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Weak association of *Staphylococcus aureus* hemolytic activity with reduced FEV_1 in older children with cystic fibrosis

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Abstract
This study aimed to determine the role of staphylococcal hemolytic activity in the pathogenesis of cystic fibrosis (CF) lung disease. We collected 50 *Staphylococcus aureus* isolates and pulmonary function data from CF patients. The hemolytic activities of these isolates and lung functions were analyzed. In addition variation in the *hla* promoter region of these isolates was determined following PCR amplification and sequencing. Wide ranges of hemolytic activities were observed in these isolates. A weak negative correlation between the hemolytic activities and the FEV_1 percent predicted was shown in CF patients greater than ten years old. Moreover, we found a significant difference in the FEV_1 percent predicted and FEV_1/FVC ratio in *S. aureus* CF patients older than 10 years of age compared to those less than 10 years of age, suggesting a greater burden of obstructive lung disease in the older group. DNA alignment analysis on the DNA sequences revealed eight single nucleotide polymorphisms (SNPs) within the *hla* promoter region, which did not correlate with hemolytic activity. Our results suggest that higher levels of hemolytic activity may contribute to the pathogenesis of CF, resulting in lung function deterioration. However, SNPs in different locations of *hla* promoter regions do not direct hemolytic activity.

Keywords: *Staphylococcus aureus*, hemolytic activity, cystic fibrosis, FEV1%, FVC%

1. Introduction
Cystic fibrosis (CF) is an autosomal recessive disorder that causes impaired airway luminal epithelial salt and water balance. Mutations in the gene of CF transmembrane conductance regulator (CFTR) are responsible for abnormal epithelial chloride transport and impaired airway function [1, 2]. Defects in CFTR activity result in airways to become desiccated and obstructed with viscous mucus [2, 3]. This viscous mucus elicits intense airway bacterial colonization resulting in chronic infection, inflammation, increased airway neutrophils and ultimately elicits progressive bronchiectasis and respiratory failure as individuals with CF age [4, 3, 5]. The newborn respiratory tract is rapidly colonized *Staphylococcus aureus* within days after birth. Preventing airway injury due to *S. aureus* production of soluble proteins and neutrophil chemotaxis is central to CF respiratory care [4-6].
Staphylococcal α-toxin is a critical virulence factor in mouse models of infections such as abscesses and pneumonia [7-9]. A-toxin induces apoptosis and necrosis of various cell types through specific binding to receptors on host cells, forming functional transmembrane pores, and /or activating signaling pathways [10-13]. Importantly, it was revealed that high level of staphylococcal α-toxin (*hla*) (titers > 2,000 mg/dL) is associated with low scores of chest x-ray and the development of pneumatoceles in the lung of 34% of CF patients with persistently positive *S. aureus* airway cultures [14].
α-toxin is a soluble protein that has been shown to directly damage endothelial cells and induce changes of vascular permeability resulting in leakage in rabbit models of isolated perfused lung [15]. We demonstrated that α-toxin targets airway epithelium and lead to increased permeability in airway epithelium after α-toxin is exposed to an animal [16]. Moreover, it has been reported that α-toxin is able to affect the phenotypic growth of *S. aureus* on vaginal mucosa through enhancing tissue disruption and biofilm formation [17]. Taken together, these findings suggest that staphylococcal α-toxin possibly contributes to airway disease in CF by directly injuring airway epithelium and promoting biofilm formation.
However, the prevalence of *S. aureus* α-toxin producing strains in individuals with CF is unknown. In the present study, we collected 50 *S. aureus* isolates from CF patients, examined their hemolytic activities, determined whether the hemolytic activity of CF *S. aureus* isolates is associated with decline in CF lung function, and further identified SNPs within the *hla* promoter region.

2. Materials and methods
2.1 Sample acquisition and bacterial culture
In order to identify pathogenic bacterial organisms, individuals seen in the Mayo Clinic CF Center have respiratory surveillance samples obtained for aerobic microbiologic culture during each clinic visit as part of their routine care procedure. Standard clinical diagnostic microbiologic approaches were utilized to identify and determine *S. aureus* isolates from the respiratory surveillance samples (sputum and throat) of patients with CF. Patients or

parents of patients were contacted after aerobic sputum cultures demonstrated growth of *S. aureus*. Our study protocol was approved by the Mayo Clinic Institutional Review Board (IRB: 13-001708). Informed consent was obtained by an oral consent script with return of the written consent form by the participant/participant representative.

A clinical sample of *S. aureus* was sent to Dr. Ji's laboratory at the University of Minnesota and was further evaluated. The *S. aureus* sample grew in Trypticase Soy Broth (TSB; Difco) liquid medium at 37˚C with shaking at 220 RPM, and was culture on sheep's blood agar plates (BD) at 37˚C.

2.2 Hemolysis assays

To confirm the presence of hemolytic activity clinical samples of *S. aureus* from individuals with CF were grown overnight in TSB at 37ºC with and shaking at 220 RPM. The supernantant was collected from 1 ml of the culture after centrifugation for five minutes at 13,200 RPM and discarding the bacterial cells. Then, 5 □l was dropped onto sheep blood agar plates and incubated overnight at 37°C, and subsequently was placed at 4ºC for two days then digitally photographed to determine the complete breakdown of red blood cell (RBC) hemoglobin in the vicinity of bacterial colonies.

For quantatitive analysis of *in vitro* α-toxin production we used rabbit RBCs as described [18]. Briefly, the supernatant of each culture was mixed with equal volume of a 5% rabbit RBCs solution in PBS in triplicate in a 96-well round-bottom plate and incubated at 37°C for 30 minutes. The plate was spun at 3,200 RPM for five minutes to pellet nonlysed RBCs. The supernatants were collected, transferred into a new 96-well plate, and measured for hemoglobin content by spectrophotometry at A_{450}. PBS was used as the negative control of hemolysis, whereas Saponin (0.5%) was used as the positive control of hemolysis. Percent hemolysis was calculated by averaging the A_{450} values of each sample, and by converting the values to a percent based on the following formula: Hemolysis% = (average of the A_{450} sample values - average of the A_{450} negative values)/(average of the A_{450} positive values - average of the A_{450} negative values). The hemolysis experiments were repeated at least three times.

2.3 Identification of SNPs in *hla* promoter region

To explore whether SNPs are involved in regulation of α-toxin production in CF *S. aureus* isolates, the bacterial cells were harvested from overnight cultures and genomic DNA was isolated using genomic DNA purification kit (Promega). The *hla* promoter regions were obtained by PCR and DNA sequenced. The SNPs were identified by BLAST alignment analyses of the promoter region based on the available *S. aureus* genomes in the NCBI genome database [19, 20].

2.4 Longitudinal analysis of lung function testing

Cystic fibrosis patients seen in the Mayo Clinic CF Center who had *S. aureus* and no *P. aeruginosa* detected in their surveillance CF cultures and at least two spirometry studies available were identified from the study group. This cohort consisted of 16 children with acceptable spirometry results performed between 2013 and 2014. Mixed model regression analysis was used to provide estimates of the FEV_1 (forced expiratory volume in one second), FEV_1 percent predicted, FVC (forced vital capacity) and FVC percent predicted average rate of change in an individual over time.

2.5 Statistical analysis

The median age for the entire group of CF patients studied was calculated and used to create two groups two groups of CF patients, age less than ten years old (n = 8) and age greater than ten years (n = 8). A two-sample t-test assuming equal variances was used to compare age, FEV_1 percent predicted, FVC percent predicted, and the FEV_1/FVC ratio between the two groups of CF patients. The t-statistic was significant at the 0.05 critical alpha level. The Pearson correlation coefficient was calculated to measure the strength of the linear association between hemolytic activity and FEV_1 percent predicted variables. A correlation coefficient of +/- 1 was used to indicate a perfect positive correlation.

3. Results

3.1 Different isolates of *S. aureus* from cystic fibrosis patients possess distinct hemolytic and cytotoxic activities.

In *S. aureus* cultures from CF patients we observed a significant qualitative difference among isolates in α- and □-hemolytic activities from supernatants of overnight cultures onto sheep blood agar plates (Figure 1). In addition quantitatively determined *in vitro* α-toxin production by *S. aureus* noted a range of hemolytic activities among the fifty isolates. We grouped these isolates into 4 groups: "highly toxic" (>100% hemolysis) "toxic" (71-99% hemolysis) "intermediate toxic" (11-45% hemolysis) "low toxic" isolates (<10% hemolysis) (Table 1).

3.2 Correlation between hemolytic of CF *S. aureus* isolates and pulmonary function parameters in CF patients.

The median age for the entire group of CF patients (n= 16) studied was 9.5 years. We used the median age to create two groups of CF patients, one group above the median age and one group below the median age. The first group consisted of CF patients less than ten years old (n = 8) and the second group age greater than ten years (n = 8). FEV_1 percent predicted was significantly lesser in the CF patients age greater than ten years compared to the CF patients age less than 10 years (77.4 ± 36.6 L/sec vs. 95.1 ± 7.9 L/sec, $p < 0.05$). Likewise the FEV_1/FVC ratio was lower in the older CF group compared to the younger CF group (78.1 ± 29.7 vs. 85.2 ± 10.4, $p < 0.05$) indicating mild obstructive lung disease (Table 2).

To explore whether hemolytic activity of *S. aureus* isolates contributes to the decline of lung function, we statistically analyzed the relationship between relevant hemolytic activities of the *S. aureus* isolates for hemolysis of rabbit RBCs and the rate of change in FEV_1 percent predicted. Pearson correlation was performed in two groups of CF patients, age less than ten years old and age greater than ten years. A small negative correlation (r = -0.17) between the hemolytic activities and the FEV_1 percent predicted was shown in the CF patients greater than ten years old. There was no linear relationship (r = -0.01) in the age less than ten years CF patient group.

3.3 Identification of SNPs in *hla* promoter region of *S. aureus* isolates from CF patients.

We have previously demonstrated that SNPs in the promoter region of *S. aureus* isolates from bovine mastitis are involved in regulation of hyperproduction of alpha-toxin [19, 20]. To determine whether SNPs are also associated with regulation of differential α-toxin production in CF *S. aureus* isolates, we isolated the genomic DNA, amplified, and sequenced the *hla* promoter regions from the isolates. DNA sequence alignments revealed eight major SNPs in the *hla* promoter region of CF *S. aureus* isolates, including -457C/T, -483T/C, -498G/A, -509G/A, -548T/C, -550A/G, -571C/T and -624A/C from start codon of *hla* (Figure 2). However, the nucleotide sequences of the -457, -483, -498, -509, -548, and -624 positions in *hla* promoter region were identical between toxic isolates such as CFSa1, CFSa4, and CFSa30 and less toxic isolates such as CFSa8, CFSa16, CFSa18, CFSa28, and CFSa31 (Table 1). Moreover, the nucleotide sequences of the -550 and -571 positions of the *hla* promoter region were also identical between toxic isolate CFSa4 and less toxic isolates, including CFSa16, CFSa18 and CFSa28 (Table 1). These results suggest that there was no association between hemolytic activities against rabbit RBCs and the identified SNPs in these CF *S. aureus* isolates.

4. Discussion

In present study we sought to pinpoint if *S. aureus* α-toxin producing strains correlate with a decline in lung function in individuals with CF. The prevalence and potential role of *S. aureus* α-toxin to produce changes in functional lung injury in individuals with CF is not known. Clinical decisions to treat *S. aureus* airway colonization with oral or inhaled antibiotics in CF children have changed over time and remain uncertain [21]. *S. aureus* α-toxin, also known as α-hemolysin, is the major cytotoxic agent released by *S. aureus*. It exhibits selectively hemolytic and cytotoxic activities and has a remarkable preference for rabbit RBCs. In tissues it monomeric form binds to membranes in a receptor independent fashion. Upon binding transmembrane pores are formed which are ion permeable which stimulates both cellular phospholipases and induces a Ca^{2+} influx leading to cell death [22].

In this study we found that different CF *S. aureus* isolates exhibit distinct levels of hemolytic activity, which is consistent with previous reports in human and animal *S. aureus* isolates [19, 20]. Our data show a weak negative correlation between hemolytic activity of CF *S. aureus* isolates and lung function in CF patients. This suggests that higher levels of hemolytic activity may be associated with greater lung damage in patients with CF.

S. aureus produces a variety of exotoxins, including α-, β- and γ-toxins, and leukotoxins. It is possible that these exotoxins contribution to the pathogenicity of CF *S. aureus*. Though α-toxin can include apoptosis and necrosis of various human cells including epithelial cells [23, 24], its role on CF airway epithelium is not certain. However, *in vitro* experiments indicate that recombinant *hla* has a significant impact on the metabolome of human airway epithelium as a consequence of direct recombinant *hla*-mediated alterations in plasma membrane permeability or indirect effects mediated by cellular signaling [25]. Moreover, it is possible that different CF *S. aureus* isolates produce different kinds of exotoxins. Indeed we demonstrated that among our CF patients different isolates carry different genes encoding various exotoxins [26].

Though the magnitude of correlation between the hemolytic activity of the CF *S. aureus* isolates and the decline of lung function in CF patients was weak, it is possible that the time frame over which pulmonary function monitoring was performed may not have been sufficient to reflect the change of $FEV_{1.}$ In addition routine chest physiotherapy, inhaled mucolytics and acute and chronic antibiotic use may diminish the impact of *S. aureus* infection on lung function. Thus, it will be necessary to further determine the role of α-toxin on lung function in a model lung environment *in vivo* [27].

Our previous studies demonstrated that the SNPs in the *hla* promoter region are involved in mediation of production of α-toxin through altering the activity of the SarZ, a key *hla* regulator in bovine mastitis *S. aureus* isolates [19, 20]. In this study, we also identified eight different SNPs in the *hla* region in the CF *S. aureus* isolates, however, data analysis revealed that these SNPs are not associated with regulation of α-toxin production. Further analysis revealed that these SNPs of the *hla* promoter region are located in different positions compared to those SNPs in bovine mastitis and other human *S. aureus* isolates [19, 20]. These results suggest that the SNPs of *hla* promoter region in CF *S. aureus* isolates possibly do not participate in the regulatory function of SarZ. It is likely complex regulatory systems contribute to differential expression of α-toxin in different CF *S. aureus* isolates [28, 29]. These regulators, include two-component signal regulators such as AgrABCD [28-30], SaeRS [30, 31], and ArlRS [32, 33], and transcriptional regulators Mgr [34] and SarZ [35-37], which positively regulate the expression of α-toxin; whereas Rot and SarT repress the expression of α-toxin [38, 39], and SarA mediates *hla* in both an *agr*-dependent and *agr*-independent manner. Moreover, *hla* is also modulated by sigma B factor (σ^B) and environmental stimuli [40, 37]. Taken together, our results suggest that SNPs in different locations of *hla* promoter regions possibly possess distinct functions, and that other regulatory mechanisms may be involved in the regulation of *hla* expression in the CF *S. aureus* isolates.

5. Tables and Figures

Table 1: Hemolytic activities of CF *S. aureus* isolates

Strain ID	% Hemolysis*
CFSa1	89.7
CFSa4	79.5
CFSa4_2	109.8
CFSa5	22.9
CFSa5_2	42.6
CFSa7	22.7
CFsa8	3.2
CFSa8_2	3.6
CFSa11	3.6
CFSa11_2	4.1
CFSa15	3.1
CFSa15_2	3.2
CFSa16	4.2
CFSa16_2	4.7
CFSa18	4.4
CFSa18_2	9.4
CFSa19	71.5
CFSa20	3.1
CFSa20_2	3.2
CFSa21	3.2
CFSa21-1	4.4
CFSa21_2	99.7
CFSa22	3.7
CFSa22_2	87.1
CFSa23	5.2
CFSa23_2	3.3
CFSa24	3.4
CFSa24_2	3.4
CFSa26	4.1
CFSa26_2	11
CFSa27	3.5
CFSa27_2	3.5
CFSa28	3.6
CFSa28_2	
CFSa29	3.4
CFSa29_2	3.6
CFSa30	90.2
CFSa31	3.8
CFSa31_2	3.3
CFSa33	11.1
CFSa35	109.8
CFSa35_2	109.8
CFSa36	109.8
CFSa36_2	109.8
CFSa37	3.6
CFSa37_2	4.2
CFSa38	7.7
CFSa40	4.6
CFSa40_2	4.6
CFSa42	89.4

The value is representative of three independent experiments.

Table 2: Comparison of lung function and lung function correlated with hemolysis percentage in children younger and greater than 10 years of age.

	Age <10 years (n = 8)	Age > 10 years (n = 8)
Age (years)	7.2 + 1.4	11.5 + 3.1 *
FEV_1 % predicted	95.1 + 7.9	77.4 + 36.6 *
FVC % predicted	100.9 + 14.0	90 + 39.9
FEV_1/FVC	85.2 + 10.4	78.1 + 29.7 *
Pearson lung function-hemolysis % correlation coefficient (r)		
FEV_1%/Hem%	-0.00675	-0.16606

* *P*-value ≤ 0.05

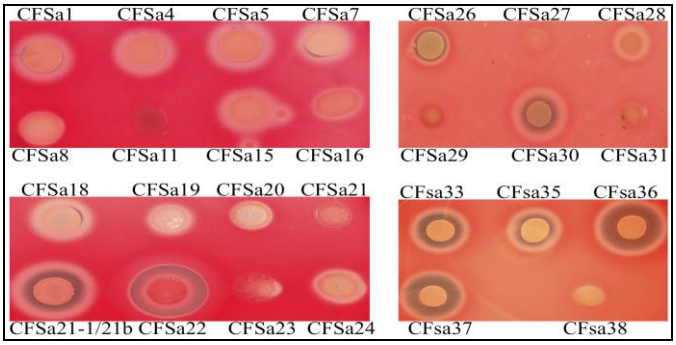

Fig 1: Hemolysis activities of the supernatants of overnight cultures of *S. aureus* isolates from CF patients. Equal volume of the supernatant from each culture was dropped onto TSA-blood agar plate (5% sheep blood). The plate was incubated at 37°C overnight, then put in 4°C refrigerator two days before taking pictures. Both α- and β-hemolysis was observed.

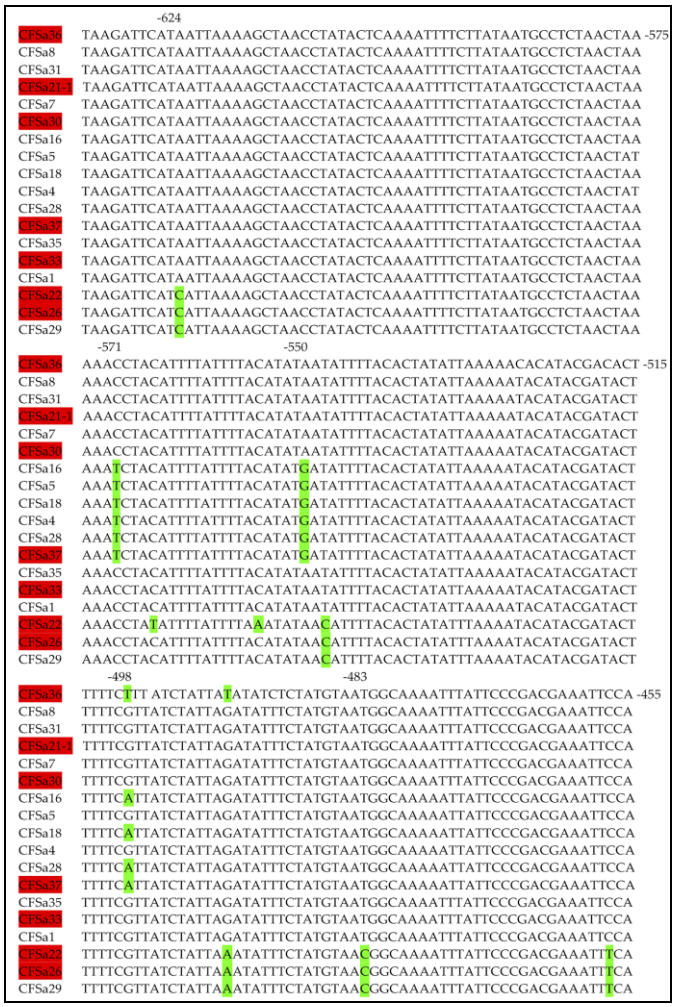

Fig 2: Identification of SNPs in *hla* promoter region of *S. aureus* isolates from CF patients. Green color indicates SNPs; Red color indicates highly hemolytic CF *S. aureus* isolates.

6. Conclusion

Our data suggest that specific isolates of *S. aureus* from CF patient's airways produce different levels of α-toxin, and that higher levels of α-toxin may contribute to more severe airway damage, resulting in greater lung function deterioration. Moreover, our data indicate that SNPs in different locations of *hla* promoter regions possess distinct functions and other regulators may be involved in the differential expression levels of α-toxin in CF *S. aureus* isolates.

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Ying Liu, Lu Ji, Junshu Yang, Dengke Zhong performed the experiments. James Phillips and Yinduo Ji designed the experiments. Ying Liu, Lu Ji, Jiang Zhang, James Phillips, Mark Wylam, and Yinduo Ji analyzed the data. James Phillips, Mark Wylam, and Yinduo Ji wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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