The Induction of Small Colony Variant Staphylococcus aureus in Artificial Sputa Media

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Abstract

Staphylococcus aureus is an opportunistic pathogen that has adapted to the respiratory tract due to its metabolic versatility, but is normally associated with the skin and mucus membranes (Parker and Prince, 2012). Small colony variants (SCV) are a subpopulation of bacteria. Characteristics of S. aureus SCV are decreased α-cytotoxin activity, auxotrophic growth deficiency, deficiency in electron transport chain, and increased resistance to aminoglycosides (Melter and Radojevic, 2010). The SCV is believed to be caused by the selective pressure of antibiotics and have increasingly been isolated from cystic fibrosis (CF) patients (Yagci et al., 2013). CF is a genetic disease that results in increased mucus formation in lungs and pancreas. CF patients are susceptible to S. aureus infections with S. aureus being the most prevalent infection in childhood and the second most common infection in adults with CF (Cystic Fibrosis Foundation, 2006). The aim of this study is to determine if S. aureus isolated from CF patients grown in artificial sputum medium will exhibit altered SCV phenotype.

Keywords: Staphylococcus aureus, Cystic Fibrosis, Small Colony Variant

Introduction

Staphylococcus aureus is a bacterium associated with the skin and mucus membranes and is an opportunistic pathogen (Parker and Prince 2012). Infections cause a variety of ailments from skin infections to endocarditis (Chakraborty et al. 2011). S. aureus has adapted to the respiratory tract due to its metabolic versatility, ability to scavenge iron, ability to coordinate gene expression, acquisition of genetic elements, and interactions with virulence factors α-hemolysin and protein A, as well as immune effectors in the lung. For these reasons, S. aureus is a successful pathogen. S. aureus affects the respiratory tracts from asymptomatic colonization to fulminant necrotizing pneumonia (Parker and Prince, 2012).

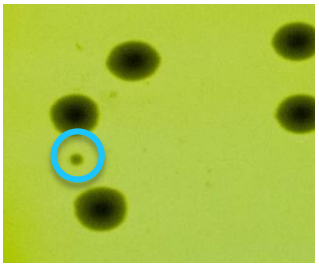


Figure 1 - Small-Colony Variant Staphylococcus aureus in Comparison to Wild-type S. aureus Morphology. The circled colony is a representation of the SCV phenotype of S. aureus. Wild-type S. aureus is much larger in size and is represented by the dark round colonies on the plate

Small colony variants (SCV) are a subpopulation of bacteria. Characteristics of S. aureus SCV (Fig. 1) are decreased α-cytotoxin activity and auxotrophic growth deficiency (Melter and Radojevic 2010). Commonly, SCV are deficient in electron transport chain but have increased resistance to aminoglycosides, which are traditional gram-negative antibiotics. There are SCV of S. aureus that are the result of mutations in metabolic genes; because large amounts of ATP are required for the creation of cell walls, the SCV colonies grow more slowly, resulting in SCV colonies being one-tenth of the wild-type S. aureus colonies. In addition to size difference, SCV S. aureus is non-pigmented and non-hemolytic. The SCV is believed to be caused by the selective pressure of antibiotics, and with prolonged exposure to antibiotics, the SCV population increases compared to the wild-type. SCVs can phenotypically be influenced by non-

*=Mentor

coding RNA present in both SCV and wild-type S. aureus.

SCV have increasingly been isolated from cystic fibrosis (CF) patients (Yagci et al. 2013). CF is a genetic disease that results in mucus formation in lungs, which blocks the airways, in the pancreas, which interferes with proper digestion, and in other organs. CF is caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR) gene. The CFTR glycoprotein is expressed in epithelial cells and acts as a chloride channel, an ion regulator, and regulates airway hydration. CF patients are susceptible to S. aureus infections, especially in childhood, but these infections persist throughout life.

The aim of this study is to determine if S. aureus isolated from CF patients grown in artificial sputum medium will exhibit altered SCV phenotype. The growth of SCV phenotype from S. aureus recovered from and compared to different age groups: children (under 13), adolescents (13-18), and adults (over 18).

Methods

Isolation:

First, previously collected growth from Mannitol Salts agar (MSA) was struck again on MSA to collect individual colonies. Twelve colonies that ferment mannitol were picked, both SCV and wild-type S. aureus, from three patients: one child (under 13), one adolescent (13-18), and one adult (over 18). Mannitol fermentation can be determined by the yellowing of MSA media caused by the acidic byproducts. Next, a freezer block was made from the individual colonies by inoculating wells of a 96-well block filled with 1.5 mL of Brain Heart Infusion (BHI) broth. The block was sealed with foil and incubated overnight while shaking at 37° Celsius at 220 RPM. After cultures had grown, the block was centrifuged at 4200 RPM for five minutes. The supernatant was discarded and the cell pellet re-suspended in 1 mL of skim milk, and stored frozen at -80°C. To create a working stock, a 48-pin replicator pin was used to stamp out cultures from the freezer block onto LB and the plate was incubated for 48 hours at 37° C. The control strain, S. aureus ATCC 12600, was struck from a frozen stock using a wooden inoculating stick onto LB and incubated for 48 hours at 37° C.

Phenotype Induction:

The negative control was growth and morphology from a minimal media (MM) broth. S. aureus ATCC 12600 was used as a positive control. One colony was grown in both a tube containing 3mL of artificial sputum media (ASM) and 3mL of MM, and incubated for 48 hours at 37° Celsius. ASM mimics the sputa of a CF patient and is made with pig mucin, salmon sperm DNA, sodium chloride, potassium chloride, diethylene triamine pentaacetic acid (DTPA), Tris base, egg yolk emulsion, and casamino acids (Diraviam Dinesh 2010). This was repeated for all colonies in the freezer block. After incubation, both the ASM and the MM was diluted in sterile H2O and the 10-3 and 10-6 dilutions were plated on MSA plates using a quadrant streak. These MSA plates were incubated for 48 hours at 37°C.

Analysis:

The MSA plates were struck with 10-3 and 10-6 dilutions from both ASM and MM cultures. To analyze the data, colony morphology was compared between MSA plates from ASM and MM for difference in size (Fig. 4). Criteria for SCV were that the colonies were punctiform after 48 hours of growth and were not close to other colonies. The total number of colony forming units and the total number of SCV were recorded. Additionally, age groups were compared to

determine if patient age correlates with a higher rate of the SCV phenotype expression. This process is summarized in Figure 2.

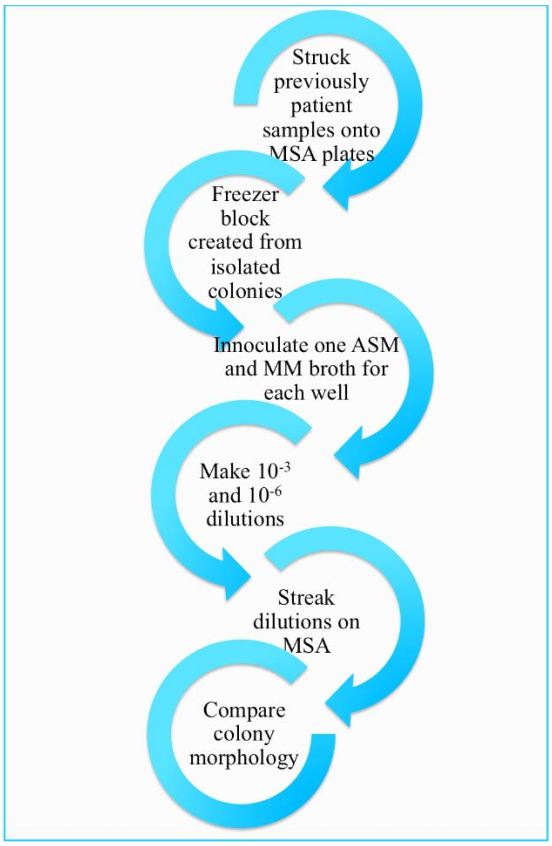


Figure 2 - This figure is a visual representation of the methods used in this study.

Results

Dilutions of liquid cultures of ASM and MM were plated on MSA plates. SCVs grew on the plates from both the ASM and the MM broths (Fig. 3A). There were more SCVs recovered from the ASM than the MM. The adult had the highest number of SCV, 33 in total, than the other ages, with twice as many colonies from ASM than from from MM. The total number of colonies grown from the adult was also the highest at 1300 colonies. The adolescent had the fewest total number of SCV, 13 colonies, with 10 of these colonies recovered from ASM. However, the adolescent had the fewest total number of CFUs, which may have resulted in the lower number of SCV growth. The child was in the middle of colony production for both SCVs and total CFUs. For the child, the amount of SCVs was 18, and 15 of those were from the ASM. Total CFUs for the child was 653, with 592 from the ASM and 61 from MM. Total number of CFUs was higher than the total number of SCV for both media in all ages (Fig. 3B). The adult had the highest total CFUs at 1300 colonies, and the adolescent had the least at 526

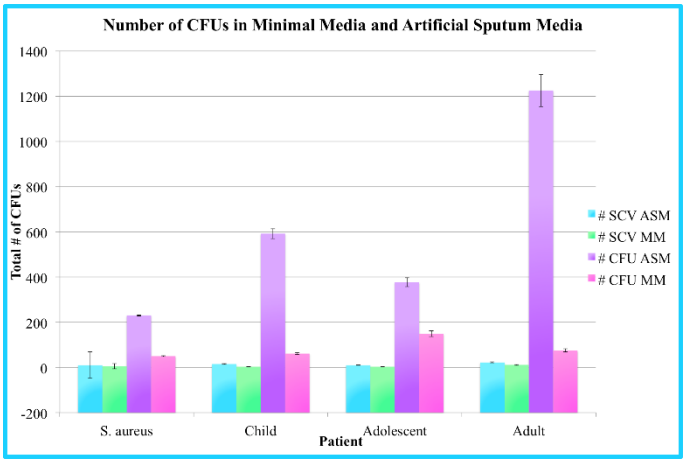


Figure 3A - This figure shows the number of SCVs formed in MM and ASM media. Error bars represent standard deviation.

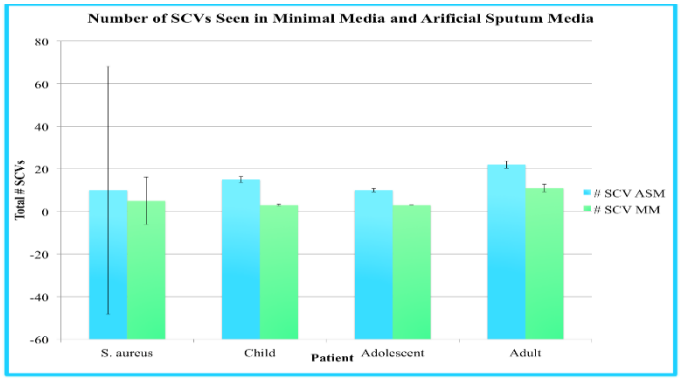
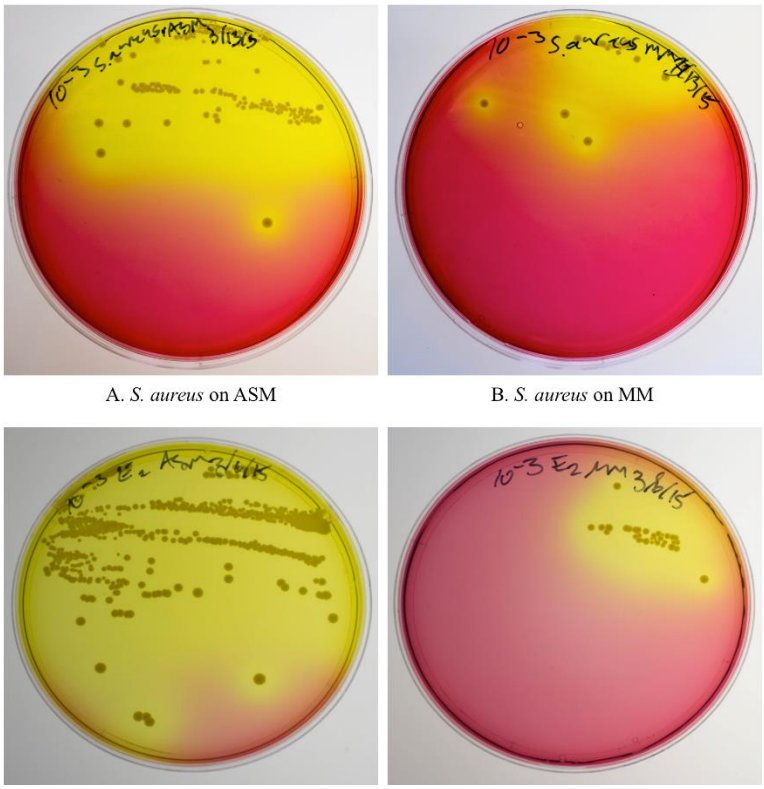


Figure 3B - This figure shows the number of SCVs formed in MM and ASM media. Error bars represent standard deviation.



A. *S. aureus* on ASM B. *S. aureus* on MM

Figure 4 - is a representation of growth seen on Mannitol Salts agar. A and B demonstrate the growth of control strain S. aureus ATCC 12600 from liquid cultures of ASM (A) and MM (B). C and D demonstrate the growth of S. aureus isolate E2 from liquid cultures of S. aureus isolate E2 from liquid cultures of ASM (C) and MM (D).

colonies with 377 from ASM. However, there was no significant induction of the SCV phenotype from ASM and from MM, as indicated by the t-test with p-values > 0.05.

Discussion

In our study, we studied the induction of the small-colony variant phenotype in S. aureus in a liquid medium that mimics the CF sputum. We did this by plating previously collected samples, picking twelve colonies from each age group, and then growing these colonies in both ASM and the MM. The two liquid cultures were plated again so colony morphology could be compared. Our results show there was more growth of both SCV and wild-type colonies from ASM than from MM. However, there was no significant induction of the SCV phenotype from either media. The number of SCVs also appeared to increase with age.

The increase in SCV with patient age could be attributed to increased antibiotic exposure with age. Antibiotics are commonly taken to clear bacterial infections, and because people with CF are immunocompromised. Antibiotics are regularly used as part of CF treatment as CF patients have chronic infections that are not cleared even with antibiotic treatment. Additionally, we saw more colonies grow from ASM than from MM. This increase can be attributed to ASM being more nutrient rich than MM.

Future studies will address questions on the induction of the SCV phenotype in mixed cultures to see if being grown in a poly-microbial community, like what is commonly found in the CF lung, could induce SCV when grown in ASM and MM.

Acknowledgements

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