins. Conversely, toxins were found in the supernatants of extracellular infected flasks. This indicates that clinical isolates that contain enterotoxin genes are capable of “switching off” their production of toxins upon entering the intracellular environment. Interestingly, both strains appeared able to switch off their toxin production, as opposed to only one of the reference strains, suggesting that these clinical isolates had adapted the capacity to downregulate their virulence as a mechanism to persist within the cellular environment.

*S. aureus* does not cause significant cellular toxicity after internalisation and develops a SCV-like growth pattern

After HBE’014 cells were infected with *S. aureus* ATCC 13565, cell culture media was changed daily and IL-6 measured using Enzyme-Linked Immunosorbent Assay (ELISA). The
IL-6 level in the culture supernatant of cells infected with *S. aureus* was significantly higher than control cells (2.24 fold increase, p=0.0002, unpaired t-test) on day 1 after infection (Fig. 4A). However, at subsequent time points up to 6 days after initial infection, a modest but significant reduction (0.75 fold, p<0.05, unpaired t-test) in IL-6 levels was found in culture supernatants of infected cells compared to controls. This indicates that HBE’014 cells react when challenged with *S. aureus* by secreting the pro-inflammatory factor IL-6, as expected, but that the intracellular bacteria do not elicit such a reaction. In order to test for toxic effects, we determined LDH levels in the same culture supernatants. When LDH measurements from infected cell cultures were analysed (Fig. 4B) against negative control cultures there were no significant differences at any time point. Cell lysates cultured on blood agar confirmed intracellular bacterial presence throughout the experiments and demonstrated phenotypic growth pattern changes from normal colony sizes at day 0-2, to SCV-like colonies identified after 3 days (Fig. 4C-D).

**Discussion**

Here, we showed that the bacterial phenotype of intramucosal *S. aureus* harvested *ex vivo* from human sinonasal tissue specimens is indicative of a SCV and have an identical clonal type as their extracellular counterparts despite variation in growth patterns. Studies have described SCV growth from tissue homogenates previously (26), however our methodology employs a series of steps that aims to eliminate surface bacterial contaminants including biofilms, and demonstrated the elimination of any surface bacterial growth post-treatment. In studies that homogenise whole tissue samples without surface cleansing, it is possible that these bacteria were cultured from surface biofilms, as decreased metabolic rate and growth is also a hallmark of the biofilm phenotype (27). Biofilms are known to play a role in CRS (28) and the phenotypic form of bacteria that reside and form a biofilm could even be that
resembling a SCV, a hypothesis that could account for their altered growth and antibiotic resistance patterns (29). This is an area that would benefit from further investigation.

We have demonstrated through our in vitro experiments that enterotoxin production can be downregulated after only 24 hours of exposure to the intracellular environment. This may represent the bacterium altering its pathogenic capacity so as to reduce the toxic effects to the cell and thus maintain its protective environmental niche. Interestingly, after infecting cell cultures with SEA and SEB producing clinical isolates, both strains downregulated their virulence state by reducing the production of SEA and SEB respectively. Conversely, the SEB reference strain was unable to reduce its enterotoxin production such that SEB was detected within the cell lysate and supernatant after internalisation. This suggests that the ability to alter the virulence state may be an important factor in achieving the indolent infection. The mechanism by which organisms sense and respond to their environmental pressures remains poorly understood. One factor that has been described as a potential mechanism is known as the “bet-hedging strategy” (30). In this, it is thought that subpopulation variations in phenotypic expression may be selectively pressured into emergence through factors such as antibiotic treatment. This may occur as a means for overall species survival in the face of hostile environments.

When we performed intracellular infections with durations of up to 7 days, it was interesting to see that although the internalisation of the organisms caused an initial inflammatory response (IL-6 production), this was rapidly reduced. The initial stimulation supports the findings of Sachse et al. (7), however, in this study the authors did not make any further supernatant recordings beyond a 12 hour time-point. This initial IL-6 secretion could be induced by a TLR2-dependent immune response occurring through the extracellular insult as reported (31). The fact that there was no significant increase in LDH at any time point suggests that despite this inflammatory insult, it did not lead to any additional toxicity.
Importantly, whereas our *in vitro* cell culture model indicates that *S. aureus* undergoes phenotypic changes upon invasion of epithelial cells, no definitive conclusions can be drawn on the relevance of this finding *in vivo*. Indeed, fundamental differences between *in vitro* cell culture models and the *in vivo* situation exist. These include the non-physiological conditions that cells are cultured *in vitro* or that pathogens are applied to the cells, the presence of only a single cell type and the lack of efficient intercellular communication (32).

In conclusion, our results strengthen the hypothesis that intracellular *S. aureus* in CRS have a SCV phenotype, and subvert the host immune response by different mechanisms. These include the abortion of a pro-inflammatory immune response, absence of toxic effects and a reversible switching of bacterial virulence factors. Regulation of *S. aureus* virulence factors is a dynamic process dependant on environmental factors; specifically, the intracellular milieu appears to provide the necessary pressures to cause these alterations in an attempt for the bacterium to survive and persist within host tissues. The SCV hypothesis is one that is applicable to bacterial persistence in CRS, strengthened by evidence from their retrieval within mucosal homogenates. Further investigation is required to ascertain whether SCVs in CRS hold a clinically relevant pathogenic role in recalcitrant disease.
Figure Legend

Figure 1. Immunofluorescence of sinonasal mucosal tissue in CRS.

**A**: Negative control slide; **B-C**: Immunofluorescent images from two representative intramucosal *S. aureus* positive CRS patients. Brightly fluorescent *S. aureus* stained in green located mainly within (B, see insert) or within and beneath (C, see insert) the cytokeratin-positive epithelium (in red). 20 X magnification.

Figure 2: Small Colony Variants in CRS mucosal samples

**A-B**: Horse blood agar plates incubated at 37°C for 48 hours and inoculated with; **A**: surface tissue swabs of sinonasal mucosa demonstrating a mixed population of normal sized and small colonies of *S. aureus* with the characteristic yellow colour; **B**: sinonasal mucosal tissue homogenate post disinfection of surface bacteria demonstrating a population of small and very small colonies of *S. aureus*, whitish in colour; **C**: intramucosal colonies subcultured onto chromogenic agar demonstrating lack of coagulase activity.

**D**: Comparison of colony size retrieved from tissue swab versus sinonasal mucosal tissue homogenate. Bars represent mean +/- standard error of the mean (SEM) (*** p < 0.0001, unpaired t-test).

**E**: Pulse Field Gel Electrophoresis dendrogram demonstrating differing inter-individual clonal types of *S. aureus* harvested from surface tissue swabs from two different CRS patients (1A/2A). These clonal types were identical within the same individual when isolated from intramucosal small colonies (1B/2B) and subcultured intramucosal colonies (1C/2C). Vertical bars demonstrate fragments of bacterial DNA cut by restriction enzymes.
Figure 3: Staphylococcal enterotoxin detection from *in vitro* infection experiments

**A-C:** Horse blood agar plates grown overnight at 37°C inoculated with; A: culture media from HBE’014 cell flasks intracellularly infected with *S. aureus* ATCC 13565 demonstrating the absence of viable bacteria; B: HBE’014 cell monolayer lysed in water 24 hours post infection with ATCC 13565 demonstrating presence of intracellular bacteria; C: extracellular culture media infected with ATCC 13565 demonstrating significant bacterial growth.

**D-I:** 3M TECRA Staphylococcal enterotoxin A (SEA) and B (SEB) identification test; experiments using ATCC 13565 (SEA, D-F *(black)*); experiments using ATCC 14458 (SEB, G-I *(blue)*); D/G: culture media from intracellular infected epithelial cells demonstrating reduced production of SEA but not SEB; E/H: cell lysates of intracellular infected epithelial cells demonstrating reduced production of SEA but not SEB; F/I: culture media from extracellular infected epithelial cells demonstrating evidence of SEA and SEB.

Figure 4: Effects of long-term intracellular infection with *S. aureus* ATCC 13565 on airway epithelial cells.

**A:** IL-6 ELISA of culture media post intracellular infection demonstrating a statistically significant rise on day 1 followed by a reduction to levels lower than non-infected cells at subsequent time points up to day 6; *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired t-test.

**B:** Lactate Dehydrogenase (LDH) measurements of culture media post intracellular infection demonstrating no significant difference compared to non-infected cells. Bars for A/B represent mean +/- standard error of the mean (SEM);

**C/D:** Intracellular colonies retrieved from epithelial monolayer; C: Day 3 post infection demonstrating mixed population of normal and small colonies; D: Day 6 post infection
demonstrating several small colony variants, characterized by tiny (pinpoint) non-pigmented colonies (blue arrows).
References


Supplementary information

Supplementary Table 1: Patient demographic factors

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<tr>
<th>Patient</th>
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<th>Co-morbidities</th>
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Isolation of *S. aureus* clinical isolates from tissue homogenates

Mucosal samples were swabbed and then soaked in 0.03% cetylpyridinium chloride (CPC; Sigma-Aldrich) for 10 minutes. After washing three times with laboratory grade ultra pure water (UPW), samples were swabbed, immersed in phosphate buffered saline (PBS) and placed into a waterbath sonicator at medium intensity for 15 minutes. The sonicated tissue was swabbed, soaked again in 0.03% CPC for 10 minutes, re-swabbed, washed in UPW, homogenised using a Tissueruptor (Qiagen, GmbH, Hilden, Germany) and cultured on blood agar plates at 37°C for up to 72 hours and experiments performed in duplicate. The diameter of 25 individual colonies was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Bacterial strains
Liquid cultures were prepared by inoculating 5ml of nutrient broth with a single loop of bacteria and placed into a shaking incubator overnight at 120 rpm and 37°C. The liquid culture was centrifuged at 4000 rpm for 10 minutes and the supernatant collected, filtered and cryopreserved at -80 °C. Bacterial pellets were used for infection experiments or RNA analysis. Clinical isolates were obtained through routine microbiological swabs taken from patients at the time of endoscopic sinus surgery. *S. aureus* positive cultures were isolated and sub-cultured in nutrient broth before being cryopreserved at -80 °C.

**Cell culture infection model**

Human Bronchial Epithelial cells (16HBE14o-HBE’014) were a kind gift from Dr Dieter Gruenert and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% L-Glutamine and 1% penicillin / streptomycin (Gibco, Carlsbad, CA). Cells were washed in PBS and incubated for one hour with 5.0 McFarland units *S. aureus* (multiplicity of infection (MOI) of 100:1). The cells were then washed three times in PBS, incubated with media containing 4ug/ml lysostaphin for 30 minutes, washed 3 times in PBS and incubated in MEM supplemented with 10% FCS, 1% L-Glutamine and 100 µg/ml gentamicin. Control cells were inoculated with *S. aureus* but lysostaphin/gentamycin treatment was omitted. After 24 hours, culture supernatants were collected and cells were washed three times in PBS and lysed in Mammalian Protein Extract Reagent (Thermo Scientific).
**RNA Isolation**

Bacterial pellets from overnight liquid cultures were resuspended in RNALater (Ambion, Carlsbad, CA) and stored overnight at 4°C. This was centrifuged at 4000 rpm for 10 minutes and the bacterial pellet lysed in Tris-EDTA (TE) buffer containing lysostaphin at 500 µg/ml for 1 hour at 37°C in a rotary heating block. Into the bacterial suspension was added 350 µl of RLT buffer (Qiagen) and 250 µg of acid washed glass beads (Sigma-Aldrich, St. Louis, MI). Mechanical lysis was performed at 30 beats/second in a Tissueruptor (Qiagen) for 10 minutes. RNA was purified using RNeasy columns (RNeasy Mini Kit, Qiagen) following the manufacturer’s instructions and included an on column DNAse treatment (Qiagen). RNA quantification was performed with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Australia).

**qRT-PCR**

Real-time qRT-PCR was performed with RNA isolated from the bacterial strains. Firstly, cDNA was produced using the Quantitect RT kit (Qiagen) and qRT detection was with the IQ Sybr green supermix (Biorad, Hercules, CA). Oligonucleotide primer sequences were as follows; SEA /1F 5’-CCTTTGGAAACGGTTAAAAC-3’; SEA/1R 5’-CTCTGMACCTTYCCATCAA-3’ (34), SEB/1F 5’-ACACCCAACGTTTTAGCAGAGAG-3’; SEB/1R 5’-CCATCAAACCAGTGAATTACTCG-3’ (35). Reactions were heated for 30 seconds at 95°C followed by 35 cycles of 30 seconds at 95°C and 60 seconds at 60°C. All experiments were performed in duplicate using an iQ5 Cycler (Biorad, Hercules, CA).
IL-6 ELISA

Human Bronchial Epithelial cells (HBE’014) were seeded into 6 well plates and infected as per the previously described protocol. Culture media was changed every 24 hours and frozen to -80°C for subsequent analysis using IL-6 Enzyme-Linked Immunosorbent Assay (ELISA) and Lactate Dehydrogenase (LDH) assay. Comparison was made to negative controls that had daily media changes with no S. aureus infection. All experiments were performed in triplicate.

A sandwich ELISA technique was carried out to detect IL-6 protein expression in cell supernatants. Briefly, ELISA plates were coated overnight with rat anti-human capture antibody (BD Biosciences, California, USA), diluted in 0.1 M NaHCO₃, at 4°C. After washing with PBS containing 0.05% tween-20 (Sigma-Aldrich), plates were incubated with blocking buffer (PBS containing 2% bovine serum albumin (Sigma-Aldrich)) for 2 hours at room temperature. Samples and standard concentrations (BD Biosciences) were applied to the wells in duplicates and incubated at room temperature for another 2 hours. After washing, biotinylated rat antihuman IL-6 antibody (BD Biosciences) was added and incubated at room temperature for 30 minutes. After washing, Horseradish Peroxidase (HRP)-conjugated streptavidin (Thermo Scientific, Australia) was incorporated and incubated at room temperature for 30 minutes. TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution (Thermo Scientific, Australia) was applied and the reaction stopped with 0.16 M sulphuric acid after 10 minutes. Light absorbance was measured at 450 nm using spectrometry. The values obtained were corrected by dilution factor and expressed in pg/ml. The lower limit of detection was 12.5 pg/ml.
Lactate Dehydrogenase (LDH) Assay

Culture supernatants were centrifuged then passed through a 0.22 µm filter before being processed by a commercially available LDH assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI) as per the manufacturer’s instructions. Briefly, in a 96 well plate, 50 µl of filtered supernatant was mixed with 50 µl of substrate mix and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 µl of Stop Solution and absorbance determined at 490 nm.

Superantigen detection

Briefly, supernatants and cell lysates were centrifuged at 13,300 g for 10 minutes before being passed through a 0.2 µm filter and 200 µl was added to 10 µl of buffer solution. Wells were pre-soaked with wash solution for 30 minutes followed by incubating 50 µl of supernatant or lysate for 2 hours at 37°C. Following washing, 200 µl of substrate solution was added and incubated for 1 hour and then washed again. 200 µl of conjugate was added and the plate read after 30 minutes. The 3M TECRA SIDVIA kit (3M, North Ryde, NSW, Australia) uses a visual assessment that either classifies a sample as enterotoxin positive or negative with a sensitivity of 1 ng/ml.

Indirect immunofluorescence
Antibodies used were a mouse IgM monoclonal antibody to *S. aureus* (Millipore, Billerica, MA) and a rabbit polyclonal anti-human pan-cytokeratin (Invitrogen Corp., Carlsbad, CA). Negative controls were prepared with normal rabbit IgG (Sigma-Aldrich, St. Louis, MI). Secondary antibodies were goat anti-mouse IgM-Alexa488 (Invitrogen Corp., Carlsbad, CA) and sheep IgG F(ab’) anti-rabbit IgG-Cy3 (Sigma-Aldrich, St. Louis, MI). Indirect immunofluorescence of paraffin embedded tissue sections was carried out following a previously described protocol (33). Briefly, 5 μm sections were prepared from paraffin embedded tissue blocks, mounted on glass microscope slides (Superfrost, Thermo Fisher, Waltham, MA), deparaffinised and rehydrated using xylene and graded ethanol. Slides were immersed in 10 mM sodium citrate and antigen retrieval performed using a microwave technique. Slides were transferred into a Tris-buffered saline buffer containing 0.05% of Tween-20 (TBST). Serum free protein block (SFB) (Dako, Glostrup, Denmark) was applied for 1 h at room temperature and slides were incubated simultaneously with mouse anti-*S. aureus* and rabbit anti-pan-cytokeratin antibodies overnight at 4 °C. Slides were washed in TBST and secondary antibodies were applied for 1 h at room temperature in a darkened moisture chamber. Slides were washed in TBST and coverslips mounted in 90% glycerol for microscope analysis with a Zeiss Axio Vert.A1 (Carl Zeiss AG, Jena, Germany).

**Statistical analysis**

To determine differences in colony size, IL-6 and LDH concentrations, statistical analysis was performed with Graphpad Prism 6.0 software (San Diego, CA) using an unpaired t-test and statistical significance was obtained when p < 0.05.