DETECTION AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT *S. AUREUS* FROM CYSTIC FIBROSIS PATIENT ISOLATES

By

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Abstract: Cystic fibrosis (CF) is a common genetic disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR). Mutations within this gene inhibit the function of the chloride ion channels across epithelial membranes. This leads to the formation of thick mucus within the lung airways of CF patients. Therefore, the CF lung becomes an excellent environment for bacterial colonization. *S. aureus* is the first pathogen to colonize the lungs and tends to persist throughout the lives of CF patients. *S. aureus* is known for its ability to develop resistance against antibiotics. Antibiotic resistance is one of the biggest problems faced in medicine today. This study aims to detect and characterize the resistance of *S. aureus* obtained from CF patients of various age groups to a panel of clinically relevant antibiotics. Based on findings from previous studies, there are nine antibiotic resistance genes in *S. aureus* that have been correlated with CF patients. Using PCR amplification, we checked if any of these resistance genes are present in the CF isolates. In addition, we performed antibiotic susceptibility tests to determine if these isolates exhibit a resistant phenotype. Minimum inhibitory concentrations (MICs) of each antibiotic to each isolate were determined to further confirm resistance. In conclusion, the presence of resistance genes and susceptibility to antibiotics differ among CF patients. CF isolates showed both susceptibility and resistance to the tested antibiotics, but the percentage of resistant isolates was higher. The interesting finding was that resistance to antibiotics, in some isolates, did not correlate with the presence of resistance genes. This study expands our understanding of *S. aureus* as a CF pathogen and its resistance within the CF lung. This will aid in enhancing treatment options for CF patients to help them live longer and more productive lives.

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CHAPTER I

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that is caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (O'Sullivan et al. 2009; Riordan et al. 1989). Cystic fibrosis is a common genetic disorder within the Caucasian population in the United States. The disease occurs in 1 in 2,500 to 3,500 Caucasian newborns; CF is less common in other ethnic groups (Cohen 2012). The CFTR gene is located on the apical membrane of epithelial cells in organs across the body, and it encodes for a phosphate-regulated chloride ion channel (Welsh and Smith 1993). CF presents itself pathologically as a multi-systemic disease that can affect the lungs, salivary glands, pancreas, liver, kidneys, sweat ducts, and reproductive tract (Davis 2016). However, the lungs are most affected by the mutation and in turn, present the most challenges in acute and chronic management of the disease. To serve as a general prognosis of life span, the current guidelines utilize the health status of the lungs to determine the predicted life span as well as the quality of life for patients with CF (Cohen 2012).

Unique to pulmonary disease as a sequel of CF, is the formation of hyper-viscous mucus in the lung airways of patients. Additionally, CF patients suffer from decreased mobility of the mucocillary escalator. Taken together, these faulted mechanisms provide for the over production of mucus, and the inability to eject, or cough up, mucosal secretions. This dynamic provides for an ideal environment allowing for bacterial and fungal growth and colonization.

Patients with CF are infected with a wide range of bacterial and fungal pathogens. Thus, CF is a polymicrobial infection (Razvi et al. 2009).

Staphylococcus aureus is a major component of the pathogenic bacteria that colonize the CF lung. Specifically, *S. aureus* is frequently isolated from children. As such, it is considered to be a primary, first colonizers of the CF lung (Dasenbook 2010; Goss and Muhleback 2011). *S. aureus* dominates the bacterial population within the lungs during childhood, and remains the leading pathogen for the first decade of life of CF patients. *S. aureus* remains present throughout the entire life span of CF patients and, despite treatment with antimicrobials, it is never completely eradicated. *S. aureus* was the leading cause of morbidity and mortality in the pre-antibiotic era (Anderson, 1999). Yet, today, amidst the availability of antibiotics, which may or may not be helpful in the treatment of infections; *S. aureus* is a CF pathogen worth continued investigation due to the development of antibiotic resistance. The emergence of both nosocomial and community acquired methicillin-resistant *Staphylococcus aureus* (MRSA) is on the rise; MRSA infections observed in patients with CF are now more than 25% (CFF 2017). The detection of MRSA in the lungs of patients with CF is associated with poor prognosis (Dasenbrook 2010). Nosocomial acquired MRSA refers to the acquirement of MRSA in a specific location such as hospital settings, given that MRSA was not present prior to hospitalization. In contrast, community acquired MRSA refers to the acquirement of MRSA outside of a hospital setting or the presence of MRSA on time of admission.

S. aureus in the context of a CF pathogen is poorly studied as most research efforts are focused on *Pseudomonas aeruginosa*, simply the dominant pathogen in adulthood (CFF 2017). Consequently, research efforts devoted to studying the childhood and adolescent stages of CF are scarce. Therefore, efforts within this study are focused on obtaining CF samples from all age groups and building a repository that can be used for research purposes related to CF infections. Antibiotic resistance is a major concern within the medical community today. Furthermore, *S.*

aureus infections are difficult to treat and are rarely eradicated due to antibiotic resistance. The aim of this study was to detect and characterize antibiotic resistant *S. aureus* isolates obtained from CF patients of various age groups. This was accomplished by: evaluating resistance to antibiotics by determining minimum inhibitory concentrations (MICs) of clinically relevant antibiotics; and profiling resistance genes by looking for the presence or absence of antibiotic resistance genes. Overall, the hypothesis of this study was that *S. aureus* isolates from adult patients would be resistant to more antibiotics than isolates from younger patients.

CHAPTER II

REVIEW OF LITERATURE

I. Cystic Fibrosis

Etiology of CF:

Cystic fibrosis (CF) is the single most common, yet lethal genetic disorder. The name "cystic fibrosis" came from an autopsy of two young children who died because of this disease due to the formation of fibrosis, or buildup of connective tissue, with cysts localized in the pancreas (Anderson 1938). Since the first account of CF, it is now known to be an autosomal recessive disorder that mainly affects the Caucasian ethnic population (Cohen 2012). CF is caused by various mutations in the cystic fibrosis transmembrane conductance regulator gene, CFTR. Currently, there are approximately 2000 mutations of CFTR that are cited, all of which allow for the onset of CF pathologies. However, the most common CFTR mutation is ΔF508 (Riordan et al. 1989). It is approximated that in the United States alone, 30,000 individuals are currently living with CF, and about 1,000 new cases are diagnosed each year (CFF 2017).

The CFTR:

CFTR is located on the long arm of chromosome 7 and it encodes for a phosphate regulated chloride ion channel. CFTR is located on the apical membrane of epithelial cells in organs across the body, thus CF is a multi-systemic disease (Rommens et al. 1989;

Welsh and Smith 1993). The two major systems that are affected in CF are the lungs and the gastrointestinal tract. That is due to bacterial infections in the lungs that eventually deteriorate lung function and the altered transport of electrolytes across the GI tract that lead to insufficient enzyme production and fibrosis. The CFTR protein allows chloride ions to pass through epithelial cells that produce mucus; water follows this gradient of chloride ions making the mucus thin (Rafeeq 2007). When mutations occur within CFTR, it results in the formation of hyper-viscous mucus in the lung airways due to the inhibition of chloride ion transportation across the epithelial membranes. Therefore, water is drawn into the lumen leading to the accumulation of dehydrated thick mucus and the impairment of mucocillary clearance (Donaldson and Boucher 2003). The blockage of the lung airways with mucus causes damage to the airway walls and inevitably fibrosis develops. Further, airway colonization and infection with a diverse group of pathogens occurs with these faulted mechanisms that will eventually lead to decreased pulmonary function (Cohen 2010).

Pulmonary Disease:

In healthy individuals, the lungs and airways are sterile environments, despite the presence of viruses and bacteria in the air that is routinely respired. This sterility is maintained by lung defenses that function to clean the airways of healthy individuals by coating the airways with a thin layer of airway surface liquid (ASL). ASL contains periciliary sol and mucus gels that move toward the mouth via ciliary beating (Wine 1999). ASL also consists of proteases and antiproteases, oxidants and antioxidants, as well as antibiotics and antibodies (McWilliams 1996). Collectively, the lung airways are indefinitely cleaned by the actions of these components along with mucocillary clearance and mechanical coughing (Wine 1999).

CF pulmonary disease starts in lung airways that are normal and healthy at the time of birth; however, these lungs become gradually diseased due to the formation of mucus plugs in the

airways (Martens 2011). The patterns seen in CF pulmonary disease are distinctive, the smaller lung airway infections are considered universal, impossible to clear, and lethal (Wine 1999). Airway colonization occurs with diverse microorganisms followed by clinical manifestations of evident infection with common CF pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae,* and *Burkholderia cepacia.* CF pulmonary disease tends to be of highest severity in the upper lobes of the lungs (Cohen 2010; Wine 1999). The increased severity of CF in the upper lobes of the lungs is due to bronchiectasis, which is the recurrent inflammation in CF that leads to the thickening of the walls of the lung airways, preventing mucus clearance (Redondo et al. 2016).

The Microbial Diversity in CF:

Earlier studies of CF microbiome assumed that a limited diversity of pathogens were present in the lung, of which only a few species led to the deterioration of lung function. These pathogens included: *P. aeruginosa*, *S. aureus*, *H. influenza*, and *B. cepacia* complex (Gilligan 1991). Therefore, most antibiotic treatments were administered based on the presence of these species in the lungs of patients with CF (Ramsey 1996). As culturing techniques advanced and sequencing became more available, the overall understanding of the microbial community of CF has changed. Presently, it is no longer regarded as an infection due to a single pathogen rather it is known as a polymicrobial infection (Guss et al. 2011; Sibley et al. 2008).

Major Pathogens in CF:

The formation of thick mucus in the CF lung airways creates an ideal environment for bacterial growth and colonization. That is due to the ability of bacteria to grow and multiply anaerobically while trapped by the thick mucus in the lung airways (Boucher 2002). It is observed that the initial bacterial infections occur shortly after the time of birth. Individuals with CF are exposed to repeated invasions by a wide flora of opportunistic pathogens, thus CF is

classified as a polymicrobial infectious disease (Lyczak 2002). Infants and young children are typically infected with *S. aureus* and *H. influenzae*. Adolescents and adults are more likely to be infected with *P. aeruginosa* (Jain and Smyth 2012).

H. influenzae is regularly found in the lower airways in young children around the age of one. It is important to know that *H. influenzae* in CF is non-typeable, meaning that immunization against *H. influenzae* type b in childhood does not protect against this infection. The role that this pathogen plays in CF pulmonary infection is not understood yet (Gibson et al 2003).

S. aureus is the most common pathogen found in young children aged between 11 and 15 years. In the past decade, *S. aureus* infections have become a major concern due to the rise of methicillin resistant *S. aureus* (MRSA). That is because MRSA infections are difficult to eradicate once they establish in the airways of patients with CF (Guss and Muhleback 2011). *S. aureus* as a CF pathogen will be discussed in more detail towards the end of this chapter.

P. aeruginosa is the most commonly studied CF pathogen as it is reported to infect approximately 50% of adolescent patients and 80% of adult patients. Most patients with CF are infected with different genotypes of *P. aeruginosa* this suggests that the source of the infection is environmental, however that is still under investigation (Gibson et al. 2003). Chronic infections with *P. aeruginosa* tend to occur in patients within the mid-twenty years of life. *P. aeruginosa* is an important CF pathogen due to its ability to adapt in the CF lung and has been described as being capable of causing severe lung dysfunction (Folkesson et al. 2012).

The *Burkholderia cepacia* complex, BCC, has recently become considered a prominent CF pathogen. Within the recent years, the life expectancy of patients with CF has increased, which permitted for BCC to be detected in about 10% of adult CF patients (Guatam 2011). BCC infections last for months or years and lead to severe pulmonary disease and cause death within a short amount of time. This has been attributed to the spread of BCC from person-to-person and

the poor outcome following lung transplantation (Chaparro et al, 1998). But such as any other CF pathogen, patients react to BCC infections differently and it is possible that some patients' clinical status will not be affected by BCC infections (Guatam 2011). Managing CF patients with BCC infections is very challenging because BCC is resistant to most antibiotics. In addition, CF patients can acquire BCC infections from the environment or other CF patients, which led to the establishment of control procedures to prevent the spread of BCC. Patients infected with BCC are segregated from all other CF patients and are advised to keep their distance to prevent unintentional spreading of BCC (Guatam 2011).

Current Treatments for CF:

CF differs among patients in severity and even symptoms. As a result, treatment options are specific to the needs of individual patients. As mentioned above, CF affects multiple organ systems. CF patients receive a combination of therapies to combat infections, support organ functionality, and as a result help them lead more productive lives. Currently, these therapies include antimicrobials to target bacterial infections, mechanical loosening of secretions, improved nutrition, and consistent exercise (CFF 2017).

Airway Clearance Techniques (ACTs): Mechanical loosening of the hyper-viscous mucus that forms in the lungs of patients with CF is a method that helps in clearing the lungs and airways by coughing/huffing. Patients are advised to consult their respiratory therapist to choose the best technique to clear their airways. ACTs are easy to perform as a daily routine (CFF 2017).

Enzyme Supplements: As described before, thick mucus accumulates in the CF lungs. Similarly, the pancreas of patients with CF produces thick mucus blocking the release of digestive enzymes. Therefore, the majority of CF patients take enzymes before they consume their meals. Typically, enzyme supplements are in capsule form and they are taken orally. Enzyme capsules start to work 45-60 minutes after they are taken. Taking these enzymes is

critical for the health of patients as not doing so may lead to poor digestion causing gas and pain. Also, improved lung function has been correlated with higher body weight (CFF 2017).

CFTR Modulator Therapies: These therapies are meant to correct the defect in the CFTR protein. The exact mechanism through which these modulators work is not fully explained. As mentioned before, there are many different CFTR mutations and the modulators designed so far are only effective in patients with specific mutations (CFF 2017).

Currently, two modulators have been approved for use by the FDA: *Ivacaftor* for children under the age of 6 years and having G551D mutation, a rare mutation (Rafeeq 2017; CFF 2017). This modulator has been very successful and shown an overall increased quality of life for patients. Recently, the FDA expanded its use to include other mutations as well as children between the ages 2 and 5 years. This therapy is not effective against ΔF508, the most common mutation, due to the decreased availability of the protein. This therapy is also expensive, and that is a limiting factor for its use. Another FDA approved CFTR modulator is *Lumacaftor,* which showed positive results in the ΔF508 mutation. This modulator showed increased transportation of the CFTR protein to the proper location, but no correction of the underlying functional impairment was noticed. Also, no improvements of respiratory exacerbation rates were seen (Rafeeq 2017; CFF 2017).

Preventing and Controlling Lung Infections: CF patients are more likely to develop bacterial infections due to the buildup of thick mucus in the lung airways. These infections can be short-term known as acute infections or exacerbations, or long-term known as chronic infections. Antibiotics are used to treat infections caused by bacteria. The actions of antibiotics either kill the bacterial pathogen known as bactericidal antibiotics or inhibit its growth known as bacteriostatic antibiotics. Many bacterial infections in the CF lung can be treated with antibiotics. Antibiotics can be taken orally, inhaled, or intravenously (CFF 2017). Patients with CF usually take

antibiotics on a daily basis. Antibiotics are classified into classes that dictate their mechanism of action. Antibiotics can inhibit bacterial cell wall synthesis or function; inhibit protein synthesis, or inhibit nucleic acid synthesis or function. The list below showcases the antibiotics most commonly prescribed to patients with CF:

The Beta-lactams: Beta-lactam antibiotics are structural homologs of the beta-lactam ring. Their mode of action is the inhibition of cell wall synthesis by targeting penicillin-binding proteins, PBPs, which are a group of enzymes located in the cell membrane and are involved in the peptidoglycan cross-linking of the bacterial cell wall. These enzymes include transpeptidases and carboxypeptidases that bind beta-lactam antibiotics, penicillin binding proteins, PBP, (Heesemann 1993; Yocum et al. 1980). Beta-lactam antibiotics used for the treatment of CF patients include: amoxicillin, methicillin, oxacillin, nafcillin, cloxacillin, dicloxacillin, cabenicillin, ticarcillin, piperacillin, mezlocillin, azlocillin, ticarcillin, and tazobactam. All of these antibiotics are prescribed for the treatment of bacterial infections caused by *S. aureus* and *P. aeruginosa* (CFF 2017).

The Aminoglycosides: Aminoglycoside antibiotics mode of action is the inhibition of protein synthesis. This is achieved by the binding of the antibiotic to the aminoacyl side of the 16S rRNA within the 30S ribosomal subunit. This leads to a misreading in the genetic code and inhibits the translocation step of protein synthesis. Therefore, elongation fails to occur due to disruption of the mechanisms for ensuring translational accuracy. Aminoglycosides are usually bactericidal against aerobic gram-negative bacilli (Davis 1987). Aminoglycosides used to treat CF patients include tobramycin, amikacin, and gentamicin. Primarily, these pharmaceuticals target *P. aeruginosa* infections (CFF 2017).

The Macrolides: Macrolide antibiotics mechanism of action can be observed in the process of the inhibition of protein synthesis; antibiotics in this class bind irreversibly to the 50S

subunit of the bacterial ribosome leading to the inhibition of translocation of tRNA during translation (Mazzeri et al. 1993). Macrolides used for CF patient treatment include erythromycin, clarithromycin, and azithromycin. These pharmaceuticals are utilized to target infections caused by *S. aureus* (CFF 2017). Azithromycin has an anti-inflammatory activity and it can prevent loss of lung function in CF patients. Azithromycin is now part of the routine CF care even if individuals are not infected with susceptible bacteria (Cohen 2010).

Other antibiotics prescribed for treating infections in the CF lung include: cephalosporins to treat *S. aureus* and *P. aeruginosa* infections; Sulfa to treat *P. aeruginosa* and MRSA. Tetracyclines were previously used to treat *P. aeruginosa* infections and now they are used to treat *S. aureus* and some *B. cepacia* infections; quinolones for *P. aeruginosa* infections; vancomycin for *S. aureus* and MRSA infections; linezolid for MRSA and some mycobacteria infections; imipenem and meripenem for both *S. aureus* and *P. aeruginosa* infections; aztreonam for *P. aeruginosa* infections; and colistimethate for *S. aureus* and *P. aeruginosa* infections (CFF 2017).

The Problem of Antibiotic Resistance:

As mentioned above, patients with CF are regularly treated with a combination of antibiotics that are taken daily to help them fight against a plethora of bacterial infections. With the excessive use of antibiotics, bacterial pathogens may develop resistance to the antibiotics. Resistance to antibiotics means that the pathogens are no longer affected by the mechanism of action provided by the drug. Typically, the increased use of antibiotics leads to a higher frequency of resistance development against the antibiotics among pathogens. This may even result in the development of bacterial strains that are resistant to several antibiotics, known as multi-drug resistant bacteria and those are very difficult to treat (Cystic Fibrosis Trust, UK 2017).

Antibiotic resistance is a natural phenomenon and it can be attributed to several factors that are either natural (develop without human action) or caused by the actions of humans. Bacteria naturally produce antibiotics and use them against other bacterial species. This leads to natural selection for resistance to antibiotics (Devel and Dermody 1991). Fungus, bees, and many other species also synthesize natural antimicrobial peptides. However, the more serious cause of antibiotic resistance is the overuse of antibiotics by humans. The more bacteria are exposed to antibiotics, the more likely they will develop resistance toward these antibiotics. Bacteria can acquire resistance to antibiotics using two mechanisms: The first mechanism is through a genetic mutation, which allows the bacteria to produce chemicals that render the antibiotics ineffective. Other mutations modify the cellular components that the antibiotics are designed to target. For example, some mutations change the binding affinity of the antibiotic by modifying the binding site; some mutations block the binding site of the antibiotic; and other mutations lead to the formation of efflux pumps that expel the antibiotic out of the cell and prevent it from reaching its target (Dever and Dermody 1991). The second mechanism that bacteria can use to acquire resistance is through obtaining resistance from another bacterium. There are several mechanisms for this type of resistance acquisition. The simplest way is through conjugation; in this process the bacteria are able to transfer genetic information, including resistance genes, from one bacterium to another. Resistance genes transferred via conjugation are found on plasmids and transposons. Another mechanism is through phage or viruses that infect bacteria. Viruses can transmit resistance genes among bacteria by obtaining resistance genes from the first bacterium and injecting them into a new bacterium that they attack, this process is known as transduction. Bacteria are also able to acquire resistance genes from their surrounding environment due to their ability to uptake naked or free DNA. Once bacteria uptake this free DNA, they can incorporate it into their genome and use it for their advantage. If the newly obtained DNA contains resistance genes, then, in turn, a beneficial evolutionary trait is possessed by the bacteria increasing odds of survival within the environment or in the presence of antibiotics (Dever and Dermody 1991).

In CF pulmonary disease, the problem of antibiotic resistance is a major concern when it comes to managing bacterial infections that eventually lead to a reduction in lung function. Major CF pathogens such as *P. aeruginosa* and *S. aureus* including MRSA have been named as a public health threat by the World Health Organization, WHO, due to their high rate of antimicrobial resistance. Multiple drug resistance is common among these pathogens. Multiple drug resistance, MDR, is defined as the resistance of species of microorganisms to multiple antimicrobial drugs. MDR species are considered a public health threat as they have resistance to multiple antibiotics and are difficult to treat (Magiorakos et al. 2011).

Staphylococcus aureus:

S. aureus is a Gram-positive non-motile coccoid bacterium and it is the primary pathogenic species in the *Staphylococci* genus. *S. aureus* is a commensal bacterium and it is usually found on skin surfaces and mucosal membranes. *S. aureus* grows best in saline environments and this characteristic explains its presence in the normal flora as 20% of the population is colonized by *S. aureus*, 60% are occasional carriers, and 20% of the population never carries it (Kluytmans 1997). *S. aureus* has a great potential of becoming pathogenic due to its localization in the nasal epithelium and in tissues beneath the epidermis. *S. aureus* is a major pathogen causing both sepsis and endocarditis. It also causes osteoarticular, epidermal, joint, and medical device-related infections (Otto 2010).

Virulence Factors: *S. aureus* exhibits a wide range of virulence factors that allow it to colonize tissues causing damage and disease to the host. These virulence factors also promote the ability of *S. aureus* to attach and adhere to surfaces, evade immune system recognition, and produce toxins that harm the host (Justyna 2011). The attachment of a pathogen to a surface is the initial step in the development of infection. Accordingly, *S. aureus* initiates the process of colonization by utilizing adherence factors, termed adhesins. Adhesins are proteins attached to the *S. aureus* peptidoglycan via covalent bonds. These adhesins attach to the host extracellular matrix

in a specific manner (Foster and Hook 1998). Major *S. aureus* adhesins are staphylococcal protein A (SpA), clumping factor (Clf) A and B, fibronectin-binding proteins A and B (FnbpA and FnbpB), and collagen-binding protein (Foster and Hook 1998; Lowy 1998).

S. aureus employs various mechanisms to evade both innate and acquired immunity. The majority of *S. aureus* strains produces and secretes anti-inflammatory molecules that protect them from the host innate immune system. Modified membrane lipids, surface polymers, and other components of the cell wall of *S. aureus* play a role in evading recognition by the immune system (Fedtke 2004). Biofilm formation is an important strategy that *S. aureus* utilizes to evade immune system recognition and also to inactivate antimicrobial molecules and phagocytes (Gotz 2002). *S. aureus* also secretes an array of toxins that cause severe biological damage to the host and often lead to cell death. The production and release of toxins by *S. aureus* also serves as a mechanism to escape elimination by the host immunity (Otto 2014). The toxins produced by *S. aureus* include: cytotoxic toxins, hemolysins (α, β, and γ), toxic shock syndrome toxin, enterotoxins, and exfoliative toxins A and B (Justyna 2011).

<u>Epidemiology of Resistance Genes in *S. aureus*:</u>

<u>Incidence and Prevalence:</u> According to the CFF Patient Registry, the prevalence of *S. aureus* infections in the lungs of CF patients increased steadily in the past 25 years. In 2015, *S. aureus* was isolated from respiratory tract cultures from 70.6% of CF patients. The prevalence of *S. aureus* infections is the highest among children and patients below the age of 18 years; the highest prevalence of MRSA infections occurs in patients between the ages 10 and 30 years (Cystic Fibrosis Foundation 2015). The outcome of MRSA infections tends to be worse than MSSA infections in patients with CF. MRSA clones were first reported in the 1960's in a British hospital and rapidly spread from there (Jessen et al. 1969 and Parker 1970). The *mecA* gene is the gene responsible for methicillin resistance and it is found in all MRSA strains. The *mecA* gene is

part of a mobile genetic element designated as *Staphylococcal* cassette chromosome *mec*, SCC*mec* (Katayma et al. 2000). Currently, four different SCC*mec* elements have been characterized and their sizes range from 21 to 67 kb (Hiramatsu et al. 2001). Epidemiologic studies on SSC*mec,* attribute the emergence of MRSA clones as a result of horizontal gene transfer from related *Staphylococci* species of the *mec* gene into MSSA clones.

In addition to methicillin resistance*,* resistance against macrolide antibiotics is common among *S. aureus* clinical isolates. Unlike methicillin resistance, a number of genes confer resistance to macrolide antibiotics as well as lincosmide and streptogramin B antibiotics, MLS_B. These resistance genes confer resistance in *Staphylococci* using a variety of mechanisms. The *erm* genes, which stand for erythromycin ribosomal methylase, include the *ermA, ermB, ermC,* and *ermT* genes, confer resistance to MLS_B antibiotics by methylation of the ribosomal RNA target site. The *msrA* gene also confers resistance to MLS_B antibiotics in *Staphylococci* by encoding for a macrolide efflux pump; this gene was first identified in *S. epidermidis.* Gerard et al. investigated the relative frequency of each of these MLS_B resistance genes in 294 *S. aureus* clinical isolates. A percentage of 88% of these isolates contained at least one of the *erm* genes. Resistance was mainly due to the presence of the *ermA* gene, as this gene was detected in 63.2% of the isolates. The *ermC* gene was also high in prevalence among these isolates and it was the single gene detected in 25% of the *S. aureus* isolates. The detection of resistance due to *msrA* was more frequent in coagulase-negative *Staphylococci* than in *S. aureus,* as it was present in 14.6% in coagulase-negative *Staphylococci* and 2.1% in *S. aureus.* The prevalence of other resistance genes encoding for MLS_B resistance was more frequently present in coagulase-negative *Staphylococci.* Therefore, studies indicate that resistance to MLS_B antibiotics in *Staphylococci* is mainly due to the presence of two variants of the *erm* genes depending on the type of *Staphylococci*, whether it is coagulase positive or negative, as well as the methicillin susceptibility pattern, susceptible or resistant. The *ermA* gene was present in 57.6% of MRSA

strains and 5.6% in MSSA strains. While, the *ermC* gene was present in 4.9% of MRSA strains and 20.1% of MSSA strains (Gerard et al. 1999).

Resistance to aminoglycoside antibiotics is widespread among *Staphylococci* species. *S. aureus* becomes resistant against aminoglycoside antibiotics by producing genes that inactivate the aminoglycoside modifying enzymes, AMEs. These genes are encoded on plasmids or transposons. The most prevalent AME in *Staphylococci* clinical isolates is reported to be the bifunctional enzyme, acetyltranferase and phosphotransferase AAC(6')/APH(2"), which is encoded by the *aac(6')/aph(2")* genes (Nurittin et al. 2006). Studies reported that this gene is responsible for aminoglycoside resistance in MRSA isolates as it was present in 66% of the isolates (Nurittin et. Al 2006). The prevalence of other aminoglycoside resistance genes was lower, as the *aph(3')-Illa* gene was only present in 8% of MRSA isolates. Schmitz et al. 1999, and Choi et al. 2003, also reported that *aac(6')/aph(2")* was the most prevalent resistance gene among *S. aureus* clinical isolates. It is important to note that occurrence of AME genes is correlated with the presence of the *mecA* gene encoding for methicillin-resistance. The simultaneous presence of at least one AME gene with the *mecA* gene was detected in 72% of *S. aureus* clinical isolates (Nurittin 2006).

CHAPTER III

METHODOLOGY

I. Cystic Fibrosis Patient Samples

I.a. Collection of Sputa Samples

Sputum was collected from patients at the Oklahoma Cystic Fibrosis Clinic at the OU Children's Hospital in Oklahoma City, Ok. The nursing staff collected the samples in 50 mL sterile conicals and provided the following information with each sample: the patient's age, gender, and diagnosed health condition upon sample collection. Health condition was described as either: healthy, not ill, acutely ill, no exacerbation, or exacerbation. Each patient was assigned an ID tag that is formulated from the date of the collection and a letter, which is given for the order of the patient, i.e. collection on 02/11/2017 from the first patient would be labeled as 21117A. The sputum was immediately struck onto nutrient and selective media by using a sterile cotton swab to dip into the sample and, in a zigzag pattern, inoculate the media. Samples were incubated for 72-96 hours at 37°C. A remaining volume of 150 uL of sputum was placed in a cryovial containing 50% glycerol. If sputum remained, two 150 uL aliquots were placed in an Eppendorf tube with equal amounts of Zymo DNA/RNA shield. Genomic DNA and RNA were

extracted from these tubes using the corresponding Zymo extraction kits (ZymeResearch). Extracted and purified RNA was then stored at -80°C, and extracted and purified gDNA was then stored at 4°C. It is important to note that all sputum samples were frozen down on dry ice during the transport from the clinic located in Oklahoma City to Stillwater; following arrival, all samples were stored long-term at -80°C.

I.b. Freezer Stocks

The nutrient and selective plates of media included the differential and selective medium, mannitol salts agar (MSA), which was prepared according to manufacturer's instructions (Criterion). Plates were incubated, inverted, at 37°C for 72-96 hours. Recoverable bacterial growth from all eight media, including MSA, was scraped from the agar with 10% skim milk. Bacterial suspensions in skim milk were then moved into corresponding cryovials. Cryovials were labeled with the patient's identification tag, the media, and the date the freezer stock was made. All cryovials were stored long term at -80°C.

II. Antibiotic Susceptibility Tests

II.a. Preparations of Antibiotics

All antibiotics used in this study were received as powders (Gold Biotechnology, USA); they were accurately weighed and dissolved in the appropriate diluents to yield the required concentrations. Stock concentrations were 0.05 g/mL; 1:10 dilutions were made for the working concentrations from the stock to yield working concentrations of 0.005 g/mL.

- Methicillin, diluted in H_2O, Gold Biotechnology, USA;
- Erythromycin, diluted in 95% ethanol, Gold Biotechnology, USA;
- Kanamycin, diluted in H_2O, Gold Biotechnology, USA;
- Gentamicin, diluted in H_2O, Gold Biotechnology, USA;

- Streptomycin, diluted in H_2O, Gold Biotechnology, USA;
- Tobramycin, diluted in H_2O, Gold Biotechnology, USA.

II.b. Kirby-Bauer Disc Diffusion Method

The susceptibility of *S. aureus* to antibiotics was determined by the Kirby-Bauer disc diffusion method. Using a sterile cotton swab applicator, a lawn of the *S. aureus* clinical isolate was struck onto Mueller Hinton Agar (ThermoFisher Scientific, USA) plates to cover the entire surface of the plate. Hand punched filter discs (diameter size = 6mm) were added onto the bacterial lawn, equidistant from one another. A volume of 10uL of antibiotics (methicillin, erythromycin, kanamycin, tobramycin, gentamycin, and streptomycin), were added onto the filter discs at a concentration of 0.005 g/mL. The susceptibility was interpreted based on the diameter of the zones of clearance, read in millimeter, after 24 hours of incubation at 37°C. The measured zones of clearance were then recorded. Results obtained from this method are referred to as phenotypic resistance profiles and they were compared to the genotypic resistance profiles obtained by PCR amplification of antibiotic resistance genes specific to *S. aureus*.

II. c. Minimum Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations (MIC), which are a measurement of the minimum concentration of antibiotics required for the inhibition of visible growth of bacteria. MICs were determined by the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Two-fold serial dilutions of methicillin, erythromycin, and kanamycin were prepared in a 100uL volume in 96-well microtiter plates starting at a concentration of 0.05 g/mL. The dilution series included the antibiotic diluents H_2O and 95% ethanol as controls. A volume of 900 uL of the bacterial culture was then added to each well. The MIC was interpreted by visual means. The lowest concentration of antibiotic, in which

there was a lack of visible growth of the bacterial isolate was recorded following an overnight incubation, shaking at 220 RPM, 37°C.

III. Extraction of Genomic DNA and Gene Amplification (PCR)

III.a. Extraction of Genomic DNA

S. aureus isolates were grown overnight shaking at 37°C, 220 RPM in 5 mL of Luria Bertani (LB) broth (Fisher Bioreagents, USA). The overnight cultures were centrifuged at 3,000 RPM for 10 minutes. Supernatant was discarded from the sample and the pellets were used for genomic DNA, gDNA, extraction using the ZymoResearch Fungal/Bacterial DNA Miniprep kit (ZymoResearch). The extracted gDNA was then used as a template for Polymerase Chain Reaction (PCR) to assess for antibiotic gene presence in the tested *S. aureus* isolates.

III.b. Gene Amplification (PCR)

Extracted and purified genomic DNA was used as a template in all PCR reactions to check for the amplification of the nine antibiotic resistance genes chosen for investigation within the scope of this study: *mecA, ermA, ermB, ermC, ermT, msrA, aphA3, aadC,* and *acaaphD*. PCR was also used to amplify the *16S rRNA* gene and the *rpoB* gene. The enzyme used in all PCR reactions was Taq 2X Maser Mix (BioLabs, USA). PCR was carried out using an Eppendorf Mastercycler.

The thermal-cycling conditions were standardized for the amplification of resistance genes and *16S rRNA* and produced optimum results: initial denaturation at 95°C for 30 sec, 30 cycles of denaturation at 95°C for 30 sec, annealing for 1min at 68°C, and extension at 68°C for 5 min. The annealing temperature was adjusted for the amplification of the *rpoB* primers to 55°C. All the primer sequences used for PCR reactions are listed in Table 1 below:

Table 1: Primer sequences used for PCR

Gene	Resistance for	Primers
mecA(Koukos et al. 2015)	B-lactams	**Forward:** GGGATCATAGCGTCATTATTC **Reverse:** AACGATTGTGACACGATAGCC
ermA (Jung et al. 2009)	Macrolide	**Forward:** TCAGTTACTGCTATAGAAATTGATGGAG **Reverse:** ATACAGAGTCTACACTTGGCTTAGG
ermB (Jung et al. 2009)	Macrolide	**Forward:** TTGGATATTCACCGAACACTAGGG **Reverse:** ATAGACAATACTTGCTCATAAGTAACGG
ermC (Jung et al. 2009)	Macrolide	**Forward:**GACAATTATAAGATTAAATGAACATGATAATATC **Reverse:** AAACAATTTTGCGTATTATATCCGTAC
ermT (Jung et al. 2009)	Macrolide	**Forward:** ATTGGTTCAGGGAAAGGTCA **Reverse:** GCTTGATAAAATTGGTTTTTGGA
msrA (Achour 2008)	Macrolide	**Forward:** TCCAATCATTGCACAAAATC **Reverse:** AATTCCCTCTATTTGGTGGT
aphA3 (Kim et al 2007)	Aminoglycoside	**Forward:** GAAGGAATGTCTCCTGCTAAG **Reverse:** GCAGAAGGCAATGTCATACC
aadC (Micutkova 2004)	Aminoglycoside	**Forward:** AGCATGCACAGAGCTGGAGAC **Reverse:** AAGAAAGGAATCAGGCCAGC
aacaphD (Matsuo et al. 2003)	Aminoglycoside	**Forward:** GTTCCTATTGGATATGGACAAATAT **Reverse:** TCGTTTTAACAAATTTTGTTCTTCT
16S rRNA (Xu et al. 2012)	**Forward:** GCAAGCGTTATCCGGATTT **Reverse:** CTTAATGATGGCAACTAAGC	
rpoB (Wichelhaus 1999)	**Forward:** ACCGTCGTTTACGTTCTGTA **Reverse:** TCAGTGATAGCATGTGTATC	

III.c. Visualization of PCR Products

PCR products were separated in 2% Agarose gels (Biotechnology Grade AMRESCO, USA), stained with ethidium bromide, and visualized using the Gel Doc system, VWR Variable Intensity UV Transilluminator. Amplicons were compared to known product sizes for gel interpretations. Results obtained from the amplification of antibiotic resistance genes are referred to as genotypic resistance profiles and they were compared to the phenotypic resistance profiles obtained by the Kirby-Bauer disc diffusion method.

IV. Resistance to Rifampicin

IV.a. Rifampicin-resistant Mutants

S. aureus isolates were struck from the freezer stock onto MSA plates containing 0.2 g rifampicin/L (Gold Biotechnology, USA) and incubated, inverted for 24 hours at 37°C. If growth was present, such indicated that the isolate was resistant to rifampicin. If isolates were susceptible to rifampicin (no growth), then they were driven to resistance by the following protocol adapted from Schaaf *et al*. Susceptible *S. aureus* isolates were grown overnight in 5 mL of Trypticase Soy Broth (TSB) (BD, USA) shaking at 37°C, 220 RPM. The overnight cultures were normalized to 1.0 at an optical density of 600 nm. Then, the isolates were plated onto Trypticase Soy Agar (TSA) plates containing 100 mg rifampicin/L. Plates were incubated, inverted for 48 hours at 37°C. Growth indicated that these isolates developed rapid mutations to resist rifampicin present in the medium. All isolates, already resistant or induced to resistance, were used to study mutations within the *rpoB* gene. The mutable region of the *rpoB* gene was amplified using PCR. The PCR products were cleaned and concentrated using the Zymoclean Gel DNA Recovery Kit

(Zymoresearch, USA) and sent for sequencing at the OSU Biochemistry and Molecular Biology Core Facility. Sequences were analyzed via DNAMAN version 9.108 software.

CHAPTER IV

FINDINGS

I. Introduction to Collection of Sputa Samples from Cystic Fibrosis Patients

An established interest exits in building and maintaining a repository of CF sputa samples and bacterial isolates in which easy access can be obtained. Access is granted to not only the Department of Microbiology and Molecular Genetics at Oklahoma State University, but additionally, other laboratory groups that are interested in research related to CF disease and its pathogens. The creation and maintenance of this repository became possible through the collaboration with the Cystic Fibrosis Clinic at the University of Oklahoma (OU) Children's Hospital in Oklahoma City, OK. Sputa samples were collected from patients with CF by the nursing staff. Then, these sputa samples were struck onto a total of eight different types of selective and nutritional media to allow for the recovery of a wide range of the microbial diversity that constitutes the CF lung. All samples were transported back to Stillwater on dry ice for preservation.

In order to study antibiotic resistance in *S. aureus,* the isolation of *S. aureus* from CF sputa samples was essential. Mannitol Salt Agar (MSA) was used for the selective isolation of *S. aureus* from sputum. MSA is highly selective for *Staphylococci* due to the high concentrations of NaCl (7.5%-10%). This elevated level of salt is inhibitory to the growth of most other bacterial species. MSA is also a differential medium for *Staphylococci* mannitol-fermentation; it contains the carbohydrate mannitol and the pH phenol red indicator that detects acid production by mannitol-fermenting *Staphylococci.* Since *S. aureus* is capable of mannitol-fermentation, it causes a color change in the medium, from red to yellow, due to the acid production and subsequent alteration of indicator, phenol red. *S. aureus* colonies appear yellow after growth on MSA plates. Other coagulase-negative *Staphylococci* do not cause a color change in the medium and grow pink/red colonies, allowing for easy visual identification. To further confirm that the bacteria recovered from the MSA plates was indeed *S. aureus*, the 16S ribosomal RNA of random patient isolates were sequenced for verification and analyzed via the NCBI Standard Nucleotide BLAST.

II. Results

Most of the collected sputa samples came from adult patients over the age of 18 years. Therefore, there is a lack of samples from child and adolescent patients. Partly, this is due to the rejection of medical compliance of parents/legal guardians to have their children participate in a research study. However, since *S. aureus* is a dominant pathogen in children and remains present in the lungs of adult CF patients, mostly all of the collected samples contained *S. aureus.* It is not surprising that a few patients were colonized with more than one *Staphylococcus* species. This was indicated by the difference in colony morphology on MSA plates. Typically, a *S. aureus* colony on an MSA growth medium is yellow in pigmentation with round, convex, and creamy morphology. However, several patient isolates produced colonies on MSA medium with differing morphologies, yet yellow pigmentation.

Specifically, these colonies with different morphologies were selected and tested as separate isolates from the same patient. For example, patient 12315E produced 3 different colony morphologies on MSA, each one of these colonies became a separate isolate from that patient and named as 12315E(1), 12315E(2), and 12315E(3). Also, the presence of small red to pink colonies with little to no color change on MSA plates was observed among the patient isolates. Specifically, these colonies were suspected to be *Staphylococcus epidermidis* due to the lack of mannitol fermentation and subsequent lack of differing media and colony pigmentation. Yet, no 16S sequencing was performed to further confirm the identity of these isolates. In order to increase the sample size, *S. epidermidis* isolates were used in the study and they were renamed as 81414C *epid*.

Table 2: Patient List

Sputa Sample ID	Age	Condition
21116B	6	Not ill
5814D	8	No exacerbation
21116A	8	Not ill
12116C	9	Not ill
11614D	10	Not ill
81414A	10	Not ill
5814E	11	No exacerbation
22714C	12	No exacerbation
8714A	13	Acutely ill
51514C	16	Exacerbation - does not feel ill
2515D	17	No exacerbation
6514A	19	Acutely ill
5814G	20	No exacerbation
51514D	20	Exacerbation
6514D *epid*	20	No exacerbation
22014D	21	Exacerbation
12116B	21	Ill
61214C	22	Ill
61214C *epid*	22	Ill
61214A	24	Ill
73114A	25	Not ill

22714E	26	Exacerbation
112014D	26	On antibiotics
22714B	27	Exacerbation
31314A	28	No exacerbation
9414B	28	Not ill
61214B	28	Exacerbation
51514A	28	No exacerbation
3614A *epid*	28	No exacerbation
3614B *epid*	29	No exacerbation
91715D	31	Not ill
12315E (1)	31	Ill
12315E (2)	31	Ill
12315E (3)	31	Ill
8714C	32	Not ill
5814F	32	Exacerbation
5814H	32	Exacerbation
81414D *epid*	32	Confusing
9414C	33	Ill
3614C *epid*	38	Not acutely ill
22014B	39	No exacerbation
81414C *epid*	39	Not ill
21915B	40	Not ill
32014B	41	Not acutely ill
32014B (1)	41	Not acutely ill
32014B (2)	41	Not acutely ill

Table 2 listed above is the sputa sample ID's, ages, and health conditions of patients that were a part of this study. A total of 46 *S. aureus* isolates recovered from 41 CF patients were collected from February 2014 to December 2016. Of these samples, 7 isolates came from children (under the age of 12 years); 4 isolates came from adolescents (between the ages 12 and 17 years); and the remaining 35 isolates came from adults (over the age of 18 years). Table 2 is ordered according to age. Among these patients, only 1 adolescent and 6 adults were experiencing pulmonary exacerbations at the time of sputa collection.

II. b. Growth on Mannitol Salt Agar

As mentioned above, *S. aureus* isolates were selected for on MSA plates. *S. epidermidis* can also grow on MSA plates. The following figure shows the typical colony morphology and pigmentation of *S. aureus* and *S. epidermidis* isolated from CF patients and struck onto MSA plates.

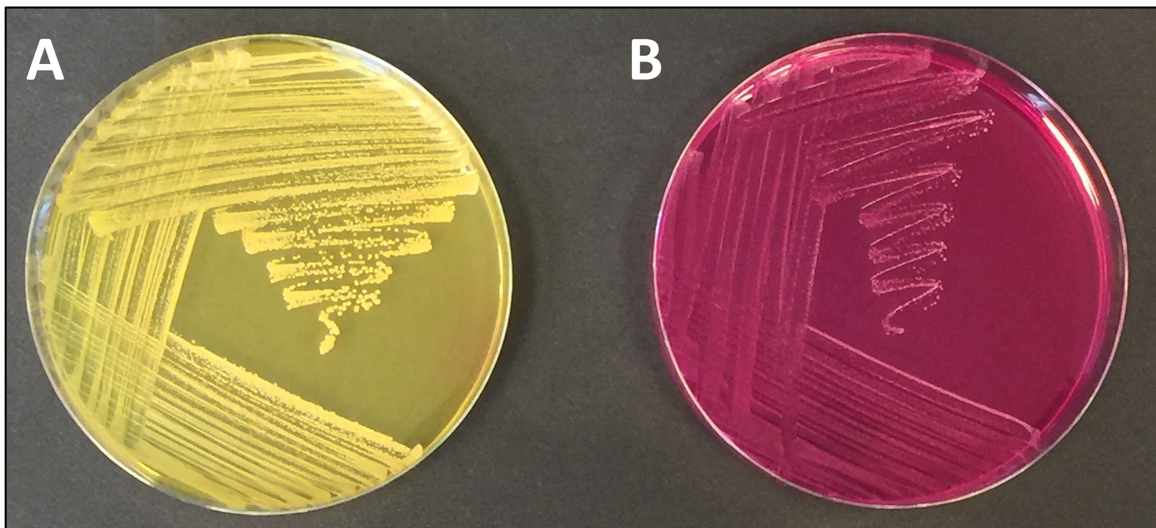


Figure 1: Colony morphology and pigmentation of *Staphylococcus* on MSA plates

Part A in Figure 1 shows the growth of *S. aureus* on MSA. The yellow pigmentation is due to mannitol salt fermentation by *S. aureus*. Part B shows the growth of *S. epidermidis* on MSA. *S. epidermidis* does not ferment mannitol salts, therefore, no change in the color of the media is observed. Also, *S. epidermidis* colonies are pink/red in color compared to *S. aureus* colonies.

II. c. *16S rRNA* Gene

The presence of the colony morphology and color change of agar upon growth of *S. aureus* on MSA medium is a good starting point as to identify the species of the bacterium by biochemical differential means. However, in order to proceed with this study, the sequencing of the 16S ribosomal RNA provided confirmation of species identity. The 16S rRNA is a component of the small 30S subunit in the prokaryotic ribosome and it is involved in the translation process of protein synthesis. The *16S rRNA* gene is often used in phylogeny reconstruction due to the extremely slow rate of evolution in this region of the DNA. The *16S rRNA* gene was amplified with PCR using oligonucleotide primers that are conserved throughout *S. aureus* strains.

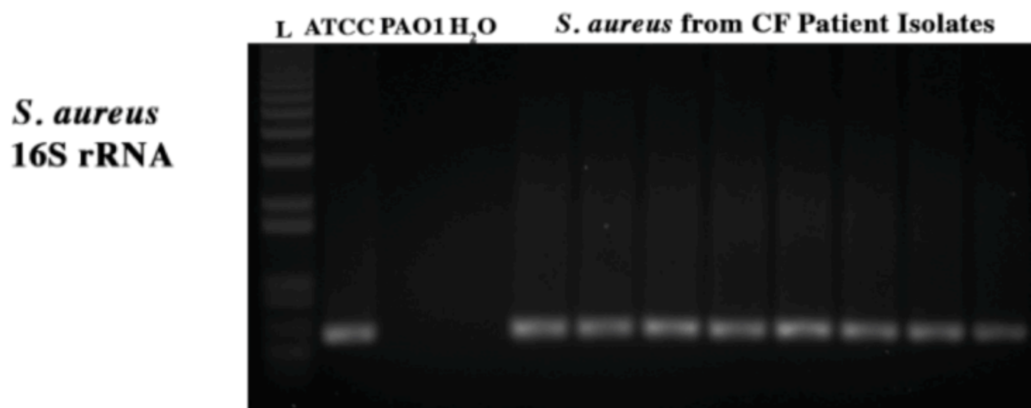


Figure 2: The amplification of *16S rRNA* gene: PCR amplification results of the *16S rRNA* gene in *S. aureus* isolates and control strains ATCC 13525, PAO1, and H_2O control.

Figure 2 shows the PCR amplification results of the 16S rRNA gene by *S. aureus* isolates obtained from CF patients' sputa. ATCC 13525 is a *S. aureus* positive control strain; PAO1 is a *P. aeruginosa* strain used as a control to show that there is no contamination in the *S. aureus* isolates; and H_2O was used as a negative control. The amplified products were sent for sequencing at the OSU Biochemistry and Molecular Biology Core Facility. Sequences were then

obtained and NCBI BLAST nucleotide was used to confirm the identity of *S. aureus,* results are shown in figure 3 below.

Organism	Blast Name	Score	Number of Hits	Description
Staphylococcus	firmicutes		102	
• Staphylococcus aureus	firmicutes	1330	69	Staphylococcus aureus hits
• Staphylococcus aureus subsp. aureus	firmicutes	1330	13	Staphylococcus aureus subsp. aureus hits
• uncultured Staphylococcus sp.	firmicutes	1330	5	uncultured Staphylococcus sp. hits
• Staphylococcus argenteus	firmicutes	1330	1	Staphylococcus argenteus hits
• Staphylococcus aureus subsp. anaerobius	firmicutes	1330	13	Staphylococcus aureus subsp. anaerobius hits
• Staphylococcus sp.	firmicutes	1330	1	Staphylococcus sp. hits

Figure 3:Analysis of *16S rRNA* sequences. The identification of isolates obtained from CF patients as *S. aureus* by analyzing the *16S rRNA* gene sequences by NCBI nucleotide BLAST.

As expected, upon analysis of the *16S rRNA* gene sequences, obtained from PCR amplification, provided by the NCBI BLAST tool, the results demonstrate that the isolates were *S. aureus* (Figure 3).

III. Introduction to Antibiotic Susceptibility Tests and MICs

Following the isolation and identification of *S. aureus* from CF patients, isolates were subjected to a survey of clinically relevant antibiotics. The goal was to characterize antibiotic resistance profiles of these patient isolates both phenotypically and genetically.

For the phenotypic characterization, the Kirby-Bauer disc diffusion method was used to measure the sensitivity of bacteria to antibiotics. Each *S. aureus* isolate was struck on Mueller-Hinton Agar plates, a non-differential medium, to create a lawn of bacteria that covers the entire plate. Antibiotic discs were added on top of the bacterial lawn, and then 10 uL of liquid antibiotics were applied onto the top of the discs at a concentration equal to 0.005g/mL as shown in figure 4 below. Susceptibility or resistance to antibiotics was determined by measuring the diameter of the zone of clearance produced around the antibiotic disc.

IV: Results

IV.a. Kirby-Bauer susceptibility test results

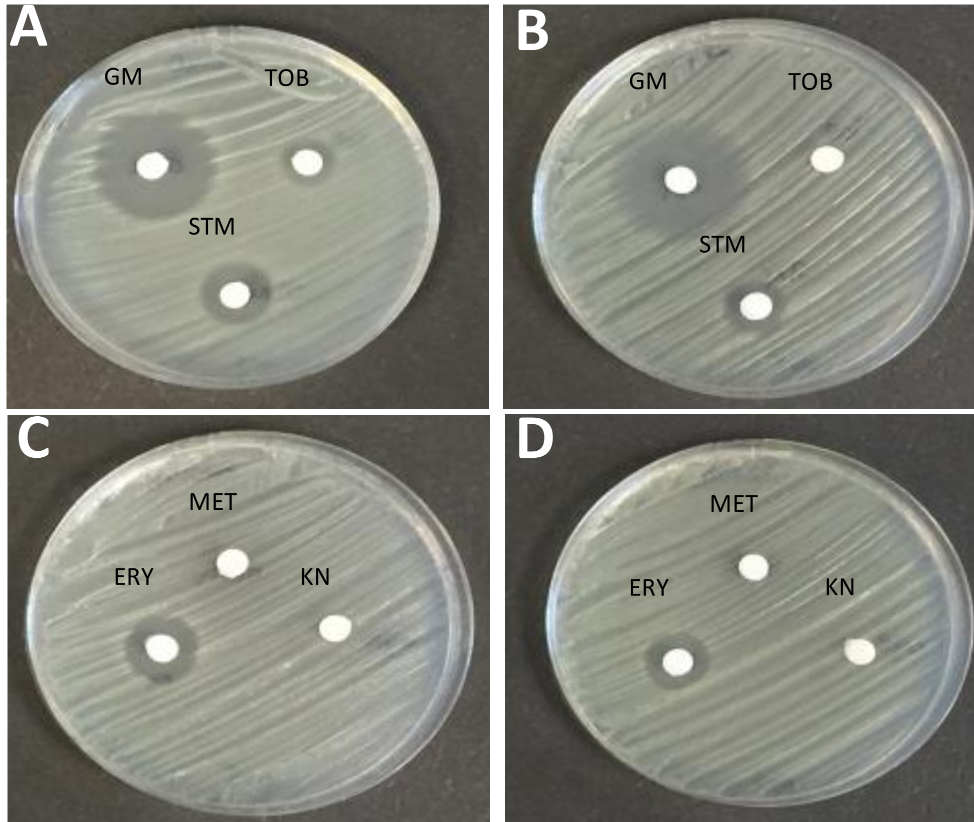


Figure 4: Kirby-Bauer Susceptibility Test Results: Kirby-Bauer results of patients' 22014D and 22714C on Mueller-Hinton Agar plates. A. 22014D tested with GM, TOB, and STM; B. 22714C tested with GM, TOB, and STM. C. 22014D tested with MET, ERY, and KN; D. 22714C tested with MET, ERY, and KN.

The Kirby-Bauer tests served as biochemical means to characterize sensitivity or resistance towards antibiotics. The diameters of the zones of clearance around the antibiotic discs produced by susceptible bacteria were measured in millimeters and recorded (Table 3).

Table 3: Resistance and Susceptibility of *Staphylococci* Isolates to Antibiotics

Isolate (Patient ID)	MET	ERY	KN	TOB	GM	STM
31314A	R	R	R	R	R	R
22014B	23 mm	26 mm	R	R	R	R
22714B	R	R	R	R	R	R
22714C	10 mm	R	R	R	R	R
22014D	15 mm	R	R	R	R	R
22714E	25 mm	R	R	R	R	R
9414B	20 mm	19 mm	R	28 mm	21 mm	19 mm
9414C	13 mm	R	R	21 mm	19 mm	23 mm
8714C	R	11 mm	R	17 mm	10 mm	R
5814D	16 mm	27 mm	R	R	R	R
5814F	R	R	R	R	R	R
11614D	16 mm	27 mm	R	29 mm	20 mm	19 mm
51514C	R	R	R	R	9 mm	R
8714A	R	R	R	R	R	R
2515D	16 mm	8 mm	R	29 mm	R	R
61214C	13 mm	10 mm	R	R	R	R
6514A	R	11 mm	R	R	21 mm	R
61214B	R	R	R	R	R	R
73114A	10 mm	R	R	20 mm	R	R
5814G	20 mm	26 mm	R	29 mm	31 mm	R
51514D	R	R	R	28 mm	R	R
91715D	R	R	R	23 mm	9 mm	R
12116B	R	R	R	R	21 mm	11 mm
51514A	R	R	R	R	25 mm	15 mm
12116C	25 mm	27 mm	R	20 mm	21 mm	19 mm
21116A	R	18 mm	R	25 mm	29 mm	11 mm
21116B	R	13 mm	R	26 mm	30 mm	10 mm
112014D	R	R	R	R	26 mm	20 mm
21915B	34 mm	R	26 mm	29 mm	29 mm	11 mm
32014B	R	R	R	R	R	R
81414A	20 mm	28 mm	R	21 mm	20 mm	14 mm
3614A *epid*	R	R	19 mm	11 mm	35 mm	17 mm
3614B *epid*	13 mm	R	19 mm	19 mm	30 mm	11 mm
3614C *epid*	20 mm	R	16 mm	11 mm	31 mm	16 mm

32014B (1)	R	R	R	R	21 mm	R
32014B (2)	R	R	R	R	24 mm	R
5814H	R	R	R	R	25 mm	R
81414C epid	40 mm	19 mm	22 mm	23 mm	39 mm	13 mm
61214C *epid*	11 mm	R	R	R	30 mm	R
6514D *epid*	R	R	R	R	33 mm	R
81414D *epid*	19 mm	R	R	R	R	R
5814E	11 mm	R	16 mm	16 mm	21 mm	18 mm
12315E (1)	10 mm	R	16 mm	16 mm	19 mm	20 mm
12315E (2)	11 mm	R	17 mm	18 mm	19 mm	21 mm
12315E (3)	12 mm	R	17 mm	16 mm	19 mm	20 mm
61214A	11 mm	R	R	R	30 mm	R

R = resistant. MET = methicillin; ERY = erythromycin; KN = kanamycin; TOB = tobramycin; GM = gentamycin; and STM = streptomycin. The diameter of the zone of clearance in susceptible isolates was measured and recorded in millimeters (mm).

Based on the results obtained from the Kirby-Bauer susceptibility tests, it is evident that the majority of the *S. aureus* isolates exhibit resistance to the tested antibiotics of the same concentration. Resistance incidences provided by isolates were enumerated and divided by the total number of isolates tested (n=46) and multiplied by 100 to provide for an overall percentage. Values are portrayed on the table below summarizing both susceptibility and resistance of the sample pool of isolates (Table 4).

Table 4: Percentages of *S. aureus* Isolates Resistant or Susceptible to a Panel of Six Clinically Relevant Antibiotics

	β-lactams	Macrolides (MLS_B)	Aminoglycosides			
	MET (MRSA)	ERY	KN	TOB	GM	STM
% of *S. aureus* isolates <u>resistant</u> to:	48.8%	67.5%	61.5%			
% of *S. aureus* isolates <u>susceptible</u> to:	51.2%	32.5%	38.5%			

Variability of *S. aureus* susceptibility to antibiotics can be observed from one patient isolate to another as demonstrated in Table 3. Some patients were susceptible and some were resistant to the same applied antibiotics. However, the percentage of resistant *S. aureus* isolates was higher than the susceptible isolates as depicted in Table 4. It can be observed that resistance to macrolide antibiotics yielded higher values compared to the beta-lactam and aminoglycoside antibiotics tested.

IV. b. Minimum Inhibitory Concentrations (MICs)

As stated above, the Kirby-Bauer susceptibility tests were the initial step in characterizing resistance to antibiotics. To further confirm resistance, the minimum inhibitory concentrations (MIC) of methicillin, erythromycin, and kanamycin were determined for each *S. aureus* isolate. A single antibiotic was chosen to serve as a representative for the class of antibiotics to which they are classified: methicillin representing the beta-lactam antibiotics, erythromycin representing the macrolide antibiotics, and kanamycin representing the aminoglycoside antibiotics. MIC is a measurement of the lowest antibiotic concentration required to inhibit the growth of an organism. MIC was carried out according to the CLSI guidelines;

using microtiter plates with two-fold dilutions of the antibiotics and inoculated with an equal bacterial inoculum ($O.D._{600} = 0.25$), incubated shaking at 37°C, 220 RPM for 24 hrs. The MIC was recoded the following day as the lowest concentration of antibiotic with no visible growth. The MIC values for each isolate are presented in Table 5 and summarized in Figure 5 below.

Table 5: Minimum inhibitory concentrations (ug/mL) of independently tested *S. aureus* isolates

Patient Isolate Tag	Methicillin	Erythromycin	Kanamycin
31314A	31.25	62.5	N/A
22014B	15.6	15.6	31.25
22714B	125	125	62.5
22714C	250	125	31.25
2515D	0.488	15.6	15.6
11614D	0.488	31.25	31.25
6514A	62.5	125	125
9414B	31.25	15.6	31.25
8714A	125	N/A	62.5
8714C	62.5	62.5	125
61214B	62.5	0.244	3.91
51514A	125	0.244	0.488
51514C	31.25	31.25	31.25
51514D	15.6	0.976	62.5
5814H	7.81	7.81	125
91715D	62.5	15.6	62.5
12116B	125	15.6	N/A
12116C	62.5	250	62.5
112014D	125	N/A	250
32014B(1)	125	250	62.5
32014B(2)	31.25	3.91	62.5
5814D	125	250	125
5814E	31.25	250	250
5814F	250	250	62.5
73114A	125	N/A	N/A
61214B	3.91	250	250
3614A	3.91	250	31.25
3614B	62.5	250	15.6
3614C	62.5	250	15.6
5814G	62.5	250	N/A

N/A indicates that the MIC was not calculated due to growth of isolate in all wells of the block.

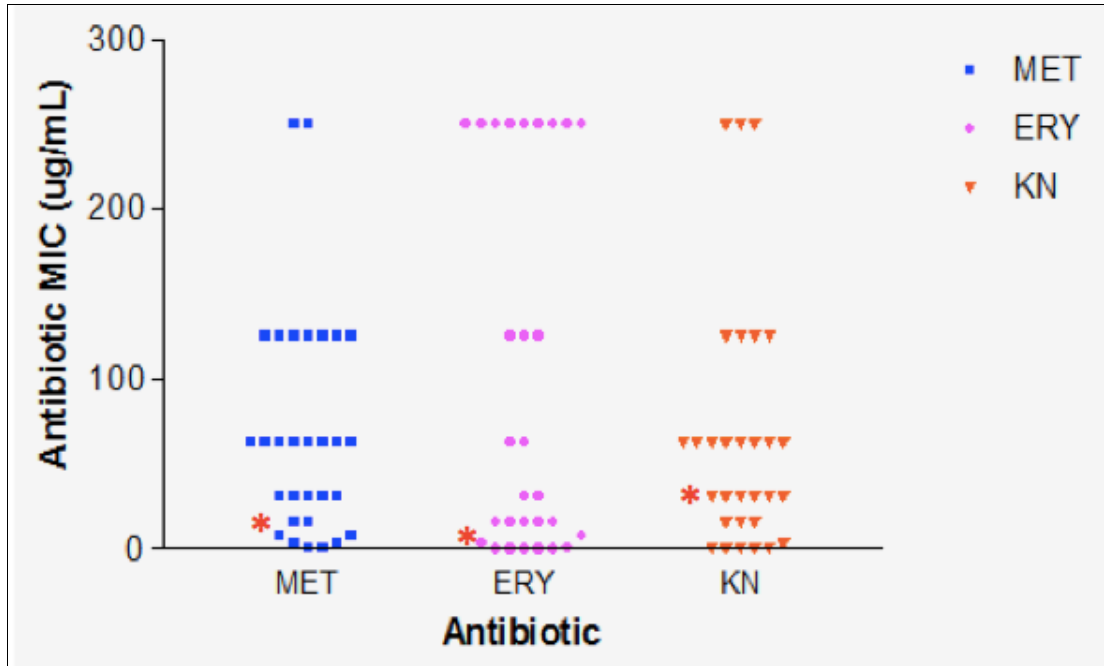


Figure 5: Antibiotic Minimum Inhibitory Concentrations

The MIC data above demonstrates that resistance of these *S. aureus* isolates when challenged with methicillin, erythromycin, and kanamycin is quite high. In fact, it is considered to be very high compared to the published MIC ranges for methicillin, erythromycin, and kanamycin. (NCCLS 2004; Matynia 2005; Chakraborty 2011). The red asterisks indicate the approximate location of published ranges of MICs for these antibiotics from clinical isolates, also shown in Table 6 below.

Table 6: Published MIC Ranges in *S. aureus* Clinical Isolates

Antibiotic	MIC Range
Methicillin (MET) (NCCLS 2004)	12.5 - > 100 ug/mL
Erythromycin (ERY) (Matynia 2005)	0.125 - > 16 ug/mL
Kanamycin (KN) (Chakraborty 2011)	> 64 ug/mL

V. Introduction to the Presence/Absence of Antibiotic Resistance Genes

Cumulatively, Kirby-Bauer susceptibility tests along with the MIC data provide phenotypic characterization of the resistance profiles of the *S. aureus* isolates collected from CF patient samples. However, genetic profiling is monumental in the understanding of antibiotic resistance among *S. aureus* isolates. Nine antibiotic resistance genes encoding for resistance to the three classes of antibiotics included in this study were hypothesized to be present in *S. aureus* isolates, as these are correlated with antibiotic resistance in CF patients (Prunier 2002; Dasenbrook 2010; and Rahimi 2016). By utilizing PCR, genes were amplified with gene specific primers (Table 1) and visualized with gel electrophoresis. Presence of amplicons demonstrated the detection of an antibiotic resistance gene. These results validate the phenotypic resistance observed by isolates obtained by the Kirby-Bauer susceptibility tests. The presence of each resistance gene in each isolate is detailed in Table 7 below.

VI. Results

VI.a. Presence/Absence of Antibiotic Resistance Genes

Each *S. aureus* isolate was tested for the presence of the nine resistance genes, which are specific for *S. aureus*. It is known that numerous resistance genes are produced by *S. aureus*; however, studies on CF indicated that the presence of these genes is associated with CF (Prunier 2002; Dasenbrook 2010; and Rahimi 2016). Furthermore, these resistance genes are found in other bacterial species, and here we are looking at the genetic variants of these genes that exclusively exist in *S. aureus,* hence the word specific for *S. aureus*. *S. aureus* isolates that have a particular gene present during screening served as positive controls for subsequent analysis. The presence of an antibiotic resistance gene from a patient, which was amplified successfully, was recorded in an Excel based format (Table 7).

Table 7: Presence or absence of antibiotic resistance genes in *Staphylococci*

Isolate ID	*mecA*	*ermA*	*ermB*	*ermC*	*ermT*	*msrA*	*aphA3*	*aadC*	*aacaphD*
21116B[c]	+						+		
5814D[c]	+		+		+	+			
21116A[c]	+						+		
12116C[c]									
11614D[c]									
81414A[c]									
5814E[c]	+								
22714C[c]	+								
8714A[t]	+								
51514C[t]	+	+							
2515D[t]									
6514A[a]	+	+							
5814G[a]									
51514D[a]									
6514D[a] -– *epid*	+								
22014D[a]	+								
12116B[a]	+	+							
61214C[a] – *epid*									
61214A[a]	+								
22014B[a]							+	+	
73114A[a]	+								

22714E[a]									
112014D[a]	+	+							
22714B[a]	+	+							
31314A[a]	+	+					+	+	
9414B[a]	+							+	
61214B[a]	+	+							
51514A[a]	+	+		+					
3614A[a]-*epid*	+								
3614B[a]-*epid*	+								
12315E[a] (1)									
12315E[a] (2)									
12315E[a] (3)									
91715D[a]									
8714C[a]	+	+					+		
5814F[a]	+	+	+					+	
5814H[a]	+	+							
81414D[a] -– *epid*	+						+		
9414C[a]	+	+							
3614C[a]-*epid*	+								
81414C[a] – *epid*									
21915B[a]	+						+		
32014B[a] (1)	+								

32014B[a] (2)	+	+							

Table is ordered according to age of patient at the time of collection of sputa sample. The superscripts indicate if the patient isolate is from a child, c, or a teenager, t, or an adult, a.

Table 7 demonstrates the results of PCR amplification when screened with nine antibiotic resistance genes in all of the *S. aureus* isolates. The (+) sign indicates that the corresponding gene was amplified from the purified gDNA from each correlating patient isolate provided by amplicon presence and size. Interestingly, not all of the isolates had resistance genes; 13 out of 46 isolates completely lacked the presence of resistance genes. For example, isolates 11614D, 61214C, and 81414A do not have any of the resistance genes. In contrast, 20 isolates had two or more resistance genes present, for example isolates 5814D and 5814F. From this data, it is evident that multi-drug resistance is present among *S. aureus* isolates recovered from CF patients. Table 7 also shows the results of PCR amplification of the resistance genes in 7 *S. epidermidis* isolates, 5 out of the 7 isolates carried the *mecA* gene encoding for resistance to methicillin. Isolate 81414D-*epid* also carried the *aphA3* gene encoding for resistance to aminoglycoside antibiotics, such as gentamicin.

Each gene which was amplified from the template genomic DNA confers resistance to a certain class of antibiotics. For example, *mecA* confers resistance to the beta-lactam family of antibiotics, which functions to inhibit peptidoglycan growth and repair. The *erm* genes, or erythromycin ribosomal methylase genes, (*ermA, ermB, ermC,* and *ermT*) confer resistance to macrolide, lincosamides, and streptogramin B antibiotics by methylation of the ribosomal RNA. Thus, the pattern of resistance to these antibiotics is known as MLS_B resistance. Macrolide efflux pumps encoded for by the *msrA* gene can also cause resistance to MLS_B antibiotics (Schmitz 2000). Resistance to aminoglycoside antibiotics is encoded by the *aphA3, aadC,* and *aacaphD* genes, which encode for aminoglycoside-modifying enzymes (Schmitz 2000). For these reasons,

genes were enumerated for total percentages and used to create a comparative Table (Table 8) to demonstrate the variability in gene presence/absence amongst isolates.

Table 8: Summary of the amplification of antibiotic resistance genes in *S. aureus* isolates

Class of Antibiotics	*S. aureus* Antibiotic Resistance Genes	PCR Results		% of Present Resistance Genes in Isolates
		Positive	Negative	
β-lactams	*mecA*	31	13	70.45%
Macrolides (MLS_B)	*ermA*	13	31	29.54%
	ermB	2	42	4.55%
	ermC	1	43	2.27%
	ermT	1	43	2.27%
	msrA	1	43	2.27%
Aminoglycosides	*aphA3*	7	37	15.91%
	aadC	4	40	9.09%
	aacaphD	0	44	0%

As shown in Table 8 above, the *mecA* gene is present in the majority (70.45%) of *S. aureus* isolates recovered from CF sputa samples. The *mecA* gene encodes for resistance to beta-lactam antibiotics such as commonly prescribed, penicillin or methicillin. Therefore, the presence of this gene in the majority of the *S. aureus* isolates is not surprising as the percentage of methicillin-resistant *S. aureus* (MRSA) among CF patients is on the rise. Typically, the presence of *mecA* provides enough evidence to classify a strain as being methicillin resistant. The presence of genes encoding for macrolide resistance came in the second place as 29.54% of isolates had the *ermA* gene present. Since this study includes 5 genes that encode for macrolide resistance, the presence of at least one gene is enough to characterize an isolate as having genotypic resistance. The same applies for the aminoglycoside resistance genes. Resistance to aminoglycoside antibiotics was the lowest compared to the beta-lactams and macrolides. The *aacaphD* gene,

which is a bifunctional enzyme (*aaC/acaphD)* that encodes for resistance to gentamicin, was never detected in our study as shown in Table 8. Although this gene was detected before in *S. aureus* clinical isolates (Matsua et al. 2003; Akpaka 2017). Therefore, the primers used in Table 1 for the amplification of this gene should not be problematic since they were the same as the primers published in the study of Matsau et al. Gradient PCR was initially performed to determine the optimal conditions for the amplification of these genes. Therefore, *S. aureus* isolates in this study do not carry the *aacaphD* gene.

VI. b. Phenotypic and genotypic resistance profiles

The detection of antibiotic resistance genes in *S. aureus* is an important tool used to understand the mechanisms of resistance that *S. aureus* may utilize in order to persist in the lungs of CF patients. Therefore, comparing the data generated from the phenotypic and genotypic studies will provide important information regarding the patterns of *S. aureus* resistance in CF patients. As mentioned above, if an isolate was resistant to a certain antibiotic in the Kirby-Bauer tests for example kanamycin, then it should also have at least one resistance gene that encodes for aminoglycoside resistance present, either *aphA3, aadC,* or *aacaphD.* As expected, most of the isolates' phenotypic resistance profiles matched the genotypic profiles, near identically. Isolates showed phenotypic resistance on the Kirby-Bauer plates as well as genotypic resistance in the PCR amplification of the resistance genes.

The more interesting results were obtained when the phenotypic resistance profiles did not correspond with the genotypic resistance profiles. Meaning that some isolates showed resistance in the Kirby-Bauer tests, but when tested for the presence of antibiotic resistance genes, no resistance genes were detected by PCR. Some *S. aureus* isolates exhibited resistance for antibiotics yet they lacked the presence of the genes that encode for the resistance to these antibiotics. These results are extremely important in the understanding of antibiotic resistance

patterns of *S. aureus* inside the lungs of CF patients. There are many ways to explain the absence of these resistance genes in some of the isolates. Importantly, and as mentioned before, *S. aureus* produces a number of antibiotic resistance genes and not all of them are included in this study. Therefore, it is possible that these isolates lack the genes we tested for and carry other genes that encode for resistance. Another explanation could be the possibility of the PCR not being sensitive enough or the presence of mutations within the primer binding regions in these isolates, which will lead to negative PCR results (Whiley 2005; Stanhouders 2010). Also, if *S. aureus* lacks the genes that encode for resistance, then this could suggest that *S. aureus* is using another mechanism to acquire resistance in the CF lung. In order to summarize these findings, the genotypic and phenotypic profiles that were matching (both resistant) or were conflicting (resistance genes absent) were enumerated in Table 9 below.

Table 9: Comparison of Kirby-Bauer susceptibility tests and PCR results

	β-lactams	**Macrolides (MLS_B)**	**Aminoglycosides**			
	MET (MRSA)	ERY	KN	TOB	GM	STM
Resistant to Antibiotics <u>and</u> Resistance Gene is <u>Present</u>	18	11			4	
Resistant to Antibiotics <u>but</u> Resistance Gene is <u>Absent</u>	2	16			18	

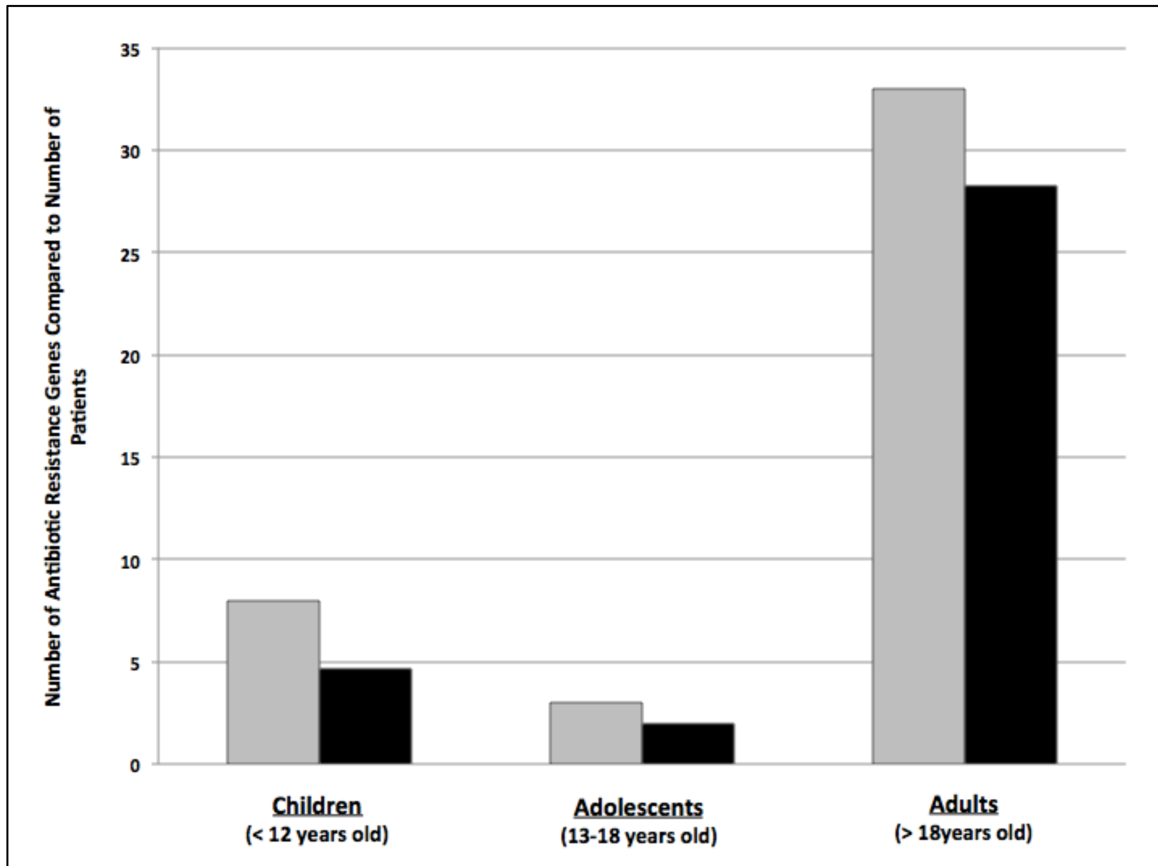
Table 9 shows the isolates with matching resistance profiles, both genetic and phenotypic profiles are in agreement, and the isolates with non-corresponding resistance profiles. Since the majority of the *S. aureus* isolates had the *mecA* gene present, resistance to methicillin often corresponded with the presence of the gene. The conflict in resistance profiles was mainly

observed in macrolides and aminoglycoside. As shown in Table 9, aminoglycosides had the lowest percentage of detected resistance genes. However, in the Kirby-Bauer susceptibility test, *S. aureus* isolates showed resistance to antibiotics in the aminoglycoside class. Even though a high percentage of the *S. aureus* isolates had the macrolide resistance genes present, the number of isolates with non-corresponding resistance profiles were more than those that have both phenotypic and genotypic resistance.

VI. c. Age and antibiotic resistance

Patients with CF are diagnosed at, or just after birth. It is known that *S. aureus* is the first pathogen to colonize the lungs of CF patients (Dasenbook 2010; Goss and Muhleback 2011). As mentioned before, it is often isolated from children. However, it remains a prevalent colonizer within the lungs of adult CF patients. Therefore, it is of interest to see if there is a correlation between the age of patients and antibiotic resistance. CF patients start receiving antibiotics to treat the chronic infections caused by *S. aureus* and other pathogens at a young age. By the time they reach adulthood, the pathogens in their lungs have been exposed to antibiotics for a long period of time. It can be speculated that the more pathogens are exposed to antibiotics, the more likely they will develop resistance.

A:



■ Total Number of Patients

■ Total Number of Antibiotic Resistance Genes in Age Group

B:

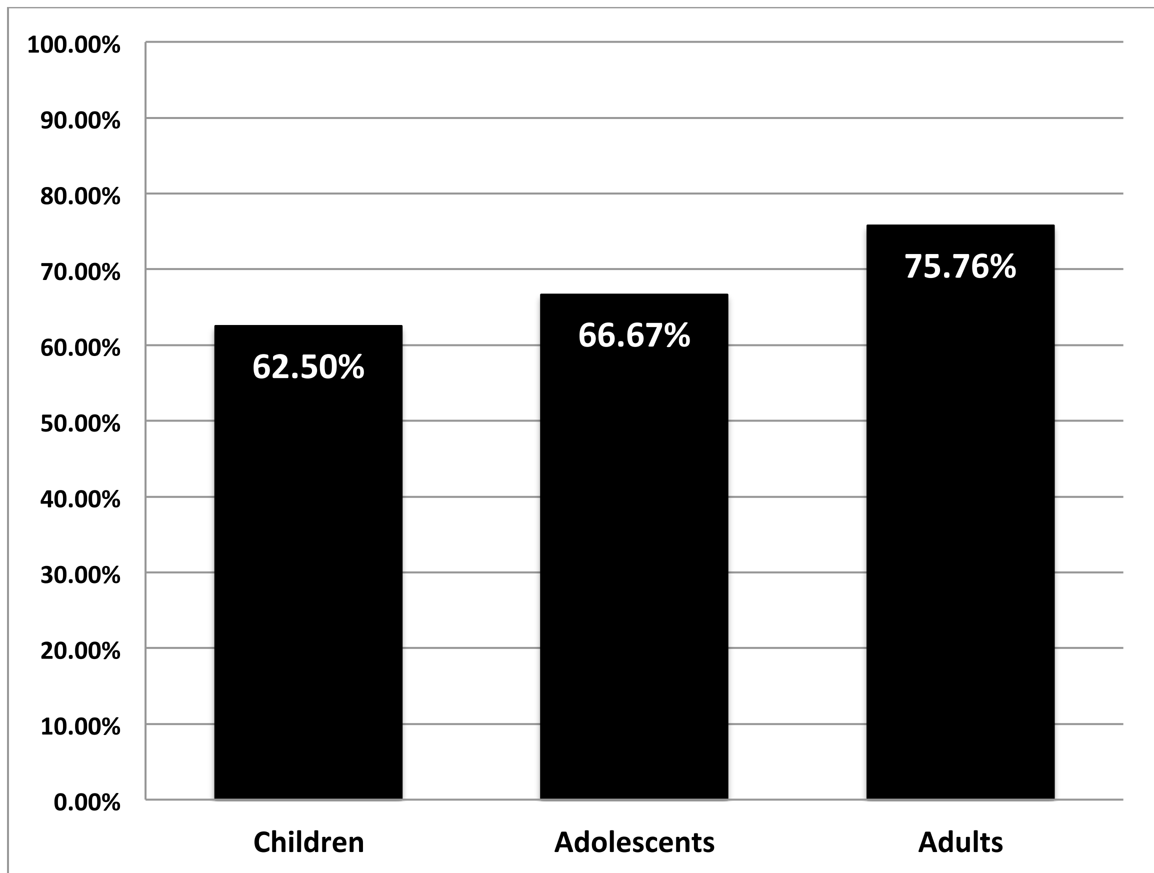


Figure 6: The relationship between age of CF patients and the presence of antibiotic resistance genes. A: Total umber of antibiotic resistance genes present in each age group compared to the total number of patients in each age group. B: The number of present resistance genes was normalized to the size of the pool from each age group.

Part A in figure 6 above shows the number of *S. aureus* patient isolates (grey) and the number of antibiotic resistance genes detected in each age group (black). It is important to note that there is a lack of *S. aureus* isolates from children and adolescents because parents and legal guardians do not consent to study participation. The total number of antibiotic resistance genes in each age group refers to the overall number of antibiotic resistance genes present among all adults, adolescents, or children, respectively.

The relationship between age and resistance cannot be fully understood due to the over-representation of adults in the study compared to other age groups. However, in this data set, isolates from adult patients, on average per isolate, exhibit more resistance genes than isolates obtained from adolescents and children as shown in part B of figure 6. As previously mentioned, some isolates (n=19) had several resistance genes present. These isolates with more than 2 resistance genes present were adults. That is, we can possibly state that *S. aureus* isolated from adult patients exhibits more resistance genes than *S. aureus* isolated from child or adolescent patients. Yet, this statement requires further confirmation with increased enrollment of child and adolescent patients. It is true that as patients with CF age their health conditions worsen and the eradication of pathogens becomes more difficult. The increased presence of antibiotic resistance genes in *S. aureus* isolates associated with older age could be a way to explain this occurrence.

VII.a. Introduction to rifampicin-resistant *S. aureus* Isolates

In the past decade, MRSA infections have increased in the United States and become even more of a serious threat. Beta-lactam antibiotics are typically used to treat *S. aureus* infections; however, when *S. aureus* is resistant to beta-lactam antibiotics, as in MRSA strains, the drug of choice is vancomycin, a glycopeptide antibiotic. Vancomycin treatment is often coupled with rifampicin; that is due to the poor tissue penetration of vancomycin as well as its slow bactericidal effect against some *S. aureus* strains (Deresinki 2009). In addition to methicillin, MRSA strains are resistant to multiple other antibiotics. More than 50% of MRSA strains are resistant to rifampicin (Voss et al. 1994). Rifampicin interacts with the beta subunit of the bacterial RNA polymerase, which is encoded by the *rpoB* gene (Aboshkiwa et al. 1995). Rifampicin resistance in *S. aureus* is caused by alterations in the target site that reduces the enzyme affinity for the antibiotic. Bacteria susceptible to rifampicin can undergo spontaneous one-step mutations to develop resistance. Rifampicin mutations are encoded by the Rif region, an 81 base pair rifampicin-resistance-determining region in the *rpoB* gene (Goldstein 2014). Here,

we have amplified this highly mutable region of the *rpoB* gene by PCR and sequenced portions of *rpoB* from rifampicin-susceptible and resistant *S. aureus* CF isolates (Wichelhaus 1999).

VII.b. Results

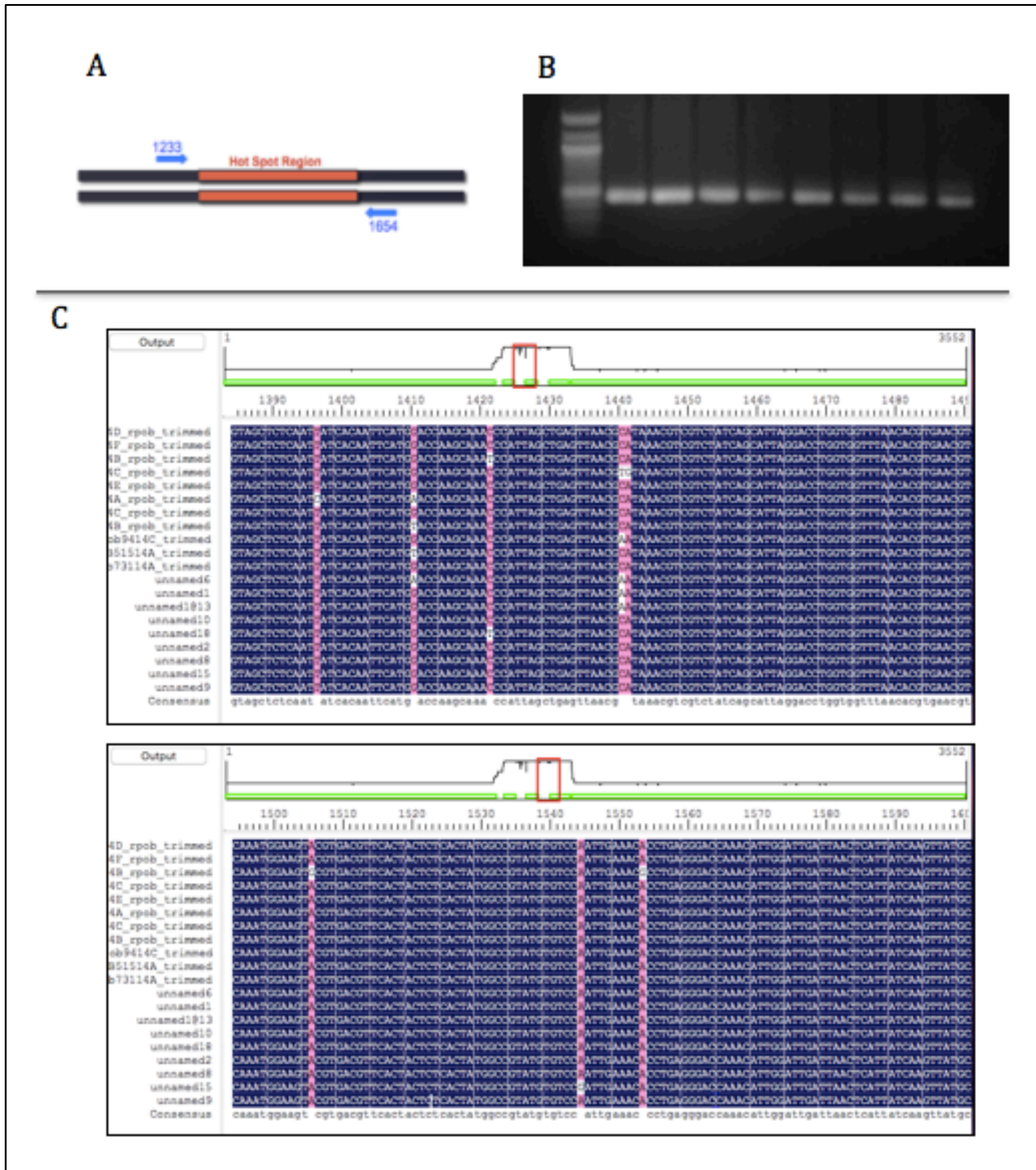


Figure 7: Amplification and multiple sequence alignment of *rpoB* gene from *S. aureus* isolates: A. The highly mutable region of the *rpoB* gene in *S. aureus* and the location of primer binding

sites indicated by the blue arrows (not drawn to scale). B. Visualization of the amplification of *rpoB* gene from *S. aureus* isolates. C. Multiple sequence alignment of *rpoB* gene sequences showing locations of point mutations.

Part A in Figure 7 above shows the region of the *rpoB* gene amplified with gene specific primers by PCR. This region is referred to as the hotspot region due to the high rate of mutation within it. The hotspot region in *rpoB* is between nucleotide base positions 1442 and 1451, the primers bind in positions 1233 (forward) and 1654 (reverse) producing an amplicon size of 460 base pairs (Wichelhaus 1999). These PCR products were purified and sent for sequencing. A multiple sequence alignment of the *rpoB* sequences from 11 isolates, including 8 rifampicin-susceptible isolates and 3 rifampicin-resistant isolates was generated. These sequences were also aligned with *rpoB* wild type sequences from *S. aureus* strains from the NCBI database.

Table 10: Mutations within the *rpoB* gene in *S. aureus* isolates

Isolate ID	Nucleotide Mutation	Amino Acid Substitution
22014B	CCC → TCC	464Pro → Ser
22014B	ACC → GCC	477Thr → Ala
22014B	ACG → GCG	473Thr → Ala
22014C	CAT → TGT	481His → Asn
31314A	ATT → ATC	471Ile → Ile
31314A	GGA → GAA	483Gly → Glu*
61214B	GGA → GTA	423Gly → Val*
9414C	CGC → CGA	458Arg → Arg
51514A	GGA → GTA	423Gly → Val*

Novel mutations within the Rif region of the *rpoB* gene are indicated by an asterisk (*).

Table 10 shows the mutations detected in the amplified portion of the *rpoB* gene. Not all of the sequenced isolates contained mutations in this region, 6 out of 11 isolates had mutations. Typically, mutations in the *rpoB* gene are characterized by a single base pair change that leads to an amino acid substitution, as observed in this data set, excluding the two silent mutations. Isolate 22014B, which is methicillin sensitive obtained from an adult patient with no exacerbations contained three point mutations. Isolate 31314A, which is methicillin-resistant obtained from an adult patient with no exacerbations possessed two point mutations. Both of these isolates were susceptible to rifampicin. The remaining 4 isolates had single point mutations and were a mix of rifampicin resistant and susceptible, all obtained from adults. Collectively, it can be inferred that the nature of the amino acid substitution affects the level of resistance. The most common amino acid substitutions in *rpoB* point mutations are 481His →Asn and Leu→Ser. The common 481His → Asn substitution was detected in our data set and it is usually associated with low-level rifampicin resistance. The 464Pro→Ser, 481His→Asn, 477Ala→Thr, and 473Ala→Thr substitutions were also previously detected in *S. aureus* isolates (Wichelhaus et al 1999; Aubry-Damon 1998). The rest of the mutations have not been previously reported in *S. aureus* isolates. Therefore, the detection of these mutations in our study is a novel finding. These mutations are designated by an asterisk (*) in Table 9 and include 483Gly→Glu amino acid substitution, and 423Gly→Val amino acid substitution. The 423Gly→Val substitution occurred twice in our data set, therefore, further investigation is required to understand the significance of this mutation in terms of antibiotic resistance. Additionally, an increased sample size is needed to conduct a more thorough screening of mutations within the *rpoB* gene in *S. aureus* isolates obtained from Oklahoma CF patients.

Table 11: Mutations within *rpoB* and susceptibility to rifampicin and methicillin

Isolate ID	Mutation to *rpoB*	Rifampicin	MRSA or MSSA
22014B	Detected	Resistant	MSSA
22014C	Detected	Resistant	MSSA
31314A	Detected	Resistant	MRSA
61214B	Detected	Resistant	MRSA
9414C	Detected	Sensitive	MSSA
51514A	Detected	Sensitive	MRSA

MRSA = methicillin resistant *S.aureus;* MSSA = methicillin sensitive *S. aureus*

Previous studies found that mutations occurring within the *rpoB* gene lead to rifampicin resistance and that was often associated with a higher level of methicillin and vancomycin resistance, respectively (Wichelhaus et al. 1999). However, no specific mutations were directly linked to this association. Table 10 shows that MRSA and MSSA are equally distributed among the patient isolates that had mutations within the *rpoB* gene in this study. Therefore, no generalizations about the association of *rpoB* mutations and higher MRSA incidents can be made using this data set.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Within the CF lung, a dynamic exists in which formation of the thick mucus in the lung airways creates an ideal environment for bacterial growth and colonization. Infections from bacterial pathogens begin shortly after birth. A wide range of pathogens are involved in the lung infections in patients with CF. Infants and children are typically infected with *Staphylococcus aureus*. *S. aureus* is the most common pathogen among young CF patients, however, it remains prevalent throughout the lives of CF patients and it is never completely eradicated. *S. aureus* is one of the most common agents in community acquired infections as well as nosocomial infections (Prabhakara et al. 2013). In addition, *S. aureus* has recently gained a lot of attention due to the increased occurrence of methicillin-resistant *S. aureus* (MRSA).

The native immune system is the first line of defense against *S. aureus*. The use of antibiotics is the second line of defense against *S. aureus* infections. It is suspected that the overuse and misuse of antibiotics led to the emergence of antibiotic resistant *S. aureus* strains.

Antibiotics have been an effective treatment for CF lung infections; however, these treatments fail to completely eradicate the lungs of the pathogens (Fodor et al. 2012). This is because CF pathogens tend to be highly adaptive within the CF polymicrobial community. *S. aureus* in particular employs several adaptive strategies including the development of antibiotic resistance. As mentioned above, *S. aureus* develops resistance to antibiotics either by genetic mutations or by acquiring resistance genes from another bacterium either by conjugation or transformation (Dever and Dermody 1991). *S. aureus* can also persist in the lung airways of patients due to phenotypic switching to a small colony variant form, also described as a persister phenotype. Persisters are basically colony variants that are dormant with a high level of resistance to antibiotics (Mitchell 2011; Yagci et al. 2013). Commonly utilized antibiotic therapy contributes to the selection of small colony phenotype, which is more frequently observed in older CF patients (Yagci et al. 2013). The formation of biofilms is also a factor that hinders the clearance of pathogens from the lungs. Biofilm formation is especially implicated, in regards to co-infection, by *P. aeruginosa.* However, recent advances in the molecular biology of *Staphylococci* allowed for the basic understanding of biofilm formation in *Staphylococci* such as *S. aureus* and *S. epidermidis* (Otto 2008).

The building of the repository of CF patient isolates allowed for studying *S. aureus* isolates from numerous CF patients of differing age groups and discrete health conditions. In general, the selection for *S.aureus* on MSA growth medium produced similar colony morphologies and pigmentations. However, *S. aureus* obtained from some patients produced colonies that were different in size and those were tested as separate isolates from that patient. It is possible for one patient to be infected with more than one strain of *S. aureus*. This was not very common among *S. aureus* in this study, but the strains that were phenotypically different responded to antibiotic challenges similarly. *S. aureus* strains isolated from CF patients have been shown to be diverse in many aspects including colony morphology (Sadowska et al 2002).

However, these variations are not very common as most CF patients are infected with phenotypically similar *S. aureus*. Our findings are in agreement with prior studies hypothesizing such (Yagci et al 2013). When we tested for the diversity of *S. aureus* isolates recovered from a single CF patient, there was not an observable difference in the size of the zones of clearance in the Kirby-Bauer susceptibility tests (See Appendix D). *S. aureus* is the predominant *Staphylococci* species in CF lungs; however other species such as *S. epidermidis* have previously been identified in CF patients (Lyczak 2002). As such, *S. epidermidis* was isolated from CF patient samples, although, infrequently in comparison to *S. aureus*.

S. epidermidis is a skin colonizer that is not known to cause harm to the host. However, in the recent years, nosocomial infections caused by *S. epidermidis* have increased in frequency. Therefore, *S. epidermidis* is currently seen as an opportunistic pathogen. The rate of infectious *S. epidermidis* in hospital settings is almost as high as infections caused by *S. aureus* (Otto 2009). In regards to CF, *S. aureus* infections in the CF lung are far more studied and documented in comparison to *S. epidermidis*. That is due to several reasons including: 1. *S. epidermidis* was not thought of as an infectious agent until recently; 2. Even though *S. epidermidis* causes infections, these infections are not life threatening (Otto 2009); and 3. *S. aureus* has been reported to cause more potent infections in the CF lung than *S. epidermidis* (Hussain 2013). Including *S. epidermidis* in this study was not initially intended, as all the *S. epidermidis* isolates were included to only increase the sample size. However, the addition of these isolates to our study provided a deeper understanding of antibiotic resistance in *Staphylococci*.

As mentioned in the results chapter, antibiotic susceptibility and the presence of antibiotic resistance genes were tested in 7 *S. epidermidis* isolates. Only 2 isolates were resistant to methicillin, and 6 out of the 7 isolates were resistant to erythromycin and gentamicin as shown in Table 3. Interestingly, the only resistance gene present in 5 out of the 7 isolates was the *mecA* gene, which encodes for methicillin resistance. The spread of antibiotic resistance genes in *S.*

epidermidis is frequent, and more importantly, resistance to methicillin is specifically high among clinical isolates and it is estimated to be between 75 and 90%, A study by Diekemia et al. in 2001, indicated that the frequency of methicillin resistance in *S. epidermidis* in clinical isolates is higher than that in *S. aureus,* which was estimated to be between 40 and 60%. One *S. epidermidis* isolate had the *aphA3* resistance gene present, which encodes for resistance to aminoglycoside antibiotics. In addition to methicillin, *S. epidermidis* is known to have resistance to multiple other antibiotics such as gentamicin and erythromycin, which are aminoglycoside antibiotics (Rogers 2008). Our findings are in line with previous studies regarding antibiotic resistance of *S. epidermidis*. Resistance to erythromycin and gentamicin was observed in our data set as indicated in Table 3. Although phenotypic resistance to methicillin was not observed in our data set, MICs for these *S. epidermidis* isolates were not measured in this study and the susceptibility observed by the Kirby-Bauer method is insufficient in determining whether these isolates are resistant or not, which will require further confirmation. The presence of the *mecA* gene among *S. epidermidis* clinical isolates has been well documented. In fact, studies indicated that the *mecA* resistance cassettes were transferred from *S. epidermidis* to *S. aureus* (Guo 2007; O'Gara 2007). Therefore, *S. epidermidis* serves as a reservoir for the transfer of resistance genes to improve the pathogenicity of *S. aureus* in human disease (Otto 2001).

The susceptibility of *S. aureus* isolates to antibiotics differed from one patient to the other. The results obtained from the Kirby-Bauer susceptibility tests demonstrated that each *S. aureus* isolate is unique. Further, though all of these patients were infected with *S. aureus*, the strain and pathology is unparalleled. As such, it is hypothesized that many factors contribute to the uniqueness of CF infections, for example, a variety of factors such as different genes and biological pathways. Environmental factors also play an important role in influencing the progression of the disease infections especially because the environmental factors are difficult to assess (Amaral 2015). This explains why no two patient isolates were similar in terms of

susceptibility to antibiotics. For example, isolate 5814E was obtained from an 11-year-old with no exacerbation yet presented as susceptible to nearly all antibiotics, for the exception of erythromycin. Isolate 22714C originated from a 12-year-old patient with no exacerbation was resistant to all antibiotics and was only susceptible to methicillin. Even though these two isolates came from children with similar health conditions, the *S. aureus* infecting their lungs responded very differently to antibiotics. In contrast, isolate 31314A came from a 28-year-old patient with no exacerbation was resistant to all of the antibiotics tested for in this study; as was the same for isolate 5814F that came from a 32-year-old patient experiencing exacerbation. These two *S. aureus* isolates were resistant to all antibiotics regardless of the health condition of the patient. Therefore, our findings agree with the notion of developing individualized treatments for CF patients as every individual with CF is unique and requires personal assessment to predict the severity of the infection and determine drug usage (Amaral 2015).

Resistance to macrolide antibiotics was the highest among all the isolates tested by the Kirby-Bauer disc diffusion method. The high rate of macrolide resistance among *S. aureus* isolates from CF patients has been documented since the 1990s. The percentage of macrolide resistant *S. aureus* recovered from CF patient was 53%, where resistance to other antibiotics was a lot lower: oxacillin at 13%, and gentamicin at 6% (n = 122) (Prunier et al. 2003). The resistance to macrolide antibiotics has been reported to increase over the years while resistance to other antibiotics remains constant (Prunier et al 2003). In this study, the representative macrolide antibiotic was erythromycin, which is one of the three most commonly used macrolides along with clarithromycin and azithromycin. These macrolides are structurally similar, therefore, the development of resistance to one antibiotic will lead to a complete cross resistance between all macrolides (Phaff et al. 2006). According to NCCLS breakpoints, *S. aureus* with an MIC of erythromycin greater than 8 mg/mL is classified as resistant. The calculated MICs shown in table 5 indicate that the majority of *S. aureus* isolates in our study are resistant to erythromycin. CF

patients receiving azithromycin for a prolonged period of time have an increased macrolide resistant *S. aureus*. Thus, physicians should be extremely careful when prescribing macrolides to CF patients, as the emergence of intrinsic resistance to macrolides in *S. aureus* is a potentially serious threat (Phaff et al. 2006).

Research studies focused on the relationship between antibiotic susceptibility patterns and antibiotic resistance genes in *S. aureus* clinical isolates found that all erythromycin resistant isolates carried at least one of the macrolide resistance genes (Duran 2012; Schmitz et al. 2000). These studies, along with others, found that the *ermA* gene is the most prevalent macrolide resistance gene among *S. aureus* clinical isolates. In our data set, *ermA,* was present in 29.54% of *S. aureus* isolates (n=46). Similarly, *ermA,* was present in 21.5% of *S. aureus* clinical isolates collected from blood, wounds, pus, urine, and other sources in Turkey over a period of two years (n=298) (Duran 2012). Our findings are in line with previous studies that the *ermA* gene is the most prevalent macrolide resistance gene. However, these studies also found that the *ermC* gene is the second most prevalent among erythromycin resistant *S. aureus* isolates. Our data shows that the *ermB* gene is the second most prevalent among erythromycin resistant *S. aureus* isolates at 4.55%, the *ermC* gene was present in 2.27% of the isolates. The prevalence of *ermC* seems to be higher in coagulase negative *S. aureus* isolates (Duran 2012); however, *S. aureus* coagulase production was not within the scope of this investigation.

Aminoglycoside antibiotics are widely used in the treatment of CF lung to target, specifically, infections caused by *P. aeruginosa*. As such, *S. aureus* is not typically treated with aminoglycosides. Nearly all *S. aureus* isolates in this study were resistant to kanamycin and streptomycin. This is not surprising, as these two antibiotics are not utilized for *S. aureus* infections. Initially, kanamycin was the only antibiotic representative from the aminoglycoside class included in this study. However, since all of the isolates in our study were resistant to kanamycin and high kanamycin resistance among *S. aureus* clinical isolates was previously

reported (Hauschild et al. 2008); expansion of the representative antibiotics from this class was performed to include streptomycin, tobramycin, and gentamicin. Tobramycin and gentamicin have similar properties and they are active in low concentrations against *S. aureus*. In fact, *S. aureus* is the only Gram-positive pathogen that tobramycin is active against. In general, the resistance against macrolide antibiotics was high, but when comparing individual patient isolates (Table 3), most isolates were susceptible to tobramycin and gentamicin and resistant to kanamycin and streptomycin. A study conducted on *S. aureus* clinical isolates that were also identified as MRSA showed that these isolates had a high level of resistance to kanamycin, tobramycin, and gentamicin (Rahimi 2016). Given that our data set contains MRSA strains, the high level of resistance to kanamycin is in agreement with these findings. However, *S. aureus* isolates in our study were susceptible to tobramycin and gentamicin as shown in Table 3. As such, the relationship between MRSA and resistance to tobramycin and gentamicin was not observed in our results.

As mentioned above, *S. aureus* gained a lot of attention in the past decade due to the increased emergence of methicillin-resistant strains, MRSA. Methicillin was first introduced in 1959; methicillin resistant strains developed only 2 years after its introduction and were only found in hospital settings (Goss and Muhleback 2012). In 2001, MRSA was found in 2% of CF patients and its frequency has increased to 25.7% in CF patients in 2011 (CFF, 2017). Thus, the presence of the *mecA* gene, which encodes for methicillin resistance, in 70% of the *S. aureus* isolates in this study was anticipated. Multiple hypotheses were proposed to explain how MRSA strains originated. One hypothesis is based on the association of the *mec* gene with genetically diverse lineages of *S. aureus*. Evolutionary genomic studies of *S. aureus* indicated that lateral gene transfer has greatly influenced the evolution of *S. aureus* (Fitzgerald 2001). The currently accepted hypothesis is that the *mec* gene has originated through horizontal gene transfer into *S.*

aureus at least five times. This implies that MRSA strains evolved multiple times in an independent manner and did not all originate from a single strain (Fitzgerald 2001).

Upon the establishment of a MRSA infection within the lung airways of CF patients, it is extremely difficult to be cleared with the usage of antibiotics. As such, large epidemiologic studies have shown that the lung function of CF patients is more likely to decline with MRSA infections (Dasenbrook et al, 2010). The detection of MRSA strains in the lung airways of CF patients was also implicated with decreased chances of prolonged survival. Logically enough, patients with MRSA tend to be hospitalized more often than patients without MRSA. Further, it is also to be expected that the use of antibiotics is higher by patients with MRSA. Despite this, it was reported that only a few MRSA were resolved in regards to infections within CF patients. As present, there are no clearly outlined strategies to treat or clear MRSA infections (Dasenbrook et al, 2010). Typically, a combination of antibiotics is used to control MRSA infections such as vancomycin, linezolid, rifampicin, and others. Yet, the rare cases of MRSA clearance indicate that the eradication of MRSA may be possible through the use of antibiotics (Goss and Muhleback 2011). Vancomycin has been considered a last resort antibiotic for the treatment of multiple-drug resistant bacteria. But in the recent years, vancomycin resistant *S. aureus* (VRSA) strains have emerged. The emergence of VRSA strains renders this last resort drug ineffective against *S. aureus* infections. Vancomycin resistance often overlaps with methicillin resistance in *S. aureus*. Thus, there is evident need for the development of new antibiotics that target *S. aureus* infections as well as new technologies that treat these infections in cystic fibrosis and other diseases caused by *S. aureus*. Likewise, more research efforts should center the focus on the mechanisms of resistance development in chronic infections.

S. aureus is a productive pathogen in terms of developing and acquiring resistance to antibiotics. This is demonstrated by the capability of the bacterium in employing a variety of mechanisms to combat the action of antibiotics. In order to understand how *S. aureus* resistance

to antibiotics occurs in the lungs of CF patients, testing was performed to determine the presence of antibiotic resistance genes within *S. aureus* isolates. These genes were present in some of the *S. aureus* patient isolates and were also in agreement with the phenotypic resistance observed by the Kirby-Bauer susceptibility tests. However, some isolates did not have any resistance genes present, yet they were resistant to antibiotics when tested with the Kirby-Bauer method. These findings were interesting due to the isolates having resistance to antibiotics phenotypically, despite the lack of resistance genes predicted to be contained by the isolate. As mentioned in chapter 3, the absence of resistance genes could be due to several factors including the possibility of the *S. aureus* isolates to carry other antibiotic resistance genes that were not included in our study. Also, this could be due to PCR not being sensitive, or even to a reduced priming efficiency due to the presence of mutations within the primer binding regions in these isolates (Standhouders, 2010). The absence of these resistance genes in phenotypically resistant *S. aureus* isolates could also suggest that *S. aureus* is using another mechanism to acquire resistance to antibiotics in the CF lung.

S. aureus has the ability to rapidly resist antibiotics with novel mechanisms. There has not yet been an antibiotic that *S. aureus* could not defeat from the first antibiotic, penicillin, to the newest antibiotics, daptomycin and linezolid (Pantosti 2007). The mechanisms that *S. aureus* uses are diverse and complex, therefore, it is difficult to predict which mechanism *S. aureus* is utilizing to acquire resistance within the CF lung airways. The use of horizontal gene transfer is highly possible especially because the chromosomal cassette *mec,* or the *vanA* operon, have been acquired by *S. aureus* via this mechanism. Also, because the polymicrobial community in CF is highly dynamic; bacteria are continuously colonizing the lungs and dying. As a result, *S.aureus* has a great opportunity to obtain and uptake exogenous DNA from its surrounding environment. *S. aureus* also develops resistance by modifying the antimicrobial target to decrease the affinity of the antibiotic; this mechanism is used by MRSA and VRSA strains. It also can inactivate

antibiotics by enzymes; for example, the penicillinase enzyme inactivates penicillins and the aminoglycoside-modification enzyme inactivates aminoglycosides. Resistance to vancomycin and possibly other antibiotics such as daptomycin occurs via trapping of the antibiotics; whereas, efflux pumps are utilized to extrude antibiotics such as tetracyclines and fluoroquinolones (Pantosti 2007).

S. aureus can also develop resistance through spontaneous mutations. These spontaneous mutations have been well studied in resistance against rifampicin (Voss et al. 1994). Rifampicin has a great ability to penetrate through host tissues, thus it is often used in the treatment of deep or systemic infections. Rifampicin interferes with transcription initiation in bacteria. Therapeutically, rifampicin is often used in combination with other antibiotics as susceptible bacteria can develop rapid one-step mutations to become resistant against it. Resistance to rifampicin arises from mutations within the *rpoB* gene, which encodes for the RNA polymerase beta subunit. It has been reported that the majority of mutations occur within a short stretch of base pairs in the *rpoB* gene termed as the Rif region (Nicholson and Maughan 2002). In this study, amplification of the Rif region in *S. aureus* isolates was performed and was followed by sequence analysis. Point mutations within the *rpoB* gene typically yield amino acid substitutions. The most common amino acid substitution from His to Asn (481His→Asn) was identified within the scope of this study. The Pro to Ser (464Pro→Ser), His to Asn (481His→Asn), Ala to Thr (477Ala→Thr) and (473Ala→Thr) substitutions were also confirmative of previously published *rpoB* mutations (Wichelhaus et al. 1999; Aubry-Damon 1998). However, a couple of mutations observed in this study did not lead to an amino acid substitution. Silent mutations have been found to be present within the Rif region of the *rpoB* gene and have been reported in MRSA isolates (Tang et al. 2015). Collectively, the rest of the mutations detected within this study were not previously described in *S. aureus rpoB* mutations, which could represent a novel identification of future mutations leading to *S. aureus* antibiotic resistances. In the past, higher

level of rifampicin resistance with MRSA has been documented in several studies (Tang et al. 2015; Wichelhaus et al 1999). However, the sample size in our study was small and that made it difficult to observe any patterns. This is partly due to the fact that the majority of the *S. aureus* isolates were already resistant to rifampicin. Considering that MRSA strains make up the majority of the *S. aureus* isolates in this study, we can conclude that there could be a relationship among MRSA prevalence and rifampicin resistance.

CF is one of the most studied genetic diseases and these research efforts have led to increasing the survival of CF patients. As of 2013, 49.7% of CF patients were over the age of 18 compared to 29.2% in 1986 (CFF 2017). The clinical understanding of CF continues to evolve; yet much is to be understood about the complexity of this systemic disease. One of the most difficult challenges in managing CF infections is antibiotic resistance. This study clearly focused on the poorly described relationship between age and the development of resistance to antibiotics. Data from the Cystic Fibrosis Foundation Registry associated increased pulmonary exacerbation rates with older age. Pulmonary exacerbation in CF is a condition that requires hospitalization or use of IV antibiotics. As patients age, they experience more pulmonary exacerbations (CFF 2014). The majority of CF studies focus on adult patients; that is because collecting CF sputa samples from children is difficult. Although the number of children and adolescents in our study is small, but the continuous collection and building of the repository of CF patient isolates will provide an avenue for further investigations related to age groups, including children. A linear relationship exists between increased exacerbation rates and aging of CF patients; this relationship in non-linear in children. In addition, as patients with CF age, the development of resistance to antibiotics becomes more frequent. This was documented in a longitudinal study conducted on Danish CF patients for over two decades; the study reported a significant rise in resistance to beta-lactam antibiotics as patients with CF became older (Goss 2006). Therefore, the tested hypothesis within this study of adult CF patients exhibiting more resistance to

antibiotics than younger patients is supported. This is not only demonstrated by previous research, but also by the findings within this study of *S. aureus* obtained from adult patients having more resistance genes present when compared to adolescents and children countertypes. This data is important due to the fact that we are looking at both phenotypic and genotypic resistance profiles of CF patients. Most studies concerned with antibiotic resistance focus on the susceptibility of patient isolates to antibiotics only.

Currently, the focus is on developing new strategies to treat CF. We now understand that every patient with CF is unique and requires a treatment strategy that is specific to his/her condition and the push for these individualized treatments should be a priority to researchers and health care facilities. The development of new antibiotics and innovative technologies that treat typical to chronic infections is also crucial. Resistance to antibiotics is a major medical concern and will be the major cause of mortality worldwide if research is not focused on averting antimicrobial resistance. A lot is to be understood about *S. aureus* infections in the CF lung specially regarding the acquisition of antibiotic resistance. The absence of resistance genes raises many questions related to the mechanisms that *S. aureus* is using to become resistant and persist in the lungs of patients with CF. This study did not include all of the resistance genes produced by *S. aureus;* thereby leaving a window open for future investigations. For instance, genes such as *aac(6)/aph(2)*, *aph(3),* and *ant(4)* encode for aminoglycoside resistance were not included (Duran et al. 2012). Several genes also encode for macrolide resistance that were not tested for within the scope of this investigation include: *vgaB, vgbB, vatC,* and *msrB* (Gerard et al. 1999). Other questions raised by the findings include: Is *S. aureus* using horizontal gene transfer to acquire resistance? Or is the cause of resistance genetic mutations? Or is it modifying enzymes and target locations? Quite possibly, *S. aureus* could be utilizing a combination or rather a novel mechanism of resistance that is still unknown.

V.a. Future Directions

The work discussed in this thesis serves as a platform for several forthcoming research projects. More research is required to understand the cause of *S. aureus* resistance to antibiotics. In the future it is important to continue the collection of CF sputa samples from the CF clinic to increase the sample size and further confirm the trends seen in this study. It is an urgent necessity to expand the number of antibiotic resistance genes to include the genes mentioned above, as they could be present in the *S. aureus* isolates. It is also worthwhile to repeat the PCR using different sets of primers for the nine antibiotic resistance genes included in this study. Using other sets of primers that have been published for the same genes might rule out the possibility of negative PCR due to mutations or gene truncation as they might bind to different locations within the gene. In addition, designing primers that specifically target these genes instead of relying on published primers can be helpful in solving the puzzle of no amplification of resistance genes. The presence of antibiotic resistance genes is only one simple step towards the understanding of antibiotic resistance of *S. aureus* during colonization and infection within the lungs of CF patients. As demonstrated by this investigation, the presence of known antibiotic resistance-encoding genes does not determine as to gene and ultimate protein functionality. As such, it is essential to follow the initial conventional PCR screening for antibiotic resistance genes with gene expression with modern *in vitro* biochemical methods.

Our findings also emphasize that *S. epidermidis* plays an important role in human disease. As previously stated, *S. epidermidis* is now accountable for many nosocomial infections and it also provides a reservoir of antibiotic resistance genes to *S. aureus*. More importantly, methicillin resistant *S. epidermidis* strains are highly transmissible. Therefore, it is important to focus on understanding both the commensal and infectious roles of *S. epidermidis* in CF in terms of pathogen-pathogen interactions. It is important to start with sequencing the *S. epidermidis* isolates. Then, look for the presence of antibiotic resistance genes among these isolates along

with conducting antibiotic susceptibility tests. Next, determine how the presence of *S. aureus* and *S. epidermidis* in the same environment affects susceptibility to antibiotics.

This study also initiated a basic understanding of the resistance of *S. aureus* isolates to rifampicin. Novel mutations were detected within this data set, and these mutations need to be further studied to see if the amino acid substitutions they caused affect the level of resistance to rifampicin. This is important because resistance to rifampicin is often associated with increased MRSA and VRSA. It is also important to investigate the relationship between rifampicin resistance and a higher level of MRSA incidents.

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APPENDICES

Appendix A: Growth of multiple *S. aureus* strains on MSA plate

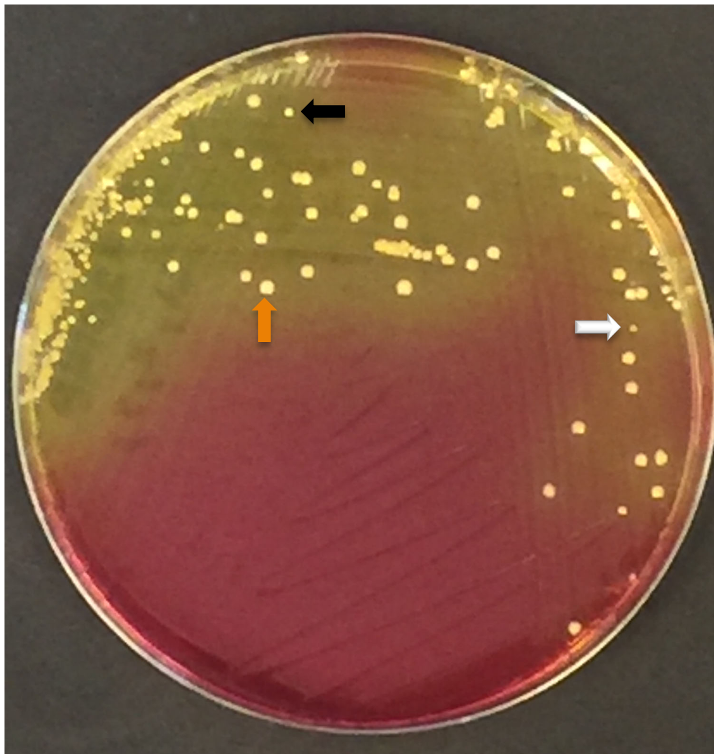


Figure 10 shows the streaking of isolate 12315E. The colored arrows point to colonies of different sizes. In particular, 3 *S. aureus* strains were selected from this one patient isolate (each color points to a different strain) and they were named as 12315E (1), 12315E(2), and 12315E (3). Each one of these strains was tested as a separate isolate from this patient.

Appendix B: Additional plate representations of the Kirby-Bauer method

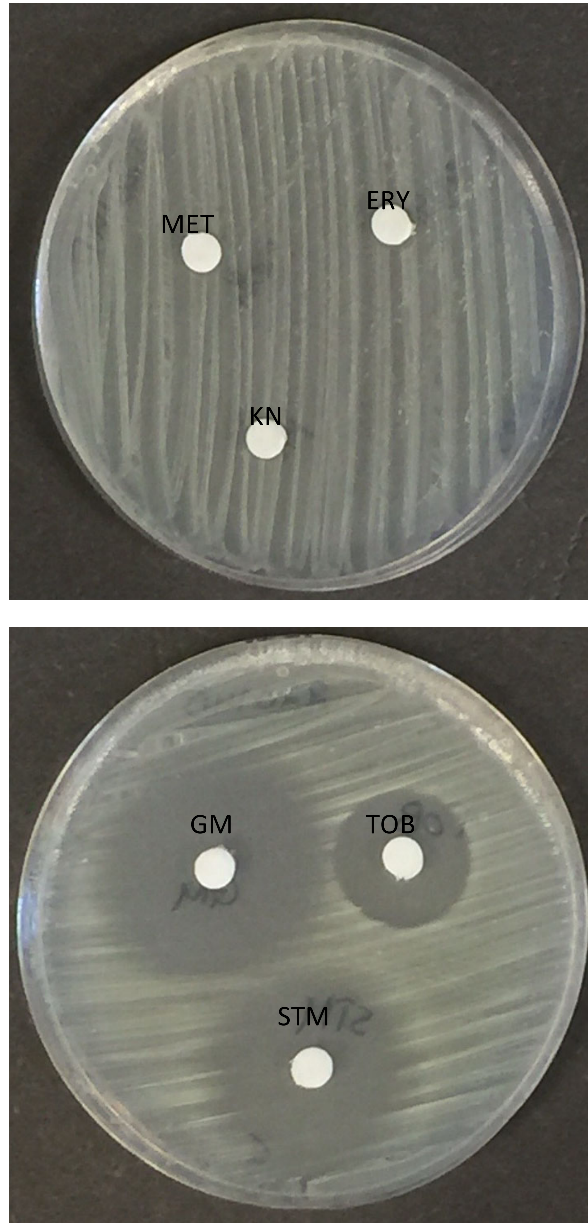
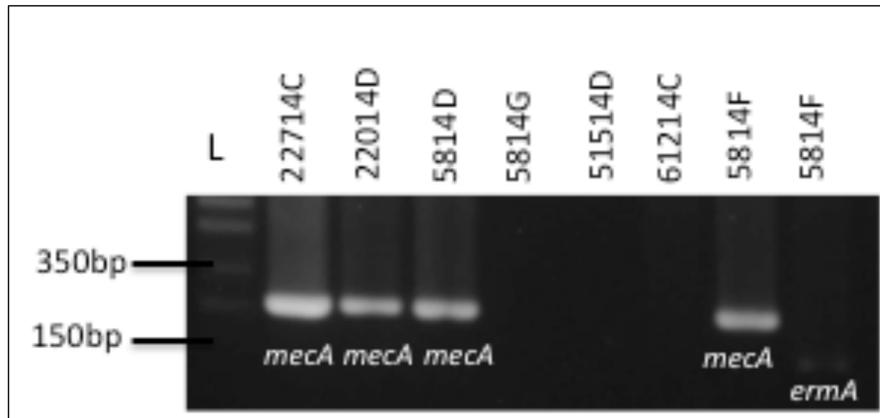


Figure 11: Complete resistance to antibiotics MET, ERY, and KN shown in the top image as no zones of clearance around the discs were observed. The bottom image shows the susceptibility to GM, TOB, and STM, which were indicated by the formation of clearance zones around the discs.

Appendix C: Gel visualization of resistance genes



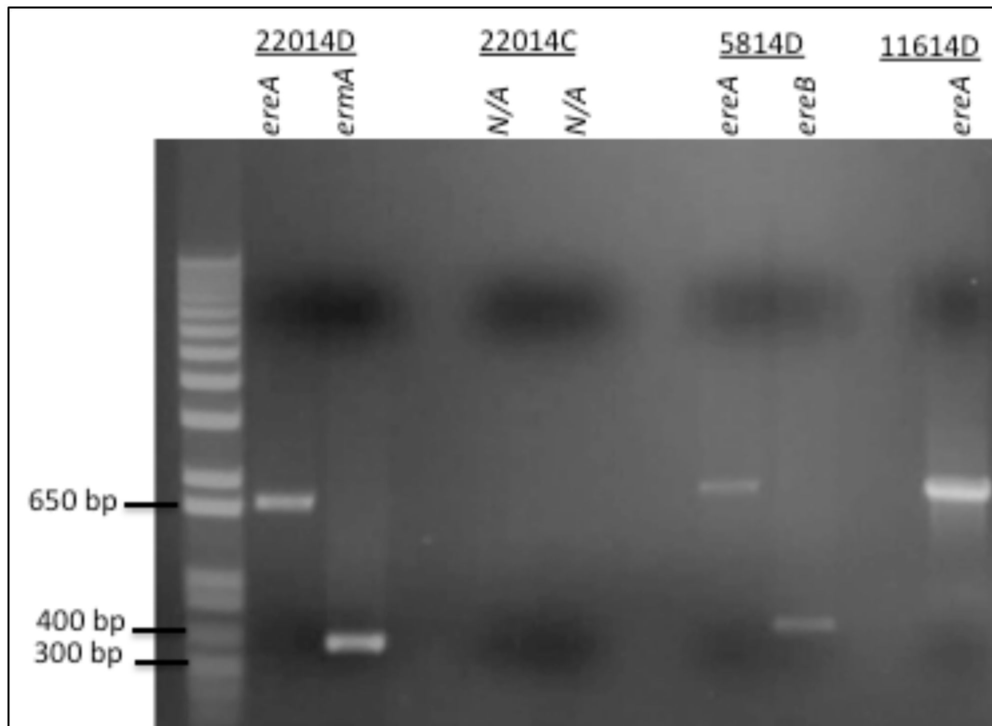


Figure 12: The gel images show the amplification results of antibiotic resistance genes in some of the *S. aureus* isolates and the sizes of the amplified genes. The images show both positive and negative results of amplification.

Appendix D: Diversity of *S. aureus* isolates within a single patient

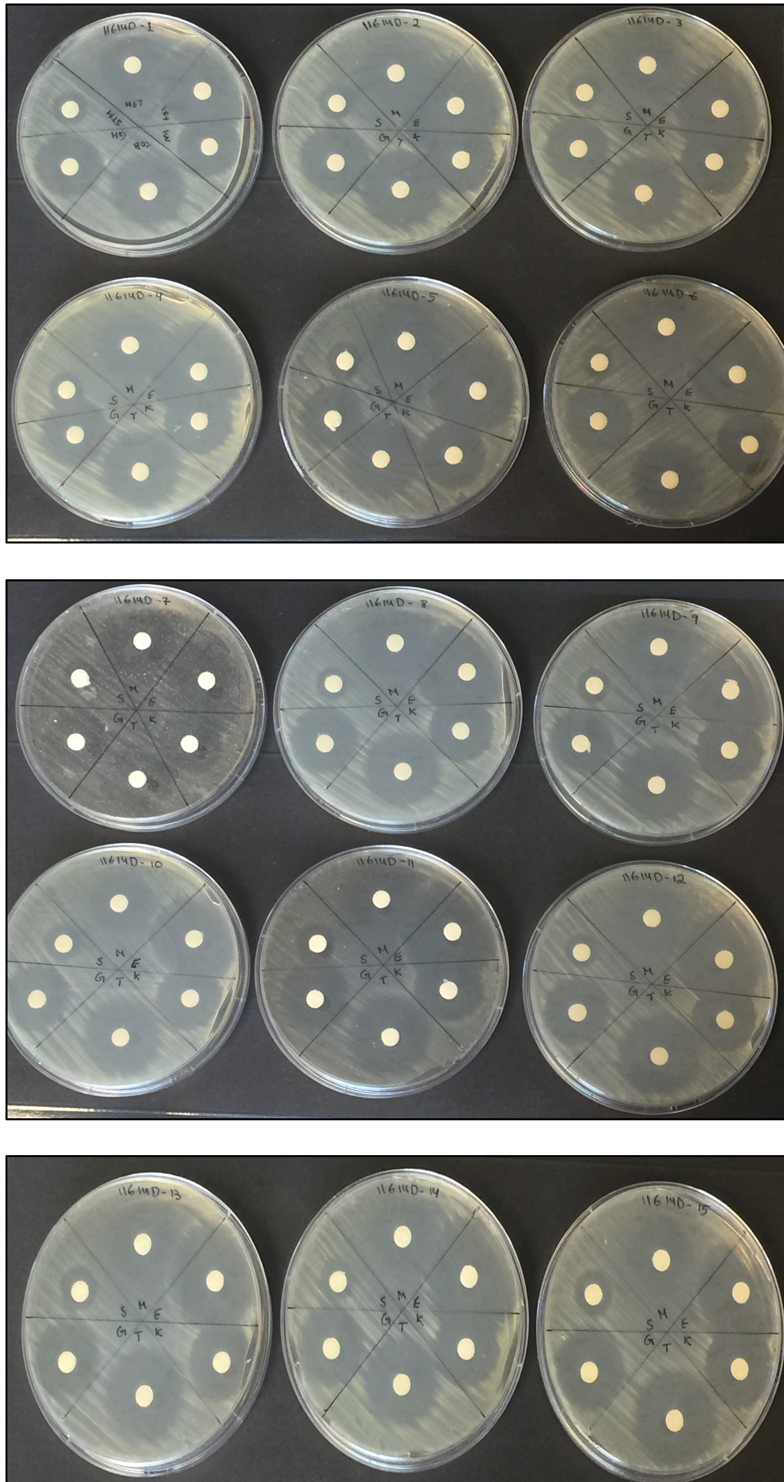


Figure 13: Diversity of *S. aureus* isolates within patient 11614D

Figure 13 shows the diversity in antibiotic resistance of *S. aureus* isolates within a single CF patient. One child patient and one adult patient were randomly selected. Fifteen random colonies were selected (each colony representing an isolate from that patient), and then performed Kirby-Bauer susceptibility tests. The plates in Figure 13 are the results of the child patient 11614D and they show that there was no difference in the zones of clearance around the discs. The same results were observed in the adult patient. This indicates that there is no diversity in antibiotic resistance of *S. aureus* isolates obtained from a single CF patient.

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