MRSA Strains with Distinct Accessory Genes Predominate

at Different Ages in Cystic Fibrosis

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Title: 92 characters with spaces	**Funding Sources:**
Abstract word count: 246	NHLBI K08 HL136927 (AJF)
Word count: 3117	University of Iowa CTSA UL1TR002537.
References: 27	Cystic Fibrosis Foundation K Boost (AJF)
Figures: 6	CFF Research and Development
Tables: 2	Program Bioinformatic Core, Iowa (AAP)
Supplemental Data: 5	CDC Advanced Molecular Detection,
	Epidemiology and Laboratory Capacity
	Cooperative Agreement (VR)

Keywords: *Staphylococcus aureus*, Cystic Fibrosis, MRSA, Whole Genome Sequencing, Birth Cohort Effect

Running Title: Distinct Clusters of MRSA in CF

Key Point: Methicillin resistant *Staphylococcus aureus* (MRSA) causes persistent airway infections in cystic fibrosis. Children and adults with cystic fibrosis often have different MRSA strains, which encode distinctive repertoires of virulence factors in their accessory genomes.

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Study Design: HSP, VR, DJD, AJF. Obtained funding: VR, AJF. Collected strains: HSP, BF, DJD. Analyzed clinical data: HSP, LJM, MML, ZEK, NP, ARH, CZ, BLM, AJF. Performed and Analyzed PFGE: HSP, LB. Genome Sequencing: VR, ET. Analyzed Data: HSP, VR, ALT, AP, MML, ZEK, NP, ARH, CZ, BLM, ET, AJF. Statistical Analysis: SBS, LJM, CZ, AJF. Archived Data and Specimens: AJF, HSP, ZEK, NP, ARH, CZ. Deposited Sequencing Data: ARH, CZ, AJF. Wrote Initial Draft: AJF, HSP. Edited Paper: HSP, VR, SBS, DJD, BF, AAP, AJF

Abstract

Rationale: Methicillin resistant *Staphylococcus aureus* (MRSA) is prevalent and consequential in cystic fibrosis (CF). Whole genome sequencing (WGS) could reveal genomic differences in MRSA associated with poorer outcomes or detect MRSA transmission.

Objectives: To identify MRSA genes associated with low lung function and identify potential MRSA transmission in CF.

Methods: We collected 97 MRSA isolates from 74 individuals with CF from 2017 and performed short-read WGS. We determined sequence type (ST) and the phylogenetic relationship between isolates. We aligned accessory genes from 25 reference genomes to genome assemblies. We classified the MRSA by accessory gene content and correlated the accessory genome to clinical outcomes.

Results: The most prevalent ST were ST5 (N=55), ST105 (N=14), and ST8 (N=14). Closely related MRSA strains were shared by family members with CF, but rarely between unrelated individuals. Three distinct clusters of MRSA were identified by accessory genome content. The first included ST5 and ST105 strains and was common among older patients with lower FEV_1. The second cluster included ST8, which was generally identified in younger patients. Sputum density of MRSA and *Pseudomonas aeruginosa* was higher in cultures from patients with ST5/ST105 compared to patients with ST8 at similar ages.

Conclusions: In this CF cohort, we identified MRSA subtypes that predominate at different ages and differ by accessory gene content. ST5 and ST105 represented the

most prevalent cluster of MRSA. ST8 MRSA was more common in younger patients

and thus has the potential to rise in prevalence as these patients age.

Introduction

The vast majority of patients with cystic fibrosis (CF) develop chronic bronchitis with

Staphylococcus aureus.[1] Infections with *S. aureus* begin as early as infancy and are

difficult to eliminate despite use of anti-staphylococcal antibiotics.[2] Moreover, methicillin

resistant *S. aureus* (MRSA) prevalence has increased significantly in the United States

over the past two decades.[1,3] Patients infected with MRSA require increased treatment

and suffer worse outcomes.[4-6] These observations raise questions about how MRSA is

acquired in these patients and which bacterial virulence factors might accelerate

disease progression.

Whole genome sequencing (WGS) may help us understand the origins of MRSA within

this population. WGS facilitates the phylogenetic comparison of MRSA strains to

determine whether there is an unexpectedly strong relationship between bacteria

isolated from unrelated individuals. Because CF is a genetic disorder, sibling dyads who

live in the same household may be infected with closely related strains of MRSA. The

phylogenetic distance between MRSA strains cultured from siblings would provide a

benchmark for identifying potential transmission of MRSA between unrelated patients

with CF during clinical care.[7,8] We hypothesized that WGS would identify person-to-

person transmission of MRSA between unrelated individuals within our CF center.

Another advantage of WGS is that it allows classification of *S. aureus* isolates on the

basis of sequence type and provides detailed information about genomic structure,

including the presence or absence of virulence factors. In particular, we are interested in

5

the prognostic significance of genes encoding staphylococcal superantigens, as we have observed high prevalence of enterotoxin gene cluster superantigens in an earlier de-identified set of *S. aureus* isolates from this center.[9] These secreted toxins have the potential to increase inflammation and interfere with the adaptive immune response to *S. aureus*, hindering clearance by the host.[10,11] We hypothesized that strains of *S. aureus* that encode enterotoxin gene cluster superantigens may be associated with poorer clinical outcomes.

In this study, we used WGS to analyze MRSA strains isolated from children and adults attending CF clinic in the year 2017. We compared sequences to identify potential transmission of MRSA between patients. Additionally, we classified the MRSA isolates based on detection of accessory genes to determine whether differences in genomic structure correlated with worsening clinical outcomes.

Methods

Ethics statement. The Institutional Review Board (IRB) of the University of Iowa approved this study # 201905718. Informed consent was waived because the study was minimal risk.

Conflicts of Interest. The authors have no financial conflicts of interest regarding this work.

Subjects. To examine changes in incidence and prevalence of CF pathogens, we studied 337 patients with a diagnosis of CF and cared for in the adult or pediatric CF centers at the University of Iowa, as recently described.[12] The previous study excluded two groups of patients who may be at risk for MRSA because they attend CF clinics: patients with CFTR related metabolic syndrome (CRMS) and patients with CF who had a lung transplant. For this study, we examined MRSA strains isolated from patients with CRMS or following lung transplant to assess for potential MRSA transmission, as these patients attended CF clinics and could be at risk of acquiring MRSA. However, we excluded patients with CRMS or post-lung transplant from calculations of incidence, prevalence, and outcomes such as FEV_1.

Respiratory culture results. We examined respiratory culture results for methicillin susceptible *S. aureus* (MSSA), MRSA, and *Pseudomonas aeruginosa* using the electronic medical record.[12] We determined annualized prevalence of each organism as the number of individuals positive for an organism divided by the number of individuals

tested during the same calendar year. We report annualized prevalence between 2004 – 2017 due to inconsistent reporting of *S. aureus* prior to 2003. We calculated the annualized incidence of MRSA as the number of individuals with a new MRSA infection divided by the number of individuals susceptible to MRSA during the same calendar year. We defined individuals as being susceptible to MRSA if they were newborn or if they did not have a positive respiratory culture for MRSA during their first year of observation. When individuals acquired an incident infection or had no further recorded data, they were removed from the denominator of susceptible individuals.

Pulmonary function testing. We obtained pulmonary function test results from the electronic medical record. For each patient, we determined the best FEV_1 % predicted for each calendar year as the highest FEV_1 % predicted measurement with or without bronchodilator.

Selection of Bacterial Isolates. *S. aureus* clinical isolates were previously stored in TSB with glycerol freezing medium by the clinical microbiology laboratory. Records from all 2017 banked *S. aureus* isolates were reviewed to determine if the patient was diagnosed with CF. Isolates were phenotypically characterized by antimicrobial susceptibilities. The first unique MRSA isolate per patient was selected resulting in 97 *S. aureus* with methicillin resistance phenotype from 74 patients (56 patients - 1 isolate, 14 patients - 2 isolates, 3 patients - 3 isolates, and 1 patient - 4 isolates). One additional isolate was originally thought to be methicillin resistant *S. aureus* based on the clinical diagnosis. However, we learned that its genome sequence matched *S. epidermidis*, and

8

it was removed from further analysis. When several isolates were archived per patient and all had identical antimicrobial susceptibility profiles, only one representative isolate was selected. Isolates were streaked on blood agar plates and isolated colonies were subcultured. DNA preparation was performed with the EZ1 DNA Tissue Kit on a Qiagen EZ1 instrument following manufacturer instructions. The genetic relatedness of the isolates was first determined by pulsed-field gel electrophoresis (PFGE) after digestion with SmaI as previously described.[13] Gels were stained with ethidium bromide and pulsed field types were determined visually and with computer aided detection using BioNumerics software (Applied Maths).

Whole genome sequencing. Library preparation was performed using Illumina Nextera DNA Flex. Paired-end sequencing was performed using Illumina MiSeq Reagent Kit. Phylogenetic analysis was completed using the Utah Public Health Laboratory (UPHL) reference free pipeline and reference-based clustering was completed with Lyveset.[14]

Sequence type, virulence factor, and antimicrobial resistance gene identification. Sequences were uploaded with the Cancer Genomic Cloud interface and tested using the Staphopia API to obtain summaries for multilocus sequence type (MLST), presence of virulence factors, and predicted antibiotic resistance.[15] We used Mykrobe 0.7.0 to identify predicted antimicrobial resistance genes.[16]

Accessory gene content. Sequence reads from FASTQ files were assembled de novo using SPAdes.[17] We used HISAT2[18] to align genes derived from a set of 25 *S. aureus*

9

reference genomes[19] to these de novo assemblies. We used the detection of accessory

genes to perform unsupervised hierarchical and K-means clustering of the MRSA

isolates using R.

SCC*mec* Type Analysis. FASTQ Sequences from clinical isolates were uploaded to

the Center for Genomic Epidemiology to the SCC*mec*Finder 1.2 tool.[20] SCC*mec*

elements were recorded by type and subtype.

Statistical Analysis. We used Poisson regression to test temporal trends in incidence

and prevalence of CF pathogens and linear regression for trends in lung function. To

compare two or more groups, we used non-parametric tests (Wilcoxon rank sum test or

Kruskal-Wallis test, respectively) for continuous data and Fisher's exact test for

categorical data. We used R Studio Version 1.2.5001, Graphpad Prism Version 8.4.2,

and SAS version 9.4 for statistical comparisons.

Data Availability. This Whole Genome Shotgun project has been deposited at

DDBJ/ENA/GenBank with BioProject accession number PRJNA650389 and accessions

JACOAG000000000-JACODY000000000. Unassembled FASTQ files are available

from the corresponding authors. The prefix given for all strains named in this study is

HP20814.

10

Results

Prevalence and Incidence of respiratory infections. Following the development of inhaled antibiotics for *P. aeruginosa* and improvements in infection control, others have noted a nationwide trend towards lower incidence and prevalence of *P. aeruginosa* infections in patients with CF.[3] We observed similar trends within the University of Iowa CF center, with rising prevalence of MRSA and a slow decline in the prevalence of *P. aeruginosa*, Figure 1. MRSA infections are persistent in CF;[12] the rise in MRSA prevalence has occurred despite a slow decline in MRSA incidence, Figure 1 and Supplemental Data 1-2.

We suspected that the rise in MRSA prevalence over the past decade may have been hastened by either person-to-person or healthcare worker-to-patient transmission. To test this possibility, we obtained clinical isolates of MRSA cultured from patients with CF in the year 2017 and compared them by PFGE and WGS.

PFGE data analysis. PFGE analysis revealed a diversity of MRSA isolates obtained within the CF population, Supplemental Data 3. Individuals with multiple MRSA cultures usually had the same pulse type on different culture dates. We observed few instances of shared pulse types between different patients. Some isolates did not digest with SmaI and were tetracycline resistant, traits suggestive of ST398 livestock associated MRSA.[21]

WGS typing and cluster analysis. Using nucleotide variations, we constructed reference-free phylogenetic trees of the CF MRSA isolates. We observed three well-

11

defined clades, Figure 2. 13 sequence types (ST) were represented. ST5 (57%) and ST105 (14%) were the most prevalent in clade 1 and ST8 (14%) in clade 2. One isolate was identified as the livestock-associated ST398, possibly the first documented case in a North American patient with CF. We are not aware that this subject had any direct agricultural exposures that would have increased the risk of acquiring ST398 *S. aureus*.

Sharing of related MRSA strains between individuals. MRSA isolates derived from the same patient usually clustered closely, with less than 60 SNPs difference, Figure 3. In one individual we observed two isolates with 678 SNPs difference (indicated in blue brackets within Figure 3). WGS suggested possible MRSA transmissions between related individuals, including a parent and child (Figure 3, purple box) and siblings (red box). However, related individuals did not always share closely related MRSA. Isolates from a child (046) and the child's parent's sibling (041) had distantly related MRSA, with a difference of 238 SNPs. There was one instance in which unrelated patients (grey box) shared MRSA separated by only 34 SNPs. We did not identify shared hospital encounters between these unrelated individuals, but noted that they lived in geographic proximity.

Antibiotic resistance. Most MRSA are resistant to methicillin because of an alternate penicillin binding protein, encoded by *mecA* within an SCC*mec* element. However, four phenotypic MRSA isolates were predicted to be methicillin susceptible by WGS because they lacked an SCC*mec* element. Each of these isolates encoded the *blaZ* gene, which could confer resistance to methicillin when overexpressed. None of the

isolates encoded *mecC*. Most of the CF MRSA isolates (97%) were resistant to erythromycin, consistent with the chronic use of azithromycin in CF. Some MRSA isolates displayed resistance to tetracycline, which is also commonly prescribed for people with CF. Ciprofloxacin is commonly prescribed for treatment of *P. aeruginosa*. Many MRSA isolates had mutations in *gyrA*, predicting fluoroquinolone resistance.

Accessory gene content correlates with sequence type. We considered the possibility that different *S. aureus* sequence types encode distinct repertoires of virulence factors in their accessory genomes. To determine the gene content of these isolates, we downloaded reference sequences for 25 annotated *S. aureus* strains,[19] comprising a set of 65,557 genes. We reduced this gene set to 6,112 by removing redundant genes. Of these, 1,879 genes were not detected in any of the isolates and 1,092 genes were present in all 97 isolates. We used the remaining 3,141 genes that were detected at least once but were not in every isolate as our accessory genome set. We aligned this set of accessory genes to de novo genome assemblies for the 97 MRSA isolates. After determining which accessory genes were detected, we classified the isolates based on their gene content, Figure 4. As we observed when clustering based on SNPs, three distinct clusters of *S. aureus* emerged. The dominant cluster, Cluster A, included ST5 and ST105 isolates, Table 1. Cluster B was represented mainly by ST8 isolates.

The most prevalent MRSA cluster encodes SCC*mec* type II and EGC toxins. We examined for genes that differed significantly between MRSA clusters, Table 1. Cluster

13

A usually possessed SCC*mec* type II and was positive for toxin genes of the enterotoxin gene cluster, which we have observed is also highly prevalent in CF-associated MSSA.[9] By contrast, Cluster B was usually positive for SCC*mec* type IV, which are sometimes described as community acquired MRSA. The third cluster, Cluster C, had the fewest and most distantly related strains. Additional genes that were differentially detected by cluster are shown in Supplemental Data 4 and 5.

Different MRSA clusters predominate at different ages. We determined which *S. aureus* clusters were identified in each patient. Although some patients had multiple isolates, each subject was positive for only one of the three MRSA clusters during 2017. We compared the patients based on which cluster of MRSA was identified, Table 2. Patients who were infected with MRSA belonging to Cluster A were older on average, and their initial positivity for MRSA was documented earlier in time compared to patients infected with Cluster B. In exploratory analysis, we found that patients with cluster A MRSA generally had lower lung function compared to those with Cluster B MRSA, Figure 5. Patients positive for Cluster A MRSA had more pronounced annual declines in FEV_1 % predicted compared to patients with Cluster B. However, these differences were confounded by the underlying age difference between these groups.

Sputum density of CF pathogens in patients infected by different MRSA clusters. Quantitative respiratory culture studies were routinely ordered by the University of Iowa Pediatric CF center until 2017, whereas this practice was discontinued for adult patients in 2016.[12] This allowed us to perform an exploratory quantitative analysis of CF

14

pathogens in a restricted age range in 2017. We compared MRSA and *P. aeruginosa* culture density for patients with Cluster A MRSA vs. Cluster B MRSA, Figure 6. Sputum samples collected from patients infected by Cluster A MRSA had a higher median density (in CFU/mL) of both MRSA and *P. aeruginosa* compared to sputum derived from patients infected with Cluster B MRSA who were of similar age.

Discussion

In this study of a CF center with high MRSA prevalence, we observed that most MRSA were unique to the individual as determined by WGS. Household contacts sometimes shared similar MRSA isolates, differing by under 60 SNPs. We did not identify hospital-based transmission of MRSA, suggesting that these infections were most likely community-acquired.

MRSA strains collected from CF respiratory cultures displayed genetic diversity (13 sequence types and up to 24,400 SNPs difference). When we classified these isolates by their accessory genome content, we found three distinct sub-populations of MRSA within this CF center. The most prevalent group included SCC*mec* type II isolates on ST5 and ST105 backgrounds. These isolates encoded the enterotoxin gene cluster, and were more common in patients who were relatively older and had lower lung function. These patients experienced more rapid declines in lung function and had higher titers of both MRSA and *P. aeruginosa* in their sputum. The second cluster was mainly represented by ST8. Patients with this type of MRSA were younger and had higher average lung function. Although these differences in outcomes between MRSA clusters

15

were intriguing, they require further study because the patient ages in this cohort were discordant. Our observation of differences in age between patients infected with different MRSA subtypes suggests a birth cohort effect—patients born in different eras likely had different environmental exposures, including the prevailing *S. aureus* strains in the community at the time they were initially infected by MRSA.

Most MRSA isolates taken from the same patient were closely related (4-51 SNPs). In contrast to PFGE, WGS shows greater capability to detect transmission of MRSA isolates between CF patients, especially in households with multiple patients. Genome sequencing also identified possible livestock-associated MRSA infecting a patient with CF. ST398 is commonly isolated from pigs and can infect farm workers.[22] Furthermore, several strains displayed resistance to tetracycline, an antibiotic used as a prophylactic and growth stimulant in animal feed. We were surprised to find some MRSA isolates that did not encode SCC*mec* elements. Other mechanisms could account for the MRSA phenotype, such as over-production of beta-lactamases by the *blaZ* gene.[23,24]

Comparison to previous studies. The results of this study are consistent with a recent WGS study by Long and colleagues from the University of Washington,[25] who found evidence of *S. aureus* strain sharing by siblings and similar sequence types of *S. aureus* account for MRSA in children with CF. Our results also confirm previous observations made by Muhlebach and colleagues, who studied MRSA infections in adult and pediatric patients with CF at the University of North Carolina and as part of a multi-centered study of children with CF in the US. The most prevalent SCC*mec* type in our

16

study was type II, similar to previous reports.[6,26,27] In a single-center study of children and adults with CF, patients with SCC*mec* II PVL-negative MRSA were older than those with SCC*mec* IV PVL-positive MRSA.[6] In a multi-center study of pulmonary outcomes related to MRSA in children with CF, SCC*mec* II MRSA was associated with more pulmonary exacerbations.[27]

However, these observations raise several questions. Are the adverse outcomes experienced by patients infected with SCC*mec* II MRSA driven by the SCC*mec* type itself, by virulence factors encoded elsewhere in the genome, or are these MRSA subtypes simply markers of more intensive treatment and greater hospital exposure? Our dataset does not fully address these questions, but provides some context. MRSA that carry SCC*mec* II usually have different repertoires of accessory genes compared to MRSA with SCC*mec* IV, making it difficult to attribute differences in outcomes to the SCC*mec* type per se.

Advantages. This study examined a cross section of both children and adults with CF, allowing us to identify how different age groups may harbor distinct types of MRSA. We used complementary analytical techniques of comparing both SNPs and gene content. Using our existing longitudinal database of respiratory cultures, we could estimate the duration of MRSA infections. Most of the patients had pulmonary function data, allowing us to examine potential associations of MRSA subtype with adverse clinical outcomes.

17

Limitations. This study has limitations. Because it is a single centered study, these patients could have different risk factors for MRSA acquisition and maintenance compared to other patients with CF. Because the study design is cross-sectional, we are unable to determine whether any differences in pulmonary function could be causally related to the type of MRSA.

Conclusions

The prevalence of MRSA in cystic fibrosis has increased over the previous decade. We observed two major clusters of MRSA within this cohort based on accessory genome content, with the prevalence of each cluster dependent upon age. Family dyads often shared MRSA isolates, but transmission between unrelated individuals was not common. Better understanding of the origins of MRSA in patients with CF could help limit acquisition of these resistant bacteria and improve patient outcomes.

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