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The Clinical Utility of Molecular Typing of Multiply-resistant *Pseudomonas aeruginosa*
in Children with Cystic Fibrosis

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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April 2010

ACKNOWLEDGEMENT

I'd first like to thank my committee for their patience and flexibility throughout this process: Dr. William Korzun, Dr. Teresa Nadder, Dr. Diane Dodd-McCue, and Dr. James Versalovic. As a group, they were a tremendous resource in ensuring the quality of the end product, and they always gave me the time I needed when "life" halted our forward progress. I'd also like to thank everyone in the VCU Health-related Sciences doctoral program for their support, especially Dr. J. James Cotter, and of course, I relied heavily on the strong bonds within our 2004 cohort. I'd like to specifically acknowledge Dr. Versalovic for his role as my mentor. His vision for my future and willingness to allow me to pursue this degree was instrumental in my success, and I am very grateful for the opportunities he has given me.

Finally, I give great thanks to my family. When I started this program, it was just the two of us and our dreams of the future. Without my husband Patrick's unconditional love and support, I would have never survived this journey. Beyond the typical rigors of pursuing a graduate degree, life had additional struggles in store for us. From losing my grandfather while I was hundreds of miles away on campus at VCU to the multiple losses and roadblocks we were met with while trying to start a family, it was Patrick that kept me focused, sane, and ready for the next obstacle. So here I sit almost six years later, a Ph.D. in hand, two beautiful miracle children, and one amazing husband...I wouldn't change one step of the journey because this truly is my happy ending.

To Larkin Clara and Kellen Patrick, I was determined to not miss any time with you (which made for lots of late nights), and you were my motivation to finish. This is dedicated to you and all that you represent.

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ABSTRACT

THE CLINICAL UTILITY OF MOLECULAR TYPING OF MULTIPLY-RESISTANT *PSEUDOMONAS AERUGINOSA* IN CHILDREN WITH CYSTIC FIBROSIS

Ruth Ann Luna, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: William Korzun, Ph.D.
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Chronic infection with *P. aeruginosa* is expected in patients with cystic fibrosis (CF), but the ability to delay, prevent, or better manage infection with multiply-resistant *P. aeruginosa* (MRPA) can potentially increase quality of life and extend survival. The Texas Children's Hospital CF Care Center has identified an endemic MRPA strain (dominant clone), and this study aimed to identify risk factors for acquisition of the clone as well as determine differences in patient outcome associated with subsequent infection with the clone.

The study included 71 patients with CF with documented MRPA infection. Designation of patients as members of the dominant clone or a non-dominant clone group was based on molecular typing by rep-PCR of MRPA isolates from respiratory cultures.

Patient data was collected from Port CF, the national patient registry of the CF Foundation. Patient demographic information and clinical parameters prior to MRPA infection were analyzed by logistic regression as potential risk factors. Differences in patient outcome including change in BMI, change in FEV₁, and hospitalization rate were evaluated by MANOVA.

Recent hospitalization (≤ 90 days) was a statistically significant ($p = 0.035$) risk factor for acquisition of the dominant clone. Patients hospitalized ≤ 90 days prior to MRPA diagnosis were four times more likely to be infected with the dominant clone, and patients hospitalized 91-180 days prior were almost three times more likely. Increased hospitalization rates were seen in the dominant clone group both pre- (11 more days/year) and post-infection (14 more days/year) as compared to the non-dominant clone group. Patients infected with the endemic strain exhibited poorer outcomes in terms of nutritional status (3.73% decrease/year in BMI %ile) and lung function (3.7% decrease/year in FEV₁ %ile). Significant overlap in hospitalization episodes of patients known to be infected with the dominant clone and patients subsequently infected with the dominant clone was observed.

Recent hospitalization was a significant risk factor for infection with the dominant MRPA clone, and following infection, patients infected with the endemic strain exhibited declines in nutritional status and lung function and increased hospitalization rates. The results suggest potentially increased virulence and transmissibility of the endemic MRPA strain.

CHAPTER 1: INTRODUCTION

Multiply-resistant *Pseudomonas aeruginosa* (MRPA) is a common respiratory pathogen found in patients with cystic fibrosis (CF) (Patient Registry 2008 Annual Report, 2009). The bacteria routinely leads to chronic pulmonary infection, and the infections have proven difficult to treat due to the organism's resistance to many of the commonly prescribed antibiotics (Falagas, Koletsi, & Bliziotis, 2006). The life expectancy for patients with CF has dramatically increased in the past 20 years, but persistent antibiotic-resistant infections continue to impact quality of life as well as survival. The Cystic Fibrosis Care Center at Texas Children's Hospital (TCH) in Houston, Texas is a large CF Foundation-Accredited Care Center. However, while the national average of multiply-resistant *Pseudomonas aeruginosa* infection in patients with CF in 2006 was 16%, the average at the TCH CF Care Center was 30.1% (Patient Registry 2006 Annual Report, 2008). This trend towards higher MRPA infection rates at TCH remained present through the latest CF center-specific data for 2008 with a national MRPA average of 17.9% and an average of 28% at TCH (Patient Registry 2008 Annual Report, 2009). Prevention or delay of MRPA infections could significantly improve long-term patient prognosis and prolong the projected lifespan of patients with CF. Infection with multiply-resistant *P. aeruginosa*, rather than a susceptible strain, has been associated with greater risk of death or lung transplantation, and infection with specific *P.*

aeruginosa epidemic strains has been implicated in increased patient morbidity (Al-Aloul et al., 2004; Edenborough et al., 2004; Lechtzin et al., 2006; Nixon et al., 2001; O'Carroll et al., 2004). Identification of risk factors for acquisition of a specific MRPA clone or information regarding projected outcomes for patients infected with a specific MRPA clone would be valuable tools for clinicians and infection control practitioners.

The Cystic Fibrosis Foundation Consensus Conference on Infection Control has provided specific infection control guidelines in the hopes of reducing the number of patients infected with MRPA in each of the CF clinics in the United States (Saiman & Siegel, 2003). Various scenarios including social contact during hospitalization and proper sterilization and decontamination during routine visits and procedures were addressed. These infection control procedures are meant to decrease the possibility of patient-to-patient transmission of MRPA and common source acquisition of MRPA.

Numerous investigators have employed strain typing technology to determine whether the *Pseudomonas aeruginosa* strains infecting patients with CF in one treatment center were genetically, or clonally, related (D. Armstrong et al., 2003; Jones et al., 2001; Nixon et al., 2001; Scott & Pitt, 2004). Molecular typing allows for the identification of different strains of a particular bacterial species. While all of the strains tested are the same species (ex., *Pseudomonas aeruginosa*), subtle differences in the bacterial genomes allow differentiation between strains that are unrelated and those that may have originated from a common source. Clonally related strains will create a cluster, a group of highly similar molecular fingerprinting patterns, when analyzed by molecular methods. Various molecular methods that employ the polymerase chain reaction (PCR) including

pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) PCR, repetitive element PCR (rep-PCR), as well as other technologies have been utilized for identification of related *Pseudomonas aeruginosa* strains. While some studies did not yield evidence of clustering of *Pseudomonas aeruginosa* strains in patients with CF (Agarwal, Kapil, Kabra, Das, & Dwivedi, 2005; da Silva Filho, Levi, Bento, Rodrigues, & da Silvo Ramos, 2001; Silbert, Barth, & Sader, 2001; Spencker et al., 2000; Tubbs et al., 2001), the majority of studies have discovered related strains of *Pseudomonas aeruginosa* in their CF patient population (Edenborough et al., 2004; Kersulyte, Struelens, Deplano, & Berg, 1995; O'Carroll et al., 2004; Scott & Pitt, 2004; Szymis et al., 2004). Several studies employing molecular typing of *Pseudomonas aeruginosa* strains in their CF population not only found significant clustering but also suggested that patients infected with the epidemic strain were more adversely affected and required more treatment than patients infected with unique strains (Al-Aloul et al., 2004; Edenborough et al., 2004; Nixon et al., 2001; O'Carroll et al., 2004).

Patients with CF infected with multiply-resistant *Pseudomonas aeruginosa* will likely suffer worse outcomes than those with susceptible *Pseudomonas aeruginosa* isolates. Patients infected with MRPA exhibit more rapid decline in routine pulmonary function tests such as FEV₁, the forced expiratory volume in one second (Lambiase et al., 2006). One study reported that patients with CF infected with MRPA were 14 times more likely to die or require a lung transplant than patients infected with *Pseudomonas aeruginosa* that was not multi-drug resistant (Lechtzin et al., 2006).

Research has been conducted comparing CF patient outcomes with MRPA versus susceptible *Pseudomonas aeruginosa* and to determine relatedness of *Pseudomonas aeruginosa* strains in patients with CF. However, to date, no study has been performed that specifically analyzes CF patient-related variables and patient outcomes as they relate to the clustered MRPA strains. If significant relationships were found between CF patient-related variables and the acquisition of a specific MRPA strain, then preventative measures could possibly stop or suppress the transmission of MRPA. Moreover, if significant relationships were found between CF patient outcomes and a specific MRPA strain, then treatment plans could be tailored based on the prognosis for that specific MRPA strain.

Purpose Statement

The purpose of this study is twofold: to determine if there is a relationship between CF patient-related variables and infection with a specific MRPA strain; and to determine if there is a relationship between infection with a specific MRPA strain and CF patient outcomes.

Research Questions

Is infection with the dominant MRPA clone related to patient-related variables prior to MRPA infection?

Hypothesis: Certain CF patient-related variables are significantly related to infection with the dominant MRPA clone. The CF patient-related variables to be studied included the following: age at time of CF diagnosis, age at time of MRPA infection diagnosis, body mass index (BMI), forced expiratory volume in one second (FEV₁), days from last

hospitalization to MRPA diagnosis, CFTR genotype, mother's educational level, gender, days from last clinic visit to MRPA diagnosis, tobramycin use, and respiratory culture results. In addition to evaluating the entire group of patient-related variables, smaller groups of variables related to patient demographics and clinical parameters at the time of MRPA infection (including subsets related to chronology and disease state) were analyzed.

Is CF patient outcome related to infection with the dominant MRPA clone?

Hypothesis: Patient outcomes such as death, change in FEV₁, change in BMI, and hospitalization rate are related to infection with the dominant MRPA clone.

Significance of the Study

The morbidity associated with MRPA infections in patients with CF is well documented, but not all patients suffer the same decline in quality of life. While studies have shown significant differences in patient outcomes with MRPA strains versus antibiotic susceptible *Pseudomonas aeruginosa* isolates (Al-Aloul et al., 2004; Lambiase et al., 2006; Lechtzin et al., 2006; Nixon et al., 2001), no research has been performed to determine if there are significant differences in patient outcomes within the patient population infected with MRPA.

If a significant relationship was discovered between CF patient-related variables prior to MRPA infection and the subsequent MRPA strain, then clinicians may be able to develop strategies to prevent transmission and new infections. If prevention is not possible, then improved management or treatment plans may be implemented for patients identified at risk for MRPA infection. If no significant relationship was discovered

between CF patient-related variables and infection with a specific MRPA strain, then the study would suggest that monitoring these characteristics prior to MRPA infection is not necessary and that these characteristics should not be a focal point for infection control guidelines and practices. If a significant relationship was discovered between a specific MRPA strain and CF patient outcomes, then clinicians could tailor their treatment efforts based on disease severity associated with that particular MRPA strain. More aggressive and customized treatment or management plans could be implemented. If no significant relationship exists between the MRPA strain and CF patient outcomes, then the study would suggest that molecular typing data is not useful in prospective patient care.

Delimitations

The study included only pediatric patients with cystic fibrosis who were treated at the CF Care Center of Texas Children's Hospital. Patients in the sample population included only those children who did not have a MRPA infection at the time of their first visit to the hospital.

Assumptions

The following assumptions were made while conducting this study:

- 1) All patient-related variables and outcome data were correctly entered into Port CF (version 1), the electronic national repository for CF patient data.
- 2) Measurement of patient data, such as lung function and weight, was accurate and precise.
- 3) All MRPA infections were correctly diagnosed from respiratory cultures.

- 4) All rep-PCR molecular fingerprinting profiles were sufficient to discriminate between clonal groups.
- 5) All MRPA isolates from patients with cystic fibrosis who are eligible for the study were sent to the Molecular Microbiology laboratory for DNA typing.

Definition of Terms

Multiply-resistant *Pseudomonas aeruginosa* (MRPA). A strain of *Pseudomonas aeruginosa* that is resistant to at least two different classes of commonly used antibiotics. The Cystic Fibrosis Foundation defines MRPA isolates as being resistant to all antibiotics in at least two of three of the following antibiotic groups: aminoglycosides, fluoroquinolones, and beta-lactams.

Cluster or clonal group. A group of bacterial isolates that yield highly similar molecular fingerprinting patterns. By molecular typing, these isolates belong to one group that represents a single clone. The terms bacterial clone and strain will be used interchangeably in this study.

Organization of the Study

The study is presented in five chapters followed by a bibliography. Chapter Two provides a review of the literature concerning cystic fibrosis, MRPA infections, molecular typing techniques, and the clinical application of molecular typing. Chapter Three describes the ex post facto research design and methodology of the study including selection of the appropriate patient-related and patient outcome variables, details of the molecular techniques utilized, and explanation of statistical analyses. Chapter Four presents the results of the study. Chapter 5 concludes with a discussion of the data

including a summary of the work and recommendations regarding how the information could be used in future studies and current medical practice.

CHAPTER 2: LITERATURE REVIEW

The following chapter will present the background necessary for a discussion of the application of molecular typing for multiply-resistant *Pseudomonas aeruginosa* in patients with cystic fibrosis. General information regarding the disease of cystic fibrosis such as underlying genetics, diagnostic testing, symptoms, and treatment strategies will be provided. Commonly used patient outcome predictors for cystic fibrosis will also be presented. Infection control as it relates to *P. aeruginosa* and cystic fibrosis will be discussed as well as utilization of molecular typing technologies for epidemiology studies in this special patient population. Finally, supporting information for the variables employed in this study will be presented including potential risk factors for acquisition of MRPA and patient outcome indicators.

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease that affects 1 in 3,500 children born in the United States (Patient Registry 2008 Annual Report, 2009). Approximately 30,000 people live with a diagnosis of cystic fibrosis in the United States, and while most patients are Caucasian, CF can affect individuals of all ethnicities. CF occurs at a rate of 1 in 9,500 Hispanics and less than 1 in 50,000 in native Africans and Asians. African Americans and Asian Americans have a higher incidence of 1 in 15,300 and 1 in 32,100 respectively (National Institutes of Health, 1997). CF is an autosomal

recessive disease, and an individual must have two mutations of the CFTR gene (one on each copy of chromosome 7) in order to be clinically affected with cystic fibrosis; therefore, individuals identified as CF carriers are more common. Carrier rates for cystic fibrosis are 1 in 29 for the Ashkenazi Jewish and European Caucasian populations, 1 in 46 for Hispanic Americans, 1 in 65 for African Americans, and 1 in 90 for Asian Americans (Grody et al., 2001).

Dr. Dorothy Andersen first described CF as a separate disease in a case report in 1938 (Andersen, 1958). However, even as early as the Middle Ages, a childhood disorder characterized by salty sweat and early death was described (Rudolph & Rudolph, 2002). In 1953, physicians discovered that patients with cystic fibrosis produced sodium and chloride concentrations in sweat that were 2-4 times greater than values found in patients with a variety of other conditions (Di Sant'Agnes, Darling, Perera, & Shea, 1953). The high concentrations of sodium and chloride in the sweat of patients with CF was subsequently found to be due to poor reabsorption of sodium chloride in the sweat duct (Quinton, 1983).

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was first characterized in 1989. The CFTR gene is located on the long arm of chromosome 7 (7q.31.2) and spans approximately 250,000 base pairs (Rommens et al., 1989). Defective CFTR alleles contain a variety of mutations (B. Kerem et al., 1989). DeltaF508, by far the most common mutation present in 70% of CF patients, was discovered during the initial sequencing of the gene (Riordan et al., 1989). The deltaF508 mutation is a three-

nucleotide deletion in the tenth exon of the CFTR gene that causes the loss of a single phenylalanine residue within a protein of 1480 amino acids.

Experiments correcting defective chloride transport led to the discovery that mutations in the CFTR gene were responsible for the phenotypic defect (Rich et al., 1990). The CFTR gene undergoes transcription into mRNA and then translation into protein in the endoplasmic reticulum. The CFTR protein is then glycosylated in the Golgi apparatus and folded into the proper conformation that allows it to assume the appropriate place in the apical membranes of epithelial cells (Rudolph & Rudolph, 2002). The CFTR protein functions as a chloride channel, which is regulated by cyclic AMP, and the interaction of two CFTR nucleotide binding domains is crucial to the protein's viability (Anderson et al., 1991). The CFTR protein not only conducts chloride, but it also regulates other chloride secretory pathways. The various mutations in the CFTR gene affect the protein's function in different manners causing varied effects on the protein's ability to both conduct and regulate conduction of chloride ions (Devidas & Guggino, 1997). For instance, the phenylalanine deletion at position F508 results in improper folding of the nucleotide binding domains of CFTR. The altered protein is retained within the endoplasmic reticulum and is targeted for degradation (Ko & Pedersen, 1997). However, research has shown that it is possible for a mutant protein to assume a functional conformation when conditions are altered appropriately in vitro (Qu, Strickland, & Thomas, 1997).

As of October 2008, 1,604 mutations were implicated in cystic fibrosis, and the most common types of defects were missense (42%) and frameshift (16%) mutations

(www.genet.sickkids.on.ca/cftr/app). As mentioned previously, the single most common mutation found on 70% of CF chromosomes is deltaF508, and that mutation has a high degree of association with increased symptoms leading to greater disease severity (Riordan et al., 1989). Researchers have grouped the various mutations into classes that describe how the mutations affect the presence of the CFTR protein in the apical membranes of epithelial cells, and these classes (Table 1) are also generally associated with disease severity (Pilewski & Frizzell, 1999; Zielenski, 2000).

Table 1.

CFTR Mutation Classes and Their Effect on the CFTR Protein and Disease Severity

Mutation class	Effect on CFTR protein	Disease phenotype
Class I	Defective protein synthesis due to translation failure	Severe
Class II	Premature degradation of CFTR by proteases in the endoplasmic reticulum	Severe
Class III	CFTR protein is nonfunctional	Severe
Class IV	Normal amount of CFTR protein with residual function	Mild pancreatic insufficiency
Class V	Reduced amount of functional CFTR present at the cell membrane	Mild
Class VI	Functional but unstable version of CFTR	Severe

CFTR genotypes, which include two copies of a defective CFTR gene, can provide physicians with valuable information on expected disease severity such as exocrine pancreatic function (Kristidis et al., 1992). Genotype alone does not predict severity of pulmonary disease among patients with cystic fibrosis due to the variability within groups of patients with the same genotype (The Cystic Fibrosis Genotype-Phenotype Consortium, 1993). However, it is still believed that heritability factors

beyond the CF genotype may play a significant role in pulmonary disease severity based on comparisons of sibling data (Vanscoy et al., 2007).

Cystic fibrosis is generally characterized by copious mucus secretions present on mucosal surfaces, especially the gastrointestinal and respiratory tracts, leading to the classic CF phenotype of chronic obstructive lung disease, exocrine pancreatic insufficiency, and elevated sweat chloride concentrations (Zielenski, 2000). In addition, infertility is extremely common in patients with CF due to thick cervical mucus in women and congenital absence of the vas deferens leading to obstructive azoospermia in men (Jequier, Ansell, & Bullimore, 1985). In 10-20% of patients with CF, thickened intestinal mucus and pancreatic insufficiency can result in bowel obstruction at birth, termed meconium ileus. Distal intestinal obstruction syndrome occurs later in life for 20-25% of patients with CF (Rudolph & Rudolph, 2002). Pulmonary disease is worsened by the bacterial colonization of the lungs. Inhaled bacteria are trapped and cleared more slowly due to the reduced water content in the mucociliary secretions (Davis, 2006). Additional complications for patients with cystic fibrosis include CF-related diabetes, bone disease, liver disease, and nasal polyps that often require surgery (Patient Registry 2006 Annual Report, 2008).

Patients with cystic fibrosis have seen major improvements in life expectancy during the past 40 years. In 1962, a patient diagnosed with CF was only expected to live an average of 10 years (www.cff.org). In the most recent Patient Registry Annual Report, the Cystic Fibrosis Foundation stated that the median survival age for a patient with CF was 37.4 years of age in 2008 (Patient Registry 2008 Annual Report, 2009). Interestingly,

a study by Kulich in 2003 found that the greatest improvement in life expectancy was seen for patients between 2 and 15 years of age, and while both genders experienced extended life expectancies, female patients continued to have poorer survival rates than male patients in the category of 2 to 20 years of age (Kulich, Rosenfeld, Goss, & Wilmott, 2003).

Diagnostic Testing for Cystic Fibrosis

In the United States, the median age of diagnosis for patients with cystic fibrosis is 5.3 months. Patients who have undergone newborn screening are diagnosed at a median age of 15 days, patients with meconium ileus are diagnosed at a median age of 6 days, and patients with other symptoms are diagnosed at a median age of 14.5 months. Notably, patients diagnosed after the onset of symptoms as opposed to newborn screening results are at a greater than 2-fold risk of medical complications before diagnosis (Accurso, Sontag, & Wagener, 2005).

Newborn screening is generally performed by a biochemical test for immunoreactive trypsinogen in plasma collected at birth, and the initial screen is followed by repeat biochemical testing and CF mutation detection (Davidson, Wong, Kirby, & Applegarth, 1984). Localities in the United Kingdom have been performing newborn screening for CF since the mid-1980s, and Australia and France both have comprehensive national neonatal screening programs (Brice, Jarrett, & Mugford, 2007). The United States has recently mandated nation-wide newborn screening for CF, and in 2010, all 50 states now offer newborn screening for cystic fibrosis (www.cff.org). Newborn screening enables much earlier diagnosis of cystic fibrosis when compared to

symptomatic presentation. This early diagnosis occurs approximately one year earlier in life and allows for earlier nutritional treatments leading to improved growth, improved cognitive development, and a reduction in the number of days hospitalized (Grosse et al., 2004).

Beyond newborn screening, several other diagnostic approaches for cystic fibrosis include the sweat test, measuring nasal potential difference, and mutational studies of the CFTR gene. Various testing algorithms are based on initial clinical indications and require a combination of methods for the final diagnosis of cystic fibrosis (De Boeck et al., 2006).

The gold standard for the diagnosis of cystic fibrosis remains the sweat test. Sweating is induced by pilocarpine iontophoresis in which a colorless, odorless compound (pilocarpine) is placed on the skin, and a small electric current is applied. The sweat is collected onto preweighed gauze or filter paper that is then reweighed, and the chloride is eluted into an exact volume of deionized water (L. E. Gibson & Cooke, 1959). A volume of 50-100 μL of sweat is adequate for the sweat chloride concentration to be measured. Macroduct collection systems are also available and allow the sweat to be collected directly from the skin into a capillary tube, following the pilocarpine iontophoresis procedure (Hammond, Turcios, & Gibson, 1994). Sweat chloride concentrations less than 40 mmol/L essentially rule out CF, concentrations between 40-60 mmol/L are considered borderline, and concentrations greater than 60 mmol/L are considered consistent with a diagnosis of CF (De Boeck et al., 2006). Prior research has found that 98% of patients with cystic fibrosis have sweat chloride concentrations greater

than 60 mmol/L (Koch & Hoiby, 2000). Another study found that 23% of patients with intermediate sweat chloride levels (30-60 mmol/L) contained known mutations in both CFTR alleles (Lebecque et al., 2002).

Another tool for the diagnosis of cystic fibrosis is measurement of nasal potential difference. The characteristically increased chloride secretion and elevated sodium reabsorption causes abnormal nasal potential differences across apical cell membranes in patients with cystic fibrosis, compared with healthy individuals and patients with a variety of other disorders (Knowles, Gatzky, & Boucher, 1981). Nasal potential difference is generally used as an additional method of confirmation when sweat tests and mutation studies are inconclusive.

Mutation studies for cystic fibrosis are important in the diagnosis of the disease, but genetic tests are also used for prenatal screening of either the parents or the fetus in utero. In 2001, a joint effort between the American College of Medical Genetics, the American College of Obstetricians and Gynecologists, and the National Institutes of Health produced recommendations for population-based cystic fibrosis carrier screening (Grody et al., 2001). The recommendations stated that testing should be offered to all non-Jewish Caucasians and Ashkenazi Jews as well as all other ethnic groups. The standard screening panel detailed in the document included 25 mutations, all with an allele frequency greater than 0.1% in the US population. In 2004, the recommended panel was revised slightly, removing two mutations (Watson et al., 2004). The first mutation removed, 1078delT, was found to be present in only 0.03% of the US population, and the second mutation removed, I148T, was determined incapable of causing classical CF as a

single defect. The updated recommendations also noted six additional mutations not listed in the panel that were found at an allele frequency of 0.1-0.17%, but additions to the revised 23 mutation panel for cystic fibrosis screening were not recommended.

Various molecular testing platforms for cystic fibrosis are available, and the vast majority of the assays employ multiplex PCR targeting of the CFTR gene. All assays target a minimum of 23 recommended mutations, but several panels include additional mutations to increase detection in ethnic groups other than Caucasians. Detection technologies include but are not limited to oligonucleotide ligation assays, line probe assays, liquid bead arrays, and microarrays (Johnson, Yoshitomi, & Richards, 2007; Krafft & Lichy, 2005). Additionally, laboratories offer complete sequencing of the CFTR gene and may detect up to 98% of mutations for all ethnic groups (www.genzyme genetics.com).

Treatment of Cystic Fibrosis

The Cystic Fibrosis Foundation recommends that patients visit their physician at least four times per year in clinics specializing in treatment of CF patients. In addition, pulmonary function tests should be performed two times or more per year. At least one respiratory culture should be collected per year (although many clinics collect respiratory culture specimens at each visit), and creatinine levels, glucose levels (if older than 14 years of age), and quantitative liver enzymes should be tested at least once per year (Patient Registry 2008 Annual Report, 2009). Research has shown that patients with cystic fibrosis attending dedicated CF centers had better clinical outcomes such as higher body mass index and better lung function than those receiving care at other types of

centers (Mahadeva et al., 1998). In caring for patients with CF, home-based medications account for the greatest proportion (47%) of the total cost of care (Baumann, Stocklossa, Greiner, von der Schulenburg, & von der Hardt, 2003). Healthcare costs rise with age, and chronic infection with *Pseudomonas aeruginosa*, which will be discussed in greater detail later, is associated with healthcare costs three times higher than that of uninfected patients with CF.

Malnutrition is common in patients with cystic fibrosis, primarily due to chronic pancreatic insufficiency (Bines, Truby, Armstrong, Phelan, & Grimwood, 2002). Nearly all patients with CF require pancreatic enzyme supplements with meals to prevent nutritional deficiencies (Koch & Hoiby, 2000). Supplementation of fat-soluble vitamins A, D, E, and K is also recommended due to fat malabsorption (Davis, 2006). Nutritional supplements such as additional vitamins, pancreatic enzymes, and calorie-dense dietary additions are crucial to the treatment of patients with CF due to the documented relationship between nutritional status and pulmonary function (Milla, 2007).

In 2008, 3.8% of patients with cystic fibrosis 6-12 years of age were on ibuprofen therapy (Patient Registry 2008 Annual Report, 2009). Konstan, et al., reported that children with mild lung disease treated with ibuprofen for an average of four years had a 29% less decline in lung function than those not treated with ibuprofen (Konstan, Schluchter, Xue, & Davis, 2007). The addition of anti-inflammatory therapy to a thorough treatment program may decrease morbidity and improve quality of life for young patients with mild lung disease (Chmiel & Konstan, 2007).

Respiratory therapy for patients with cystic fibrosis includes airway clearance techniques and aerosolized medication delivery. Most patients use a combination of airway clearance techniques including exercise, positive expiratory pressure, coughing, and active cycle breathing exercises among others. While there is acceptable adherence to airway clearance schedules by patients with CF, the most common reasons for non-adherence were being too busy and too tired (White, Stiller, & Haensel, 2007). The cleaning of nebulizers and airway clearance devices is especially important and is generally performed by either boiling, soaking in bleach/rubbing alcohol/hydrogen peroxide, microwaving, or placing in the dishwasher (Reychler et al., 2005).

Hypertonic saline and Pulmozyme[®] are important inhaled treatments for patients with cystic fibrosis. By restoring hydration to the airway epithelium, inhaled hypertonic saline allows increased airway clearance and improves lung function (Elkins et al., 2006). Pulmozyme[®], or dornase alfa, is a recombinant human DNase that thins mucus by hydrolyzing the DNA released from neutrophils (Shak, Capon, Hellmiss, Marsters, & Baker, 1990). The aerosolized drug is used by 76.1% of patients with cystic fibrosis who are older than six years of age and have mild to severe lung disease (Patient Registry 2008 Annual Report, 2009). Pulmozyme[®] was the first drug developed specifically for cystic fibrosis, and its use is associated with a slower rate of decline in lung function (Konstan, 2008).

There are varying antibiotic treatment regimens including continuous treatment, on-demand treatment, and early aggressive treatment (Koch & Hoiby, 2000). Continuous treatment risks the development of antibiotic resistance, and on-demand treatment could

possibly lead to under treatment. Antibiotic treatment in cystic fibrosis is commonly directed specifically at infection with *Pseudomonas aeruginosa*. A multi-year study at a Danish CF center evaluated an early anti-*Pseudomonas aeruginosa* treatment regimen. Initial results showed that the treatment resulted in improved survival for their patients (Frederiksen, Lanng, Koch, & Hoiby, 1996). Continued evaluation of the treatment at their center found that those treated with high-dose colistin by inhalation and oral ciprofloxacin showed the best results in delayed onset or prevention of chronic *P. aeruginosa* infection (Frederiksen, Koch, & Hoiby, 1997). The treatment plan from the Danish study also resulted in the maintenance of increased pulmonary function after initiation of treatment. The groups credited cohort isolation based on *P. aeruginosa* infection status for reducing the number of patients chronically infected with *P. aeruginosa* (Frederiksen, Koch, & Hoiby, 1999).

The early and aggressive treatment protocol has been adopted and modified by many cystic fibrosis centers. For instance, one center describes an increasingly aggressive treatment protocol each time *P. aeruginosa* is isolated (Koch, 2002). The treatment plan begins with three weeks of inhaled colistin upon the first isolation of *P. aeruginosa*. A second positive *P. aeruginosa* culture justifies three more weeks of colistin with double the usual dose. Treatment for a third positive culture supports doubling the dosage of colistin for three months, and all treatment plans for repeated positive cultures also include oral ciprofloxacin therapy.

With acute *P. aeruginosa* infections, early antibiotic therapy can eliminate *P. aeruginosa* for a short period of time (approximately 18 months by one study) and thus

delay lung function decline (Taccetti, Campana, Festini, Mascherini, & Doring, 2005). In contrast, chronic infections with *P. aeruginosa* are difficult to eradicate (Doring et al., 2000).

With continued advancements in antibiotics, azithromycin has become the drug of choice for treating *P. aeruginosa* in patients with cystic fibrosis. In 2008, a total of 65.6% of patients with cystic fibrosis who were older than six years of age, positive for *P. aeruginosa* infection, had moderate to severe lung disease, and weighed more than 55 pounds were on azithromycin therapy (Patient Registry 2008 Annual Report, 2009). Initial trials with azithromycin found that treatment reduced the rate of decline in lung function, reduced the number of respiratory exacerbations, and improved nutritional status and quality of life (Wolter et al., 2002). In 2003, the Cystic Fibrosis Foundation recommended administration of azithromycin for patients with cystic fibrosis greater than six years of age and chronically infected with *P. aeruginosa* (Saiman et al., 2003). Interestingly, while azithromycin resulted in clinical improvement, a significant reduction in bacterial density was not observed; and this phenomenon suggests that the antimicrobial properties of azithromycin are the reason for the clinical response (Nguyen et al., 2007). More recent studies also suggest that while improvements in pulmonary function were seen in the first year of azithromycin treatment, pulmonary function declined with longer courses of therapy (Tramper-Stranders, Wolfs, Fler, Kimpen, & van der Ent, 2007).

Inhaled forms of antibiotics have shown the most recent promise for the treatment of bacterial infections in patients with cystic fibrosis. Tobramycin solution for inhalation

(TOBI) was used by 67.4% of patients with cystic fibrosis in 2008 who were older than six years of age, positive for *P. aeruginosa*, and had moderate to severe lung disease (Patient Registry 2008 Annual Report, 2009). Although TOBI use is recommended for patients with cystic fibrosis, concerns persist regarding the emergence of resistant organisms with use of the drug (Hagerman, Knechtel, & Klepser, 2007). Cheer, et al. reported a decline in the susceptibility of *P. aeruginosa* infections treated with TOBI, but this reduced susceptibility did not cause adverse outcomes in the patients with CF (Cheer, Waugh, & Noble, 2003). The most recently approved inhaled antibiotic is aztreonam, which was FDA-approved in 2010. Aztreonam treatment has been combined with the new eFlow electronic nebulizer delivery system that is capable of providing 1 mL of the drug in two to three minutes (R. L. Gibson et al., 2006). This new treatment option provides concentrations of aztreonam in the sputum of patients with CF that exceed the concentrations necessary to inhibit bacterial growth of *P. aeruginosa*.

The selection of appropriate antibiotic treatment for patients with cystic fibrosis is based on susceptibility patterns of the bacterial organisms (Doring et al., 2000). Recently, concerns have surfaced that antibiotic combinations effective in vitro will not be as effective in the lungs of patients with CF, especially in the presence of biofilms (Hill et al., 2005). In addition, the selective stress of antibiotic treatment has been shown to increase the rate of organisms that are resistant to multiple antibiotics (Alonso, Campanario, & Martinez, 1999). The clinical significance of multiply-resistant *P. aeruginosa* and *Burkholderia cepacia* is well established, but the significance of other emerging multiply-resistant organisms such as methicillin-resistant *Staphylococcus*

aureus (MRSA) and *Stenotrophomonas maltophilia* is still unknown (Waters & Ratjen, 2006).

Once all medication options have been exhausted and lung function reaches a critical level, lung transplants must be considered for patients with cystic fibrosis. In 2008, a total of 157 patients with cystic fibrosis received lung transplants (Patient Registry 2008 Annual Report, 2009). Referral criteria for lung transplants in patients with cystic fibrosis includes severe decline in lung function, life-threatening pulmonary complications, and increasing antibiotic resistance of bacterial pathogens infecting the lungs (Yankaskas & Mallory, 1998). Infection with *Burkholderia cenocepacia*, pan-resistant *Burkholderia multivorans*, or pan-resistant *Burkholderia vietnamiensis* is considered a contraindication to lung transplantation in patients with cystic fibrosis in the United States due to poor survival rates post-surgery for patients infected with the pathogen (Chaparro et al., 2001). However, pulmonary infection with multiply-resistant *Pseudomonas aeruginosa* is not a contraindication for lung transplants in these patients (Goldberg & Deykin, 2007).

Beyond the conventional treatment plans to alleviate symptoms and improve quality of life, a cure for cystic fibrosis remains elusive. As in many genetic diseases, gene therapy holds promise of providing a cure. Gene therapy targeted at cystic fibrosis began with the correction of CFTR-dependent chloride transport by the use of nonviral vectors in transgenic mice with cystic fibrosis (Hyde et al., 1993). Several routes of administration of gene therapy have been evaluated since the initial studies. One study utilized compacted DNA nanoparticles instilled into the nose. This approach to gene

therapy did seem to provide partial to complete chloride transport correction in 8 of the 12 patients involved in the study (Konstan et al., 2004). Adeno-associated virus transmitted via nasal installation or bronchoscopy has also been explored for delivery of gene therapy; however, chloride transport correction was only observed in a few patients (Flotte, Schwiebert, Zeitlin, Carter, & Guggino, 2005). Human clinical trials have included recombinant adenovirus, recombinant adeno-associated virus, cationic liposomes, and condensed naked DNA. So far, human clinical trials have not revealed a single successful potential strategy for gene therapy in cystic fibrosis, but the possibility of using embryonic or adult stem cells is also being explored (Sueblinvong, Suratt, & Weiss, 2007).

Predictors of Outcome in Cystic Fibrosis

Several parameters have been used to predict patient outcomes in cystic fibrosis. Interestingly, the presence of meconium ileus at birth was not found to be a significant predictor of survival (Munck et al., 2006). However, genotypic determinations at birth were found to be predictive of disease phenotype. The various mutation groups mentioned earlier have been associated with different disease severities. Studies have found significant differences in survival and median age of death between high-risk and low-risk CF genotype classes (McKone, Goss, & Aitken, 2006). Even within genotypes that include deltaF508, mortality rates and disease severity differ for patients that are homozygous and heterozygous for the mutation (Johansen, Nir, Hoiby, Koch, & Schwartz, 1991).

Body-mass index (BMI) is a factor closely documented by physicians to monitor patients with cystic fibrosis. The standards set by the Cystic Fibrosis Foundation state that BMI is a percentile of weight and height compared to healthy children of the same age and gender (Patient Registry 2008 Annual Report, 2009). Because BMI conveys valuable information regarding nutritional status and overall patient health and prognosis, several studies have identified BMI as an important predictor of survival in patients with CF (Durie & Pencharz, 1992; Kraemer, Rudeberg, Hadorn, & Rossi, 1978). The CFF states that patients with CF should exhibit growth consistent with age-matched individuals who do not have the disease (Patient Registry 2008 Annual Report, 2009).

Another important measure in determining the health of a patient with cystic fibrosis is forced expiratory volume (FEV₁). FEV₁ is “the volume of air that can be forced out in one second after taking a deep breath” (Merriam-Webster's New World Medical Dictionary, 2003). FEV₁ can be expressed as volume in liters or as a percent predicted based on healthy, non-smoking people of the same age and gender (Patient Registry 2008 Annual Report, 2009). FEV₁ has also been deemed a significant predictor of survival, and one study reported that patients with a value less than 30% forced expiratory volume in one second had a 50% chance of dying within two years (Corey, Edwards, Levison, & Knowles, 1997; Kerem, Reisman, Corey, Canny, & Levison, 1992).

Pulmonary infection with bacterial pathogens is another significant factor in the health and survival of patients with cystic fibrosis. Variants of *Staphylococcus aureus* have been associated with more advanced lung disease (Besier et al., 2007). However, *Pseudomonas aeruginosa* is the most significant respiratory pathogen in patients with CF,

and infection with multiply-resistant *P. aeruginosa* is associated with a significant decrease in FEV₁ (Lambiase et al., 2006). Relative to demographic information, a mother's educational level has been identified as a potential risk factor for the acquisition of *P. aeruginosa* (Kosorok et al., 1998; Watts, Seshadri, Sullivan, & McColley, 2009). Interestingly, patients with a late diagnosis of cystic fibrosis (at 24 years of age or later) were less likely to be infected with *P. aeruginosa* than those diagnosed at an earlier age (before 15 years of age) (Rodman et al., 2005). Another study suggests that pulmonary infection with *P. aeruginosa* or *Burkholderia cepacia* may have a greater effect on survival than genotype (Courtney et al., 2007). When looking exclusively at *P. aeruginosa*, patients infected with a multiply-resistant strain rather than a susceptible strain were 14 times more likely to either die or need a lung transplant (Lechtzin et al., 2006).

Infection Control in Cystic Fibrosis

A study in the cystic fibrosis centers of Germany found that physicians have differing opinions on the risk of bacterial acquisition, specifically *P. aeruginosa*, from the environment and suggested that strict precautions created a greater level of fear for the patient and their family (Steinkamp & Ullrich, 2003). Cystic fibrosis centers in the United States had a different view, and in 2003, the Cystic Fibrosis Foundation published a consensus statement regarding infection control in cystic fibrosis (Saiman & Siegel, 2003). The document covered isolation procedures for patients with CF and also recommended the use of molecular typing for surveillance of transmission of infections. Consensus committee members made further recommendations in 2004 and noted the

importance of monitoring infection control practices as well as tracking details on new infections (Saiman & Siegel, 2004). The recommendations stated that patients with cystic fibrosis should be instructed on containment of secretions and should maintain a distance of at least three feet from other patients with CF regardless of respiratory infection status. Cystic fibrosis centers were also instructed to standardize protocols for the cleaning of equipment used in respiratory therapies and to educate patients and families on proper care and disinfection of home use equipment. A significant proportion of home nebulizers were contaminated with *P. aeruginosa* highlighting the importance of infection control practices (Rosenfeld, Joy, Nguyen, Krzewinski, & Burns, 2001).

Isolation of hospitalized patients with cystic fibrosis infected with *P. aeruginosa* is commonly accepted due to the many studies suggesting transmission between patients. One review of the literature, found that 31 of 39 studies showed evidence of patient-to-patient spread of *P. aeruginosa* in patients with cystic fibrosis (Vonberg & Gastmeier, 2005). Multiply-resistant strains of *P. aeruginosa* are assumed to be even more transmissible, so segregation is critical for those patients (Davies, McShane, Davies, & Bush, 2003). Multiple studies have suggested that molecular epidemiologic surveillance is needed to determine if improved infection control practices limit the spread of *P. aeruginosa* between patients with cystic fibrosis (D. Armstrong et al., 2003; O'Carroll et al., 2004; Pitt, 2002; Ramsey, 2002).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a fastidious organism that prefers moist environments and surfaces. When placed on filter paper or freeze dried, *P. aeruginosa* has a survival

rate of 10-150 days. However when placed in water at 4-37°C, *P. aeruginosa* was found to survive for more than 300 days (Emmanouilidou-Arseni & Koumentakou, 1964).

Because of this extended survival, *P. aeruginosa* is a significant factor in device-associated nosocomial infections involving urinary catheters, central venous catheters, and ventilators (Pierce, 2005). It is believed that bacterial biofilms may coat these devices and account for persistent contamination.

Beyond cystic fibrosis, *P. aeruginosa* is an important pathogen in other disease states such as immunocompromised patients with cancer or HIV infection. *P. aeruginosa* is a primary consideration for patients with leukemia and in neutropenic patients (Funada & Matsuda, 1998). The frequency of *P. aeruginosa* infections in patients with solid tumors has decreased but has remained constant for patients with acute leukemia (Chatzinikolaou et al., 2000). In addition, hospitalized patients with HIV-1 infection are susceptible to community-acquired and nosocomial bacterial pneumonia commonly caused by *P. aeruginosa* (Afessa & Green, 2000).

Pseudomonas aeruginosa in Cystic Fibrosis

The Cystic Fibrosis Foundation Patient Registry Report noted that 52.5% of patients with cystic fibrosis were infected with *Pseudomonas aeruginosa* in 2008 (Patient Registry 2008 Annual Report, 2009). Earlier studies hoped to discover risk factors for the acquisition of *P. aeruginosa*. The presence of meconium ileus at birth was once found to be a significant risk factor, but advances in infection control and surgical treatment have lessened this risk (E. Kerem et al., 1989). Clinic exposures and use of aerosol medications and treatments have also been proven to increase the risk of acquisition of *P.*

aeruginosa. Interestingly, the level of education of the patient's mother was found to decrease the risk of acquiring *P. aeruginosa* presumably due to relative abilities to clean and handle respiratory therapy equipment (Kosorok et al., 1998).

Evidenced by whole genome analysis, *P. aeruginosa* genetically adapts to the airways of patients with cystic fibrosis. Genetic properties differed greatly between the *P. aeruginosa* strains that initiated the infection and the strains present late in *P. aeruginosa* infections of cystic fibrosis airways (Smith et al., 2006). *P. aeruginosa* present in acute or early infection stages have a non-mucoid appearance, are pan-sensitive to antibiotics, and are present at low densities (Rosenfeld, Ramsey, & Gibson, 2003). Several studies have shown that a window of opportunity may be present in which early intervention may allow eradication of *P. aeruginosa* from upper and lower airways (Treggiari, Rosenfeld, Retsch-Bogart, Gibson, & Ramsey, 2007). However, selective pressure has been shown to promote the transformation of non-mucoid strains into mucoid phenotypes resulting in chronic *P. aeruginosa* infections.

P. aeruginosa is observed as a biofilm in the sputum of patients with cystic fibrosis (P. K. Singh et al., 2000). In *P. aeruginosa* infections of the airways of patients with cystic fibrosis, the mucus is targeted rather than the epithelial cell surface compartment (Worlitzsch et al., 2002). The mucoid morphology associated with chronic infection is caused by the overproduction of extracellular polysaccharide alginate (Driscoll, Brody, & Kollef, 2007). Alginate production facilitates the establishment of microcolonies in biofilms, which protect pathogens from host defense mechanisms and antimicrobial agents (Koch, 2002). Mucus clearance is part of a healthy respiratory

system's defense against infection, but patients with CF are unable to clear the thick mucus characteristic of their disease leading to chronic lung infections (Worlitzsch et al., 2002). Chronic infection with *P. aeruginosa* leads to decline in pulmonary function and ultimately death in patients with cystic fibrosis (Lyczak, Cannon, & Pier, 2002).

The initial definition of chronic *P. aeruginosa* infection included the presence of this pathogen in the sputa of the patients for a period of six months (Hoiby, 1974). In 2003, the Leeds criteria proposed revised definitions for the various stages of *P. aeruginosa* infection (Lee, Brownlee, Conway, Denton, & Littlewood, 2003). "Chronic infection" was defined as positive *P. aeruginosa* cultures during more than 50% of the months when samples were obtained. "Intermittent infection" was defined as a positive *P. aeruginosa* culture during 50% or less of the months when samples were obtained, and "free of infection" was defined as no growth of *P. aeruginosa* in the previous 12 months for patients with a prior history of *Pseudomonas* infection. More recent studies have upheld the Leeds criteria and the definitions of patterns of *P. aeruginosa* infection (Proesmans et al., 2006).

Clinical laboratories employ various techniques for the identification of *P. aeruginosa* in respiratory samples of patients with cystic fibrosis. Serological tests can detect specific antigens implicated in the diagnosis of chronic *P. aeruginosa* colonization, but the test has failed to detect early colonization in young patients (Tramper-Stranders et al., 2006). The identification method recommended by the Cystic Fibrosis Foundation utilizes culture paired with agar diffusion assays for susceptibility testing of *P.*

aeruginosa, but a recent study showed that only 52% of laboratories in the United States use this methodology (Zhou, Garber, Desai, & Saiman, 2006).

In recent years, the identification of *P. aeruginosa* has been aided by new molecular assays. The discovery of a nucleotide sequence present in all isolates of the Liverpool epidemic strain led to a PCR assay capable of identifying that particular strain in colonies or directly from sputum (Parsons et al., 2002). Another laboratory developed an assay using temperature gradient gel electrophoresis paired with pyrosequencing to identify *P. aeruginosa* in respiratory samples of patients with cystic fibrosis (Kolak, Karpati, Monstein, & Jonasson, 2003). *P. aeruginosa* isolates as well as other atypical nonfermenting gram-negative bacilli in patients with cystic fibrosis are known to have atypical morphologies and metabolic properties, and these organisms often require further testing for confirmation (Ferroni et al., 2002). One laboratory designed a PCR assay to specifically identify difficult and non-typical *P. aeruginosa* in the sputa of patients with cystic fibrosis (Spilker, Coenye, Vandamme, & LiPuma, 2004). At Texas Children's Hospital, a pyrosequencing assay was implemented to identify any organism that proved difficult to definitively speciate by conventional microbiological methods. Many of the organisms that were referred for this testing were found to be *P. aeruginosa* isolates from patients with cystic fibrosis that did not perform as expected by culture and both manual and automated biochemical testing methods (Luna et al., 2007).

Infection with *P. aeruginosa* has a profound effect on the lives of patients with cystic fibrosis. On the emotional front, recent colonization with *P. aeruginosa* has been associated with a lower quality of life (Goldbeck, Zerrer, & Schmitz, 2007). *P.*

aeruginosa infections are also major predictors of morbidity and mortality. The clinical outcomes of patients positive for *P. aeruginosa* respiratory infections are significantly poorer due to lower lung capacity (FEV₁), lower weight percentiles, and higher rates of hospitalization. Most troublesome is the fact that the eight-year death risk is 2.6 times higher for patients with *P. aeruginosa* isolated from respiratory cultures than for those without *P. aeruginosa* in respiratory cultures (Emerson, Rosenfeld, McNamara, Ramsey, & Gibson, 2002).

The largest complication with *P. aeruginosa* is the emergence of multiply-resistant strains, or strains that are resistant to multiple antibiotics. Various publications attempt to define multiply-resistant *P. aeruginosa*, but the most widely accepted definition is provided by the Cystic Fibrosis Foundation (CFF) and has been adopted by CF centers throughout the United States (Falagas et al., 2006). According to the CFF, a *P. aeruginosa* isolate must be resistant to all antibiotics in at least two of three groups (aminoglycosides, fluoroquinolones, and beta-lactams) to be considered multiply-resistant. Studies have shown that treatment with certain antibiotics can affect the emergence of antibiotic resistant strains of *P. aeruginosa*. One study found that ceftazidime treatment yielded the lowest risk and imipenem yielded the highest risk of emergence of antibiotic-resistant *P. aeruginosa* (Carmeli, Troillet, Eliopoulos, & Samore, 1999). Hypermutable strains also contribute to the development of antibiotic resistance, and one study suggested that early and aggressive antibiotic treatment combined with antioxidants could prevent mutation of the strains. The study claimed that strain typing by pulsed field gel electrophoresis confirmed this hypothesis by showing patients had the

same strain throughout the chronic lung infection phase (Ciofu, Riis, Pressler, Poulsen, & Hoiby, 2005).

Molecular Typing Technology and Clinical Applications

While a variety of techniques for molecular typing exist, the goal of each methodology is to determine if isolates of a specific species are clonally related. A clonal relationship would suggest that they originated from the same bacterial cell and could indicate a lateral or point source of transmission. The discriminatory power of a technique describes the technique's ability to differentiate unrelated isolates (A. Singh, Goering, Simjee, Foley, & Zervos, 2006). Based on a review of the literature, the most commonly employed techniques for molecular typing include restriction fragment length polymorphism (RFLP), ribotyping, random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), arbitrarily-primed PCR (AP-PCR), and repetitive element PCR (rep-PCR).

Various factors of each technique determined their acceptability for clinical use. Lack of reproducibility has hampered clinical application of AP-PCR and RAPD (Struelens, 1998; Wu & Della-Latta, 2002). RFLP was too labor intensive for daily clinical use, and ribotyping only provided moderate discriminatory power compared to the other techniques (Struelens, 1998). PFGE was considered the gold standard for many years, but there are several factors that make this technology less than ideal for the clinical laboratory. PFGE requires a two to four day turnaround time, is costly to perform, is labor-intensive, and requires specialized expensive equipment (Struelens, 1998; Wu & Della-Latta, 2002). In contrast, the major complaint of rep-PCR was that the

technology lacked standardization (Wu & Della-Latta, 2002) while others suggested the improved reproducibility of the technology would lend itself to standardization as a library typing system (Struelens, 1998). The benefit is that rep-PCR is the most rapid of all the technologies currently available for molecular typing (Kang & Dunne, 2003). The issues with standardization would later be remedied by the introduction of the DiversiLab system (Healy et al., 2005). The DiversiLab system offered several improvements to the rep-PCR process including optimized PCR chemistry provided in a reagent kit format, microfluidics-based DNA amplicon detection, and internet-based computerized analysis and data storage.

When reviewing studies in molecular typing, the definitions of clones and strains become important. Clones are defined as genetically related isolates that are indistinguishable or so similar to each other that they are presumed to descend from a common parent, and a strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species (Tenover et al., 1995).

Indistinguishable or closely related isolates should have no more than a two to three band difference (absence or presence of a band), isolates that are possibly related would have a four to six band difference, and those isolates that are clearly different, considered distinguishable, would have a seven or greater band difference (Tenover et al., 1995).

Pulsed-field gel electrophoresis is performed by digestion of chromosomal DNA with rare-cutting restriction enzymes and analysis of the resulting series of fragments by agarose gel electrophoresis (A. Singh et al., 2006). The “pulsed-field” is obtained by constantly changing the direction of the electrical field during electrophoresis. PFGE has

been used in clinical molecular typing studies in many institutions and has been instrumental in the discovery of outbreak situations. PFGE typing in a neonatal intensive care unit determined a clonal relationship between the strains of *Serratia marcescens* isolated from the patients and those found in the hand washing disinfectant used by the nursing staff (Villari, Crispino, Salvadori, & Scarcella, 2001). The outbreak was contained by isolating each of the patients infected with *S. marcescens* and by more diligent hand washing and glove use by the staff. PFGE was also shown to identify two endemic strains of methicillin-resistant *Staphylococcus aureus* (Petersdorf, Oberdorfer, & Wendt, 2006) and a cluster of *Enterobacter cloacae* associated with a high mortality rate (Kuboyama, de Oliveira, & Moretti-Branchini, 2003). In another example of molecular typing of antibiotic resistant organisms, PFGE identified a strain of vancomycin-resistant *Enterococcus faecium* that was present in 85% of the samples typed (Deplano et al., 2007). The implementation of weekly surveillance cultures and the increased use of barrier precautions were effective in controlling the spread of the strain in the hematology unit of the hospital.

While PFGE has long been considered the gold standard for molecular typing, improvements in rep-PCR have made the technology more appealing to advanced laboratories. Repetitive element PCR capitalizes on the widespread distribution of repetitive DNA elements throughout the genomes of many microorganisms (Versalovic, Koeuth, & Lupski, 1991). By positioning amplification primers in these repetitive regions, amplified fragments of various lengths and quantities are produced leading to a unique molecular fingerprint for each strain of a particular species. Rep-PCR has been

significantly improved by the introduction of bioMerieux's DiversiLab system. The complete DiversiLab system offers quality-controlled reagents in a kit format, microfluidics-based amplified fragment detection, and internet-based computer assisted analysis, reporting, and data storage (Healy et al., 2005). The level of discrimination and reproducibility of the technology paired with the electronic storage of data created an ideal environment for the archiving of rep-PCR data for comparative longitudinal and epidemiological studies.

Several studies have compared the discriminatory power of rep-PCR with other techniques for molecular typing. One study compared PFGE, rep-PCR, and AP-PCR for typing of *Bartonella henselae* and found that PFGE and rep-PCR had the highest discriminatory potential (Sander, Ruess, Bereswill, Schuppler, & Steinbrueckner, 1998). Another study compared rep-PCR and RFLP for the typing of *Pseudomonas* isolates from soil and concluded that rep-PCR performed just as well as RFLP (Cho & Tiedje, 2000). A comparison of rep-PCR, PFGE, and ribotyping in *Clostridium difficile* found a high correspondence between rep-PCR and PFGE results but lesser discrimination with the ribotyping results (Spigaglia & Mastrantonio, 2003).

Multiple investigators chose to perform a direct comparison between PFGE and rep-PCR. *Candida albicans* was a specific pathogen of interest, and rep-PCR was found to be comparable to PFGE (Chen, Lo, Lin, & Li, 2005). One study showed that the DiversiLab rep-PCR system was more rapid and conducive to ongoing epidemiologic studies in *Candida* species (Wise et al., 2007). The DiversiLab system was also evaluated for typing of *Staphylococcus aureus* strains, and the study found that rep-PCR provided

more consistent results as compared with PFGE and was also less labor intensive (Shutt, Pounder, Page, Schaecher, & Woods, 2005). Further studies confirmed the comparable discriminatory power and more rapid turnaround time of rep-PCR as compared to PFGE in reports of *Listeria monocytogenes* (Chou & Wang, 2006) and vancomycin-resistant *Enterococcus* (Pounder et al., 2005).

Rep-PCR is a proven technology for typing of various bacterial species. Published studies used rep-PCR to type *Acinetobacter baumannii* (Martin-Lozano et al., 2002) and *Vibrio vulnificus* (Chatzidaki-Livanis, Hubbard, Gordon, Harwood, & Wright, 2006) as well as several other bacteria isolated from the International Space Station (Castro, Thrasher, Healy, Ott, & Pierson, 2004). One study that separated *Listeria monocytogenes* isolates into four separate clusters suggested that molecular fingerprints be considered different if there was a discrepancy (presence or absence) of at least two bands (Jersek et al., 1999). A larger study that evaluated the relatedness of several organisms (*Acinetobacter sp.*, *Enterobacter sp.*, and coagulase-negative *Staphylococcus*) between an individual's hands and the immediate environment stated specific criteria for the identification of clusters (Pancholi et al., 2005). Interestingly, greater than 25% of the bacterial isolates found on the hands and the immediate environment of a single household were found to be the same strain. Different isolates were defined as those with less than 95% similarity and a 2-3 band difference. Similar isolates were described as those with less than 97% similarity but only 1-2 bands difference; and indistinguishable isolates were defined as those with greater than 95% similarity and no band differences including intensities. Overall profile intensity differences were acceptable because that

indicated a universal change throughout the whole rep-PCR profile most likely due to efficiency of the PCR rather than actual differences between the bacterial isolates.

In addition to bacterial typing, rep-PCR has been recommended as an effective tool for the typing of fungi (Healy et al., 2004) and dermatophytes (Pounder et al., 2005). Most importantly, rep-PCR has been proven for use in infection control investigations. Rep-PCR of vancomycin-resistant *Enterococcus* was instrumental in the control of an outbreak in the neonatal intensive care unit (NICU) in one hospital (N. Singh, Leger, Campbell, Short, & Campos, 2005). In another institution, rep-PCR epidemiologically linked a NICU outbreak of methicillin-resistant *Staphylococcus aureus* to a health care worker afflicted with chronic otitis externa and nasal colonization with a specific MRSA clone (Bertin et al., 2006).

A recent study compared PFGE to rep-PCR for typing of *P. aeruginosa*, including several isolates from patients with CF (Doleans-Jordheim et al., 2009). Rep-PCR was found to be comparable to PFGE, and the grouping of isolates was consistent when adhering to a 95% similarity cut-off to define a clonal group. The CF isolates included isolates from three families, and all isolates within each family were found to be identical whereas the molecular profiles between families were found to be unique.

Molecular Typing of *Burkholderia cepacia* in Cystic Fibrosis

Molecular typing of *Burkholderia cepacia* in patients with cystic fibrosis has been performed in several centers. An early study discovered epidemic strains in a Toronto CF center using RFLP and PFGE (Sun et al., 1995). RAPD analysis confirmed an epidemic strain among multiple patients with CF across the United Kingdom and eastern Canada

(Mahenthiralingam, Simpson, & Speert, 1997). Additional studies using RAPD and rep-PCR found cross-infection of the *B. cepacia* complex across several European cystic fibrosis centers (Coenye, Spilker, Van Schoor, LiPuma, & Vandamme, 2004); (McDowell et al., 2004). Similarly, a combination of rep-PCR and PFGE was utilized to confirm clusters of *B. cepacia* complex in several CF centers in the United States (Biddick, Spilker, Martin, & LiPuma, 2003).

Molecular Typing of *Pseudomonas aeruginosa*

One of the earliest methods used to type strains of *Pseudomonas aeruginosa* was pyocin typing. Pyocin is a bacteriocin, a toxin produced by bacteria that inhibits growth of similar or related species, produced by *P. aeruginosa* (Higerd, Baechler, & Berk, 1967). Pyocin typing was based on the level of pyocin activity in the isolate and while it performed better than serotyping, it proved much less accurate than results obtained with primitive molecular methods (Fyfe, Harris, & Govan, 1984). By the mid-1990s, molecular typing relied largely on PFGE technology. Comparison studies specific to the typing of *P. aeruginosa* showed PFGE to have better discriminatory power than RFLP (Grundmann, Schneider, Hartung, Daschner, & Pitt, 1995) while a separate study confirmed that rep-PCR had a level of discrimination equal to PFGE in the typing of *P. aeruginosa* (Lau et al., 1995). Utilizing microfluidics chips in conjunction with rep-PCR was suggested as the most rapid and automated method for typing of *P. aeruginosa* (Jamasbi, Kennel, Waters, Foote, & Ramsey, 2004).

PFGE of *P. aeruginosa* uncovered eight cases of cross-colonization in an intensive care unit (Bergmans et al., 1998) as well as a clonal infection in a NICU linked

to artificial nail colonization of a health care worker (Foca et al., 2000). Across four French intensive care units, carriage of or infection with *P. aeruginosa* was determined to be a result of transmission between patients 50% of the time based on PFGE analysis (Bertrand et al., 2001). The same study documented that patients who were carriers, colonized, or infected with *P. aeruginosa* had a significantly longer median stay in the ICU, 26.3 days versus 7.4 days. *P. aeruginosa* was also identified in several water faucets in health care institutions. PFGE led to the discovery of a common clone in an ICU, and infection control measures, including the replacement of water faucets and reiteration of hand-hygiene recommendations, were found to decrease the colonization rate (Petignat et al., 2006). Similarly, a cluster of multiply-resistant *P. aeruginosa* detected by AFLP typing was eliminated only when water taps were pasteurized weekly and sterile water was employed for use with gastric tubes (Bukholm, Tannaes, Kjelsberg, & Smith-Erichsen, 2002). The MRPA outbreak strain was also found to be significantly associated with patient mortality. A molecular typing project utilizing PFGE for the typing of MRPA found one genotype at four different hospitals in Rio de Janeiro (Pellegrino et al., 2002). The researchers suspected that inadequate infection control practices were the culprit behind the spread but also suggested that certain MRPA clones may be more equipped to spread among susceptible hosts in a health care setting. These clones may exhibit prolonged survival in the outside environment and could further adapt to the geographic region or ethnicity of the host.

Molecular Typing of *Pseudomonas aeruginosa* in Cystic Fibrosis

Attempts were made to type *Pseudomonas aeruginosa* in patients with cystic fibrosis as early as the late 1980s. Initial methods included early versions of molecular technology, pyocin typing, serotyping, and ribotyping. Many of those methods proved relatively unreliable in later years, so the results of the studies cannot be considered conclusive given the advances made in the past 20 years. As expected, even the early typing studies did see related strains present in siblings (Grothues, Koopmann, von der Hardt, & Tummler, 1988) or with patients attending the same summer camps (Wolz et al., 1989). An early comparison between pyocin typing and RFLP analysis did confirm cross-colonization by indicating that 43% of patients typed shared a common genotype, and the authors agreed that RFLP was a more reliable method than pyocin typing (Fegan, Francis, Hayward, & Fuerst, 1991). However, even as late as the mid-1990s, laboratories were still evaluating strains of *P. aeruginosa* based on phenotypic differences rather than strain typing (Mahenthiralingam, Campbell, Foster, Lam, & Speert, 1996).

Since genotyping quickly proved superior to phenotyping for detecting differences between bacterial strains, RAPD and PFGE analysis became the techniques of choice for typing *Pseudomonas aeruginosa* in patients with cystic fibrosis in the 1990s; but there was lingering concern about standardization of interpretation (Renders et al., 1996). To address reproducibility of results, a study comparing RAPD typing to previous PFGE results found that the results for individual patients remained closely related but did display a shift over time (Kersulyte et al., 1995). The project also found related strains in patients that were visiting the same clinic during the same time period. As

indicated in earlier publications, PFGE identified a clone that was present in the tap water, sinks, wash basins, and creams of a CF ward only to discover that the predominant strain had spread into the adjacent non-CF control ward four years later (Bosshammer et al., 1995). In contrast, a Danish CF center utilizing PFGE documented that none of their patients were infected with strains of *P. aeruginosa* that were similar to the environmental isolates leading them to conclude that their contact isolation and other infection control precautions were effective in preventing environmental contamination with *P. aeruginosa* (Zembrzuska-Sadkowska, Sneum, Ojeniyi, Heiden, & Hoiby, 1995). When considering antibiotic resistance, a CF center in the United Kingdom found by PFGE that 85% of their patients infected with *P. aeruginosa* appeared to have the same beta-lactam antibiotic resistant strain (Cheng et al., 1996).

Social interaction both within and outside of healthcare institutions has been a documented cause of transmission of *P. aeruginosa* in patients with cystic fibrosis. PFGE detected that 12 of 40 patients who acquired *P. aeruginosa* during a period of four years contracted it from camps, clinics, or rehabilitation centers (Tummler et al., 1991). The introduction of new infection control practices led to the detection of only one case of transmission in the CF ward during a two year period, leading the researchers to conclude that nosocomial transmission significantly contributes to the prevalence of *P. aeruginosa* in cystic fibrosis. Camps specifically for patients with CF were later recognized as significant risk factors for the transmission of *P. aeruginosa*. While one study did document no related strains by RAPD analysis at a summer camp and only one case of acquisition (Hoogkamp-Korstanje, Meis, Kissing, van der Laag, & Melchers, 1995), a

more recent study found evidence by PFGE of significant transmission, 17 of 22 campers, of a pan-susceptible strain of *P. aeruginosa* by PFGE once the patients returned from camp (Ojeniyi, Frederiksen, & Hoiby, 2000). Social interaction can occur even during inpatient stays as discovered in a study stating that four patients became superinfected with the same strain of *P. aeruginosa* after inpatient social contact (McCallum et al., 2001). This finding led the clinic to type each patient infected with *P. aeruginosa* and separate the inpatients colonized with the epidemic strain. Camps for patients with CF have been discontinued in the United States, but a study of camps in the Dead Sea region of southern Israel demonstrated that cross-infection among patients could be eliminated by meticulous separation of individuals (Greenberg et al., 2004). Social circumstances where separation is impossible such as family life also contributes to colonization. Cross-transmission of bacterial pathogens in siblings with cystic fibrosis is well documented (Speert et al., 2002), but a more interesting case described the chronic colonization of two previously healthy parents with the same *P. aeruginosa* strain as their daughter with CF (McCallum et al., 2002).

While a multitude of studies confirmed genetic relationships between strains of *P. aeruginosa* in patients with CF, it is appropriate to note that several studies have not yielded evidence of cross-transmission. A CF center in Leipzig stated cross-colonization was rare by PFGE (Spencer et al., 2000). Similarly, RAPD analysis suggested that cross-infection was uncommon in an outpatient clinic in Brazil, but the study only spanned six months (da Silva Filho et al., 2001). A separate study in Brazil came to the same conclusion when PFGE typing revealed 39 distinct fingerprint patterns from 41

patients (Silbert et al., 2001). The two remaining patients were siblings harboring the same strain of *P. aeruginosa*, and the authors also noted that mucoid and non-mucoid strains of *P. aeruginosa* from the same patient exhibited the same molecular profile in the majority of cases. A separate study also confirmed that mucoid and non-mucoid *P. aeruginosa* isolates from the same patient produced the same pattern (Campana et al., 2004). Additionally, a CF clinic in North Staffordshire found no evidence of cross-infection with the environment or between patients except in the case of siblings (Tubbs et al., 2001). Even one recent study in New Delhi, India that utilized a form of rep-PCR found 26 of 27 *P. aeruginosa* isolates to be unique (Agarwal et al., 2005).

Several studies have reported lack of evidence by molecular typing of transmission of *P. aeruginosa* between patients with CF, but the majority of recent studies have found evidence of cross-infection including several publications from the United Kingdom. Analysis of patients in the Manchester Adult CF unit by PFGE discovered that 14% of patients harbored the same strain, which happened to be solely composed of multiply-resistant *P. aeruginosa* isolates in either the mucoid or non-mucoid form (Jones et al., 2001). The clinic also typed *P. aeruginosa* isolates from non-cystic fibrosis patients, and those isolates were found to be unique strains. Further research into the patients infected with the epidemic strain revealed no common demographic factor but confirmed that 23 of the 24 patients with the epidemic strain had been inpatients at least once in the previous two years. A smaller study reviewed the history of six patients from a pediatric CF center in the United Kingdom. Four of the patients had overlapping hospital stays, and PFGE analysis produced identical genotypes for those four patients

and unique profiles for the other two patients (Denton et al., 2002). In addition, a large study of CF centers across England and Wales found evidence of cross-infection between and within centers (Scott & Pitt, 2004).

Several publications documenting evidence of cross-infection of *P. aeruginosa* have also originated from Australian cystic fibrosis clinics. An initial probing of patient samples by PFGE found that greater than one third of patients were infected with the same *P. aeruginosa* strain in an adult CF center (Anthony et al., 2002). A similar study focusing on a pediatric CF center showed that 55% of patients shared a genetically related strain (D. S. Armstrong et al., 2002). Interestingly, two environmental surveys performed four years apart failed to detect the clonal strain, and analysis of patient data determined that infection with the clonal strain was not associated with adverse clinical outcomes. The results of these studies led to molecular typing, by PFGE and RAPD, across five CF clinics in Australia. An identical clone, a mucoid multiply-resistant *P. aeruginosa*, was discovered in all five clinics and was found to be the dominant clone in three of the clinics (D. Armstrong et al., 2003). A more recent analysis of an adult and pediatric CF center by rep-PCR and PFGE confirmed a dominant clone infecting 38% of the patients with *P. aeruginosa* (Syrmis et al., 2004). This particular study also defined profiles with two or more band differences as unrelated.

A recent publication described the environmental pathogen risk in an Italian outpatient cystic fibrosis clinic (Festini et al., 2007). Pathogens found on environmental surfaces included *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia*. *P. aeruginosa*

was found in 22.8% of environmental samples, and the estimated risk for a non-colonized patient coming in contact with *P. aeruginosa* from clinic surfaces was 5.4% for each visit. RAPD analysis confirmed the relationships between environmental and patient isolates, and the authors suggested complete disinfection of visiting room surfaces and availability of alcohol rubs to reduce the risk of transmission.

Newer studies have evaluated patient outcomes based on the specific clonal strain identified. Researchers in the United Kingdom developed an assay to specifically detect the Liverpool epidemic strain of *P. aeruginosa*, and analysis of patient data revealed patients with the epidemic strain had a greater loss of lung function and a deteriorating nutritional state as determined by FEV₁ and BMI, respectively. These patients also demonstrated a greater need for admittance to the hospital and additional days of antibiotic treatment (Al-Aloul et al., 2004). Another study in an Australian pediatric clinic documented that 43% of patients with cystic fibrosis acquired *P. aeruginosa* before they reached seven years of age. The study also identified a strain of MRPA infecting seven children, four of whom died due to the increased morbidity associated with the strain (Nixon et al., 2001). Overall the study confirmed that patients with a history of *Pseudomonas aeruginosa* infection had lower average FEV₁ values and spent more time in the hospital. Interestingly, the study also found no difference in patient outcomes for mucoid versus non-mucoid forms of *P. aeruginosa*. Analysis of a patient group in the United Kingdom by PFGE also identified two epidemic strains, the Liverpool and Sheffield strains, in patients that led to higher morbidity and more treatment as opposed to patients infected with unique strains (Edenborough et al., 2004). A large study across

several adult and pediatric CF centers in Australia revealed 59% of patients shared a common *P. aeruginosa* strain not found to be MRPA (O'Carroll et al., 2004). When compared to unique strains, those infected with the clonal strain were younger, exhibited poorer lung function by FEV₁, and required more days in the hospital (26.5 days versus 12 days). If patients were infected with an additional strain besides the clonal strain, the patient was still classified as having the clonal strain. In addition, the researchers stated that minor clonal strains that included two or more patients should still be deemed a cluster.

The review of the literature revealed that no study specifically focused on molecular typing of multiply-resistant *P. aeruginosa* in patients with cystic fibrosis. While some studies discovered a clonal strain that proved to be MRPA, their dataset also included patients infected with susceptible *P. aeruginosa*. In addition, few of the studies described multi-year molecular analyses or used rep-PCR as their molecular technique of choice. A comprehensive study that includes the most advanced molecular techniques, several years of data, and analysis of patient outcomes based on clonal group is needed.

A preliminary study identified a dominant MRPA clone present in patients with cystic fibrosis at Texas Children's Hospital. The dominant clone included patients seen over the course of several years, and no clear pattern of association was evident between the patients. Ongoing molecular typing of MRPA isolates from patients with CF was requested by the CF Center and the Infection Control department of Texas Children's Hospital, and additional analyses of patient-related variables as they relate to clonality were desired.

The patient-related variables selected for inclusion in the study were based on multiple references highlighting the potential significance of each variable. While an ongoing large multi-center clinical trial aims to identify additional risk factors for acquisition of *P. aeruginosa* (Treggiari et al., 2009), two studies presented data supporting the inclusion of variables related to gender, CF genotype, culture results, lung function, and hospitalization rate (Levy et al., 2008; Maselli et al., 2003). Many early references noted gender, specifically females, as a risk factor for acquisition of *P. aeruginosa* (Demko, Byard, & Davis, 1995). Exposure to CF clinics, BMI percentile, and the mother's educational level were also implicated as risk factors by separate studies (Farrell et al., 1997; Kosorok et al., 1998; McPhail, Acton, Fenchel, Amin, & Seid, 2008; Watts et al., 2009). In addition to hospitalization rate and clinic visits, another study documented the added risk of tobramycin use (Merlo et al., 2007). Initial infection with other pathogens, such as *Staphylococcus aureus* was shown to contribute to infection with *P. aeruginosa* (Lyczak, Cannon, & Pier, 2000). An early diagnosis of CF has been shown to affect the time of acquisition of *P. aeruginosa* (D'Alessandro, Renteria, Fernandez, Martinez, & Segal, 2009; Kappler, Feilcke, Schroter, Kraxner, & Griese, 2009; Rodman et al., 2005), and the patient's age at the time of infection has been found to potentially impact the clone acquired (van Mansfeld et al., 2009). A large study utilizing national data from the CF Foundation patient registry selected FEV₁, BMI, and annual hospitalization rate as important indicators of a patient's overall health status (Zemel, Jawad, FitzSimmons, & Stallings, 2000). In regards to patient outcomes, *P. aeruginosa* infection has consistently been shown to affect lung function (FEV₁) and

BMI. Finally, several studies have presented data confirming that infection with a specific *P. aeruginosa* clone can negatively impact patient outcomes (Al-Aloul et al., 2004; Bradbury, Champion, & Reid, 2008; Edenborough et al., 2004; Nixon et al., 2001; O'Carroll et al., 2004).

Patients with cystic fibrosis are expected to become chronically infected with *P. aeruginosa* at some point in their lives. However, adequate diagnosis, prevention, and treatment could contribute positively to both quality of life and longevity. By evaluating patient-related variables and patient outcomes as they relate to MRPA clones, additional information will be obtained that could be translated into new treatment and prevention guidelines. Identification of risk factors for acquisition of the dominant MRPA clone could contribute to future infection control strategies. Differences in patient outcome based on infection with the dominant MRPA clone could also tailor future clinical practice guidelines, such as treatment with more aggressive antimicrobials.

CHAPTER 3: METHODOLOGY

This chapter will discuss the following topics related to the methodology of the study: research design, subjects, variables, human studies protocol, data collection, data analysis, validity, and limitations. The purpose of this study is to discover relationships between patient-related variables and infection with multiply-resistant *Pseudomonas aeruginosa* (MRPA) in cystic fibrosis. The specific research questions addressed by this study include:

- Is infection with the dominant MRPA clone related to patient-related variables prior to MRPA infection?
- Is patient outcome related to infection with the dominant MRPA clone?

The following hypotheses are proposed by this study:

HO1: Patient-related variables prior to MRPA infection are correlated to infection with the dominant MRPA clone.

HO2: Infection with the dominant MRPA clone is correlated to patient outcome.

Research Design

A non-experimental ex post facto design was employed for this study. Polit and Beck (2004) describe this type of study as when “the researcher observes phenomena as they naturally occur without intervening.” The study was a comparison of patient-related variables between two groups of patients with cystic fibrosis (CF), those infected with the

dominant MRPA clone and those infected with a non-dominant MRPA clone. Due to the potential impact on patients, a true experimental study was not possible with this research topic. The ex post facto design is ideal for retrospective studies, and while it cannot establish causality, it can estimate how much of the variation in the dependent variable can be predicted from the variation of the independent variable (Polit & Beck, 2004). For the evaluation of possible risk factors for infection with the dominant MRPA clone, the independent variables are patient-related variables prior to infection with MRPA and the dependent variable is the MRPA clone (dominant or non-dominant). Conversely for the comparison of post-infection differences between the two clonal groups, the independent variable is the MRPA clone (dominant or non-dominant) and the dependent variables are patient outcomes following infection with MRPA.

Several assumptions were made in developing this study including that all patient-related variables and patient outcome data were correctly entered into Port CF, the electronic national repository for CF patient data (FitzSimmons, 1993). It was also assumed that measurement of patient data, such as FEV₁ and weight, was accurate and precise. In regards to the accuracy of laboratory data, it was assumed that no MRPA infections were missed by respiratory culture and that all rep-PCR molecular profiles were accurate. In addition, the study assumed that all MRPA isolates from patients with CF who were eligible for the study were submitted for molecular typing.

Design Quality

Internal Validity

The following specifically addresses threats to internal validity that might compromise the ability to infer that the dependent variables are related to the independent variables. The variables included in the research design were intended to account for any history that may affect the outcome. Variables were selected for this intent based on conclusions from a thorough literature review (Al-Aloul et al., 2004; Edenborough et al., 2004; Emerson et al., 2002; Kosorok et al., 1998; Nixon et al., 2001). It is possible that additional variables exist that may contribute to the acquisition of MRPA, but the study was limited by the data fields collected and entered into Port CF. Additional factors not recorded in the patient registry that may be significant include social interaction both in and out of the hospital or clinic, specific hospital or clinic rooms utilized, and practitioners seen at each visit. The independent variables associated with the first research question document the important historical and demographic factors of the patient's illness, and these factors were then tested against whether or not the patient has been infected with the dominant MRPA clone.

Because the patients were not randomized into groups and instead were grouped by which MRPA clone infected them, no selection bias was present. The study was designed to identify variables that contributed to infection with one of the two clonal groups. The data spanned the course of a few years for some of the patients, so physical maturation or childhood development could be a concern. However, the changes in the patients that are not thought to be associated with infection by the dominant MRPA clone

should be random for patients in either group as these changes would be part of the natural disease progression, ex., steadily declining lung function that is characteristic of CF. In addition, quantitative patient outcome variables were normalized for the passage of time by calculating hospitalization rates per year and changes in BMI and FEV₁ percentiles per month or per year.

The results of the study cannot be affected by testing because all testing was performed as part of routine clinical care for patients with CF. Instrumentation threats were controlled by using clinically validated technologies and previously evaluated commercially available reagents and data analysis software (Healy et al., 2005). The utility of rep-PCR in differentiating strains of the same bacterial species is well documented in the literature with multiple comparisons to PFGE, the gold standard (Chou & Wang, 2006; Doleans-Jordheim et al., 2009; Jamasbi et al., 2004; Pounder et al., 2005; Shutt et al., 2005). At the time of the proposal of this study, a thorough literature review showed that no published study specifically focusing on MRPA had utilized rep-PCR. However, there have been studies that confirmed the performance of rep-PCR for *P. aeruginosa* in patients with CF (Agarwal et al., 2005; Doleans-Jordheim et al., 2009; Syrmiss et al., 2004). In addition, rep-PCR technology continues to meet the College of American Pathologist's guidelines for utility in a clinical setting, including quality assurance metrics and proficiency testing.

Patient mortality should not threaten the integrity of the study. While patients in the study succumbed to illness, data obtained before death was included in the analysis, and death was measured as a dependent variable for the second research question (which

asks if patient outcome is related to infection by the dominant MRPA clone). Death would not affect the other patient outcome variables because clinical data would have been collected at the next evaluation following MRPA infection, and hospitalization rate accounts for the number of days hospitalized per year (including fractions of a year). The only rare situations where patient mortality would affect the ability to collect patient data would be if a patient died less than one month after MRPA diagnosis (preventing the collection of the post-MRPA infection data). No patients in the study met this criterion.

External Validity

The study design also showed few weaknesses with respect to threats to external validity. Because there was no interaction with the patients beyond routine clinical care, no expectancy effects, novelty effects, experimenter effects, or measurement effects were identified with respect to testing. The most significant threat noted with external validity was the possibility of interaction of history and its effect on treatment due to undocumented contact between patients, such as social interactions. While social interactions during inpatient stays are carefully monitored and discouraged by hospital staff, contact outside of the hospital is certainly plausible. Contact of TCH patients with patients from other CF centers including the adult CF Clinic is also possible and would threaten external validity as well. It is possible that the results of this study could not be generalized to other CF centers and CF populations. The dominant MRPA clone could possibly be a geographical anomaly that is only present at TCH or could be related to a specific clinic or hospital process in this center. The CF Foundation has published numerous consensus statements regarding treatment of patients, and the foundation

inspects each accredited CF center (Saiman & Siegel, 2003, 2004; www.cff.org).

However, it is still reasonable to note that subtle differences in practice could contribute to the proliferation of this MRPA clone in this specific population, which would be considered a threat to external validity due to the inability to apply the study results to other CF centers.

Statistical Validity

Missing data was a threat to statistical validity, but missing data points were randomly observed within this dataset. Patient data is required to be reported in Port CF for each hospital or clinic visit, so by only accepting patients who have consented to have their information documented in Port CF, the study controls for this factor as much as possible. Patients with missing data points were removed from the analyses involving the particular variable for which data was unavailable.

Statistical power could not be calculated prior to analysis because effect size could not be estimated. Statistical power based on the sample size that was obtained and the resulting statistical output was calculated as a post hoc analysis using nQuery Advisor 7.0 (Statistical Solutions, Saugus, MA). Each individual model tested by logistic regression was analyzed by nQuery using the resulting R^2 value and an alpha level of 0.05.

Significant predictors (patient-related variables) of infection with the dominant or a non-dominant MRPA clone could ultimately be responsible for changes in patient outcome. The scope of this study, specifically the limited patient population, does not provide the ability to definitively determine whether the predictor variables or the

specific MRPA clone were ultimately more responsible for poor patient outcomes. By determining which of the factors are significant, larger studies exploring specific predictors of patient outcomes in multiple CF centers could be performed.

Subjects

The population of interest for this study was all patients of the CF Care Center at Texas Children's Hospital from 2004 through early 2009. This study utilized a non-probability convenience sample and was specifically intended for patients of the CF Care Center that were diagnosed with a MRPA infection during the course of the study (typing data obtained from October 2004 through January 2009). Inclusion criteria were that patients have documented MRPA infection and that data relevant to particular patient-related variables are available through Port CF or through medical records at TCH. Port CF is the national patient registry established by the Cystic Fibrosis Foundation as a resource for physicians and researchers (FitzSimmons, 1993; Zemel et al., 2000). Patients were deemed eligible for the study if they satisfied the following requirements: patient background data was available through Port CF (handling of missing data points is addressed under statistical validity), patient information collected at a clinic or hospital visit just prior (no greater than 6 months) to infection with MRPA was available, patient information collected at a clinic or hospital visit following diagnosis of MRPA infection (at least one visit 1-6 months following infection) was available, and MRPA isolates were submitted to the Molecular Microbiology laboratory for DNA typing.

Variables

Pediatric patients were routinely seen at the CF clinic at least four times per year. During a patient's initial visits, patient data was captured in Port CF with their consent. The categorical variable responsible for creating the two groups analyzed in this study is whether or not the patient has been infected with the dominant MRPA clone. The clone designation was made based on results of DNA typing. In cases where a patient was infected with the dominant MRPA clone as well as another clone, patients were considered members of the dominant MRPA clone group. New MRPA isolates were added to the cumulative dendrogram produced during the course of the epidemiological study at the hospital. If a patient had a previous MRPA infection and thus a previously typed MRPA isolate, then the new MRPA isolate was checked against that patient's older MRPA isolates to ensure the patient remained in the same clonal group (i.e., dominant MRPA clone or non-dominant MRPA clone). Some patients were initially infected with a unique MRPA clone (non-dominant clone group) but later became infected with the dominant MRPA clone. Once infection with the dominant MRPA clone was confirmed, the patient data was included in the dominant MRPA clone group only.

The variables selected for the study were based on multiple references detailing clinical significance and are summarized in Figure 1. It has been noted that disease severity is highly impacted by CF genotype. In addition, gender is significant due to poorer survival rates in females. Several factors including age at the time of initial CF diagnosis, chronological age, hospitalization rate, and mother's educational level are known contributors to infection with *P. aeruginosa*. Inhaled tobramycin use is also

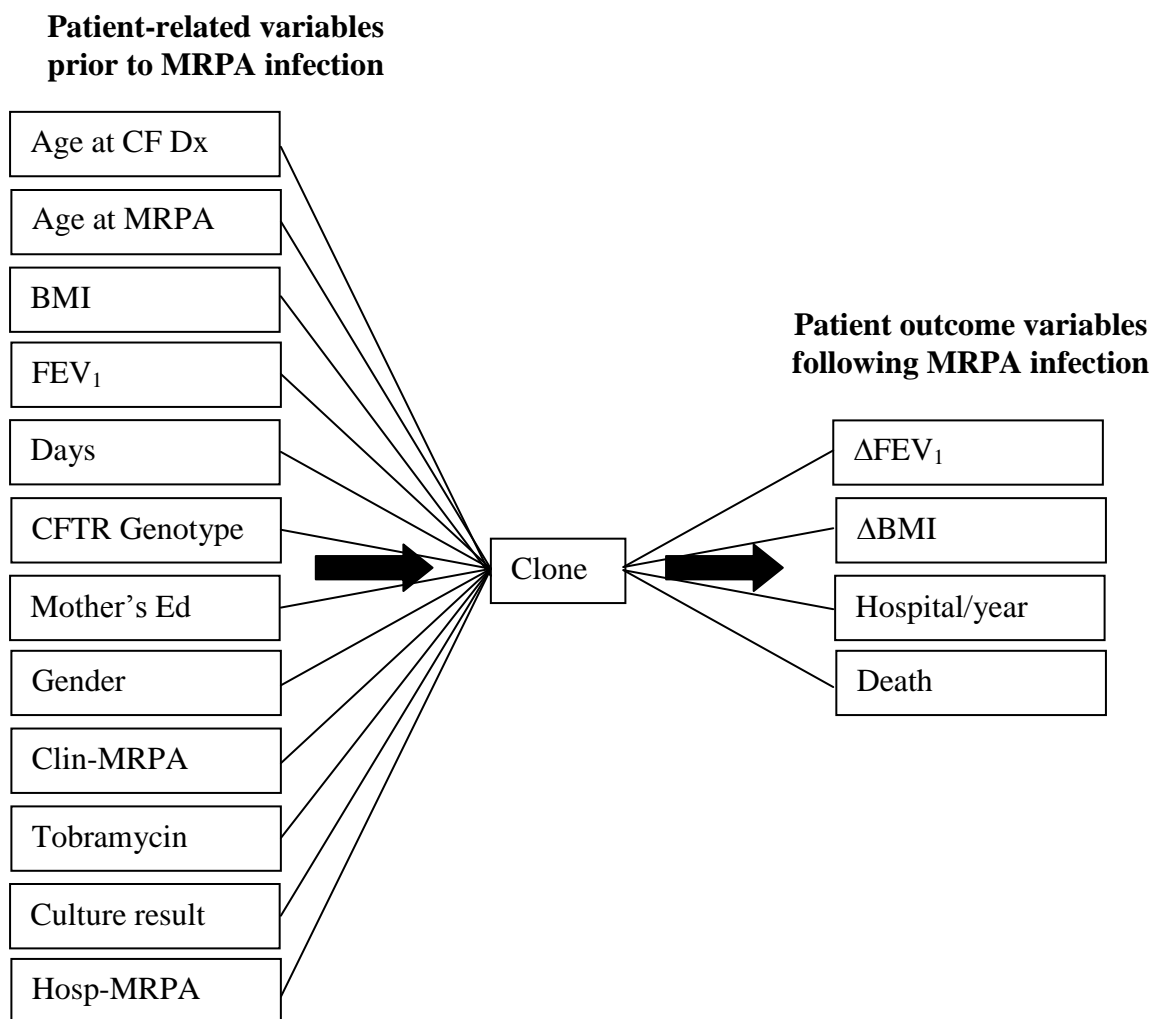


Figure 1. Patient-related Variables and Patient Outcome Variables.

Note: The patient-related variables and the patient outcome variables are collected in Port CF. The clone variable is obtained through molecular typing.

suspected as a contributor to the emergence of MRPA. Infection with MRPA is associated with a higher risk of mortality, and FEV₁ and BMI are widely accepted predictors of survival in patients with CF.

Patient-related Variables as Risk Factors for Clone Acquisition

Patient-related variables recorded prior to MRPA infection were obtained from Port CF. Data for variables collected from the clinic visit just prior to MRPA infection were required for inclusion of that patient in the study. The visit just prior to MRPA infection is defined as a hospital or clinic visit just before (but within six months of) the visit during which MRPA was diagnosed. The independent variables for this research question include a variety of patient data such as demographic information and disease-specific characteristics. The data was readily available for the majority of study patients as patients with CF are seen quarterly in addition to hospital or clinic visits due to illness. The dependent variable for this research question is the MRPA clone (dominant or non-dominant). All variables included in the analysis of patient-related variables as risk factors of dominant MRPA clone acquisition are listed in Table 2.

Differences in Patient Outcome Based on MRPA Clone

All post-MRPA infection patient-related variables were obtained from Port CF. Data for short-term patient outcome variables collected from the visit after diagnosis with MRPA infection were required for inclusion of that patient in the study. The visit after diagnosis with MRPA infection is defined as a hospital or clinic visit after (no sooner than one month, but no later than six months) the visit during which MRPA was diagnosed. Data for long-term patient outcomes were collected from the last recorded clinic visit prior to December 2009, as approved by the IRB. The independent variable for this research question is the MRPA clone (dominant or non-dominant). The dependent variables are patient outcome data involving changes in FEV₁ and BMI per

Table 2.

Variables Analyzed in Determination of Potential Risk Factors of Dominant MRPA Clone Acquisition

Variable	Definition of variable	Variable type	Unit of measurement or coded categories
Clone	Clone identified by DNA typing	Categorical	1 for dominant MRPA clone, 0 for non-dominant MRPA clone
Age Dx	Patient age at time of CF diagnosis	Quantitative	0 for < age 1, 1 for > age 1
Age MRPA	Patient age at time of MRPA infection diagnosis	Quantitative	Years
BMI	Body mass index percentile based on height and weight at visit just prior to MRPA infection	Quantitative	Percentile
FEV ₁	Forced expiratory volume in one second, volume of air (in liters) which can be forcibly exhaled from the lungs in the one second, expressed as percentile predicted based on healthy, non-smoking people of the same gender and age, at visit just prior to MRPA infection	Quantitative	Percentile
Days Hosp	Total number of days hospitalized in the year prior to MRPA diagnosis	Quantitative	Days
CFTR Genotype or Mutation	Mutation combination responsible for CF identified in the patient	Categorical	1 for delF508/delF508, 2 for delF508/other, 3 for other mutations
Mother's Ed	Mother's educational level	Categorical	0 for less than high school (HS), 1 for HS diploma or equivalent, 2 for some college, 3 for college graduate, 4 for unknown
Gender	Gender of the patient	Categorical	0 for male, 1 for female

Table 2. Variables Analyzed in Determination of Potential Risk Factors of Dominant MRPA Clone Acquisition (continued)

Variable	Definition of variable	Variable type	Unit of measurement or coded categories
Clin-MRPA	Time between last CF clinic visit and MRPA diagnosis	Categorical	1 for ≤ 60 days, 2 for 61-120 days, 3 for > 120 days
Tobramycin	Tobramycin use at visit just prior to MRPA diagnosis	Categorical	0 for no, 1 for yes
Culture result	Respiratory culture result at visit just prior to MRPA diagnosis	Categorical	1 for negative culture, 2 for non-MRPA <i>P. aeruginosa</i> , 3 for <i>P. aeruginosa</i> (PA) and MSSA, 4 for PA and MRSA, 5 for PA and another organism, 6 for other organism(s) not PA
Hosp-MRPA	Time between most recent hospital admission and MRPA diagnosis	Categorical	1 for ≤ 90 days, 2 for 91-180 days, 3 for 181-365 days, 4 for > 365 days

month (short-term outcomes), changes in FEV₁ and BMI per year (long-term outcomes), annual hospitalization rate, and mortality. All variables included in the analysis of potential differences in patient outcome based on MRPA clone are listed in Table 3.

Measurement Quality

All measurements were performed by clinically validated testing methodologies in compliance with hospital regulations pertaining to the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) and laboratory regulations pertaining to the College of American Pathologists (CAP) and the Clinical and Laboratory Standards Institute (CLSI). All measured patient data such as age, weight and height (necessary for BMI calculation), and FEV₁ were obtained by trained and

Table 3.

Variables Evaluating Differences in Patient Outcome Between the Two Clonal Groups

Variable	Definition of variable	Variable type	Unit of measurement or coded categories
Clone	Clone identified by DNA typing	Categorical	1 for dominant MRPA clone, 0 for non-dominant MRPA clone
Δ FEV ₁ /Month	Change in FEV ₁ percentile between visit just prior to MRPA diagnosis and visit after MRPA was diagnosed divided by the number of months between visits	Quantitative	Percentile
Δ FEV ₁ /Year	Change in FEV ₁ percentile between visit just prior to MRPA diagnosis and the last recorded visit divided by the number of years between visits	Quantitative	Percentile
Δ BMI/Month	Change in BMI percentile between visit just prior to MRPA diagnosis and visit after MRPA was diagnosed divided by the number of months between visits	Quantitative	Percentile
Δ BMI/Year	Change in BMI percentile between visit just prior to MRPA diagnosis and the last recorded visit divided by the number of years between visits	Quantitative	Percentile
Hosp year	Number of days hospitalized per year after MRPA diagnosis, calculated as (# of days hospitalized) / (# of years data is available), ex. 35 days hospitalized in 18 months of follow-up data post MRPA infection would be 35/1.5 = 23.3	Quantitative	Days per year
Death	Death of a patient during data collection period	Categorical	0 for still living, 1 for death during data collection period

knowledgeable staff. All patients are routinely seen by either the CF Care Center staff in the hospital clinic or the inpatient hospital staff dedicated to Pulmonology at TCH. All lung function testing (FEV₁) was performed by licensed respiratory therapists who routinely perform these tests on pediatric patients. Calculations for BMI and FEV₁ were performed using pre-determined computer-aided formulas. In addition to the clinical data, demographic data such as age of the patient, clinic visit and hospitalization dates, CFTR genotype, mother's educational level, gender, and tobramycin use were entered into Port CF by a single well-trained individual.

Sensitivity and specificity for each of the laboratory assays were documented during the initial validation phase. Biochemical testing and DNA sequencing methodologies have been validated for abilities to generate meaningful bacterial species identification (including *P. aeruginosa*). Antimicrobial susceptibility testing methods have been validated with respect to classifying isolates as susceptible or resistant to each antibiotic tested (Clinical and Laboratory Standards Institute, 2005). DNA typing methodologies (DiversiLab) were validated in terms of abilities to distinguish different bacterial strains with accuracy, resolution, and reproducibility (Clinical and Laboratory Standards Institute, 2007). DNA typing of MRPA isolates in patients with CF was specifically evaluated for reproducibility, and it was found that MRPA isolates from the same patient with the same clone yielded reproducible DNA profiles by the following comparisons: repeat testing of the same MRPA DNA on multiple occasions, multiple MRPA DNA isolations from the same respiratory sample, and multiple MRPA DNA isolation from different respiratory samples (in many cases, reproducible profile data

span several years). The reproducibility of molecular typing of MRPA isolates is illustrated in Figure 2.

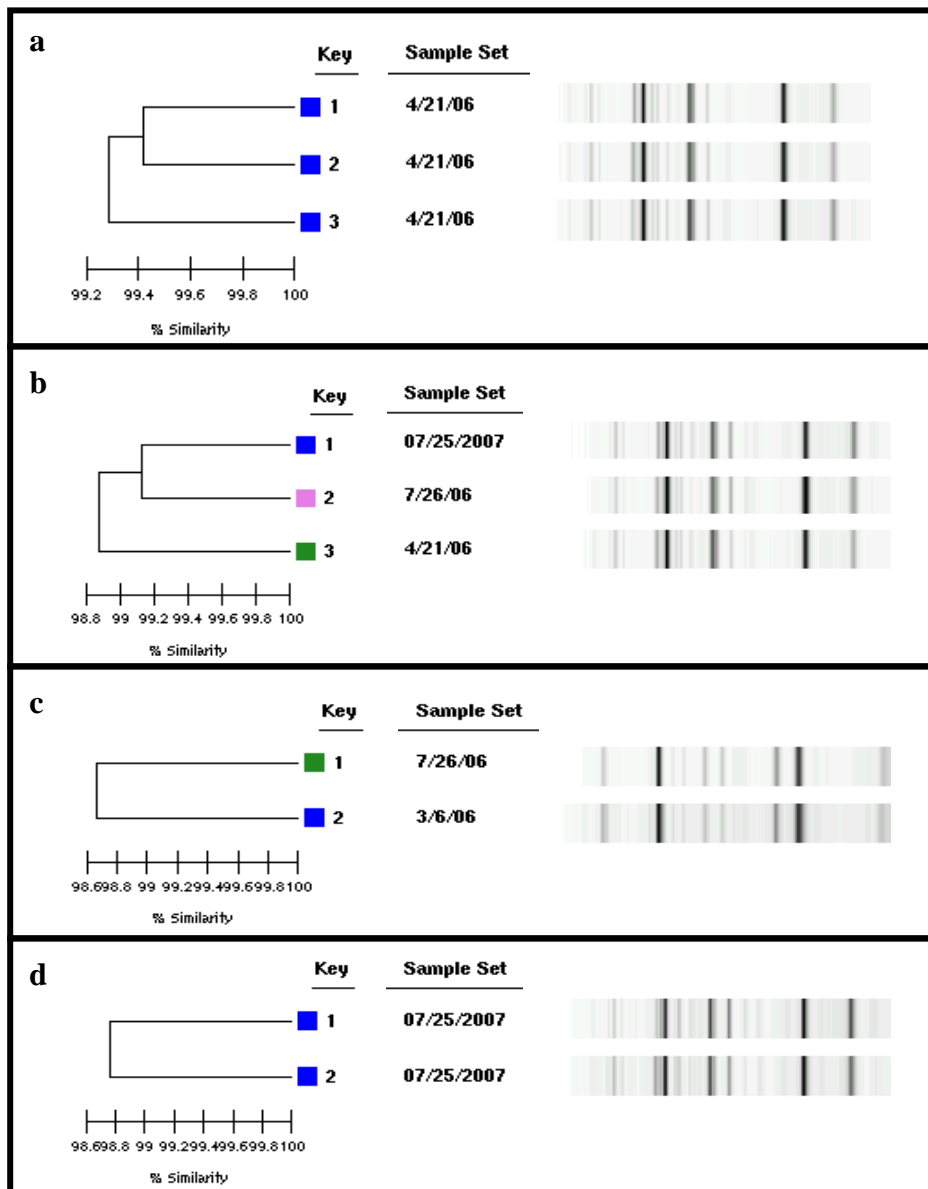


Figure 2. Reproducibility of MRPA Molecular Typing Profiles.

Note: The highly similar molecular profiles above show the same MRPA clone (strain) in (a) different specimens from one patient at one collection date, (b) different specimens from one patient on multiple collection dates, (c) a sibling pair, and (d) a mucoid and non-mucoid isolate from the same patient.

Human Studies Protocol

This study was approved by the Institutional Review Boards (IRB) of both Baylor College of Medicine (protocol H-25980) and Virginia Commonwealth University (protocol HM 12652). No patient interaction was required for this study, and no patient consent was necessary. All patient information including laboratory results were obtained through the normal course of treatment for cystic fibrosis (Bush, Alton, Davies, Griesenbach, & Jaffe, 2006). No compensation to patients was offered. Inclusion and exclusion criteria were based on availability of data in Port CF.

Patient data was collected from Port CF by the student researcher reviewing each individual patient record, and each patient was assigned a unique number that only the researcher could relate back to patient name or medical record number. All patient data was kept either on secure servers or in locked file cabinets in locked offices. Compliance to HIPAA standards was maintained at all times. No one besides the researcher, physicians, and CF Center personnel was allowed access to the patient data. The document linking patient information with the assigned study number was destroyed at the completion of the study. No patient identifiers will be reported in any published version of the study. The results of this study will only be reported as aggregate data.

Data Collection

Laboratory Data

Isolation and Identification of P. aeruginosa

Respiratory samples such as sputa, throat/nasal cultures, and bronchoalveolar lavages were collected from patients at each visit to the CF clinic and as part of inpatient

care as recommended by the CF Foundation (Patient Registry 2006 Annual Report, 2008; Saiman & Siegel, 2003). The samples were submitted to the Microbiology laboratory at Texas Children's Hospital for respiratory culture, and routine culture methods were performed by certified medical technologists. Respiratory samples were cultured on multiple media (blood agar, chocolate agar, MacConkey agar, PC (*Pseudomonas cepacia*) agar, CNA agar (Columbia agar with colistin and nalidixic acid), and ChromeSA agar) and incubated at 35°C in a 5% CO₂ incubator for 24-48 hours. If a blue-green pigment was detected on the MacConkey or chocolate agar plates due to the presence of pyocyanin, then the isolate was reported as *P. aeruginosa*.

If no pigment was detected, then the isolate was tested in the VITEK 2 instrument (bioMerieux, Durham, NC). A suspension of organisms (density equivalent to a McFarland No. 0.50 to 0.63) was prepared in a tube of sterile saline and loaded into the VITEK 2 instrument. The instrument then inoculated a series of pre-formulated wells in the VITEK 2 Gram-Negative identification card. This particular card contains 47 biochemical tests based on established biochemical methods as well as carbon source utilization, enzymatic activities, and vibriostatic compound resistance. The VITEK 2 provides an automated result, in terms of relative probability, based on unique organism-specific biochemical profiles for the card loaded on the instrument. The relative probability is a percentage based on a comparison of the observed reactions in the patient sample to the typical reactions of each organism intended to be identified by the card. If the isolate was identified with a relative probability of $\geq 90\%$ *P. aeruginosa*, then the isolate was reported as such based on validation of the VITEK 2 system and routine

culture methods in the Microbiology laboratory at Texas Children's Hospital. If the relative probability was < 90%, then the isolate was forwarded to the medical technologists in the Molecular Microbiology laboratory at Texas Children's Hospital for DNA pyrosequencing-based bacterial identification (Luna et al., 2007).

For pyrosequencing-based bacterial identification, bacterial DNA was extracted using the Mo Bio UltraClean™ Microbial DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA). Amplification using primers specific to the V1 and V6 regions of 16S rDNA was performed in a master mix reaction containing deoxynucleoside triphosphates, magnesium chloride, 10X PCR buffer, and *Taq* DNA polymerase. The amplified product was then subjected to pyrosequencing, and sequences of 30-40 bases were produced for each region, V1 and V6. Ribosomal RNA gene sequences were used to search against a publicly available database, the Ribosomal Database Project (Cole et al., 2005). A final identification was made by combining DNA sequence and biochemical data.

Determination of Antimicrobial Resistance

Antibiotic susceptibility testing for all *P. aeruginosa* isolates was performed by disk diffusion. *P. aeruginosa* isolates were cultured on Mueller Hinton plated medium, and antimicrobial-containing disks were placed on the surface of the medium. Antibiotics tested included tobramycin, gentamicin, amikacin, ciprofloxacin, ceftazidime, meropenem, piperacillin, ticarcillin/clavulanate, and aztreonam. Based on the zone of inhibition, the organism was reported as susceptible, indeterminate, or resistant for each antibiotic tested (Clinical and Laboratory Standards Institute, 2005). MRPA was defined as being resistant to all antibiotics in two or more of the following groups:

aminoglycosides (tobramycin, gentamicin, and amikacin), fluoroquinolones (ciprofloxacin), and beta-lactams (ceftazidime, meropenem, piperacillin, ticarcillin/clavulanate, and aztreonam) (Lang, Aaron, Ferris, Hebert, & MacDonald, 2000).

Determination of MRPA Clone (Dominant vs. Non-dominant)

When a MRPA isolate was identified from a patient with CF, a glycerol stock was made and stored at -80°C by the Microbiology laboratory. Every three months, all of the accumulated MRPA samples were verified as MRPA isolates from patients with CF, cultured, and forwarded to the medical technologists in the Molecular Microbiology laboratory of Texas Children's Hospital for DNA typing.

DNA typing to determine clonal groups present among the MRPA isolates from patients with CF was performed by rep-PCR (Versalovic et al., 1991). Rep-PCR is a highly reproducible technology that generates results comparable to PFGE. Studies involving *Staphylococcus aureus* (Shutt et al., 2005), *Listeria monocytogenes* (Chou & Wang, 2006), and vancomycin-resistant *Enterococcus* (Pounder et al., 2005) have shown that rep-PCR was faster and less expensive than PFGE, making the technology more conducive to the clinical laboratory environment. The DiversiLab system, which utilizes reagent kits for rep-PCR as well as proprietary software for interpretation, is suitable for epidemiological studies due to the standardized nature of the technology (Healy et al., 2005; Wise et al., 2007). The DiversiLab system is used clinically by many molecular diagnostics laboratories and has been validated for use with multiple organisms including *Pseudomonas aeruginosa*. The DiversiLab molecular typing system has been in use

clinically as a fully validated testing strategy to determine the relative similarities of bacterial isolates (strains within a single species) at Texas Children's Hospital since 2003. In addition to validation studies performed at each individual laboratory, the technology is also subject to proficiency testing through the College of American Pathologists (CAP). Reproducibility studies were performed to assess the effectiveness of the typing strategy for *P. aeruginosa* in patients with CF prior to the initiation of this study. DNA replicates as well as organism replicates (same culture, separate extraction) yielded greater than 99% similarity. Cultures obtained from unique specimens from the same patient also exhibited greater than 99% similarity, and the time between collection of those specimens varied (from minutes to years).

Quality control of molecular typing reagents was performed at each quarterly run. Positive *P. aeruginosa* controls as well as previously tested MRPA DNA extracts were tested with each batch of samples and verified for adequate profile diversity and similarity to previous results. Every change in lot number for PCR reagents was verified for quality by this system, as well as external proficiency testing and technologist competency testing. Repeat cultures from previously tested patients were also confirmed regarding previous similarity grouping.

Bacterial DNA was extracted from bacterial colonies using the Mo Bio UltraClean™ Microbial DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA). An inoculation loop (10 µL) of bacterial cells was scraped from a culture plate and suspended in the proprietary microbead solution. The suspension was processed through a series of washes and microcentrifuge spins as described in the procedure manual of the

extraction kit, and the bacterial DNA was eluted in a final volume of 35 μL . The eluted DNA was quantitated ($\text{ng}/\mu\text{L}$) and evaluated for purity based on absorbance spectrophotometry (A_{260}/A_{280} ratio) using the NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE). The NanoDrop 1000 was calibrated regularly using known concentrations of commercially obtained nucleic acid standards, and two μL of the eluted DNA was placed on the pedestal of the instrument for measurement.

Rep-PCR was performed using the DiversiLab *Pseudomonas* fingerprinting kit (bioMerieux, Durham, NC). Rep-PCR oligonucleotide primers bind to conserved, interspersed repetitive sequences throughout the bacterial genome, and these primers direct amplification at multiple primer binding sites to produce multiple DNA fragments of various lengths, yielding strain-specific DNA profiles. The details of the oligonucleotides included in the commercial primer mix are proprietary information. The amplified products were loaded into the DNA LabChip, combined with a proprietary gel-dye matrix, and size-fractionated by microfluidics and electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The Bioanalyzer uses a series of wells and microchannels to electrophoretically separate DNA fragments, and each fragment is detected by laser-induced fluorescence (excitation wavelength of 635 nm, emission wavelength of 685 nm) of a proprietary dye that intercalates into DNA. The various sizes and relative intensities of the amplified fragments yield clone- or strain-specific chromosomal DNA profiles.

Automated DNA fingerprinting results were then transferred to the DiversiLab 3.1 software. The software uses the Pearson correlation coefficient and the Unweighted

Pair Group Method with Arithmetic mean (UPGMA) to compare the presence or absence of amplified fragments as well as the intensity of each fragment. This analysis leads to the generation of a dendrogram, similarity matrix, and scatterplot that conveys the relative similarities of isolates based on the DNA profiles generated by rep-PCR (Figure 3). The dendrogram is a hierarchical tree representation that uses group averages to display relative similarities or clusters of the isolates. The similarity matrix builds upon the information provided in the dendrogram by providing a percent similarity for each node of the tree. The scatterplot is a non-hierarchical data display that uses a two-dimensional spatial representation to display relative similarities of isolates by virtue of relative distances on a grid.

Patient isolates were designated by the researcher as the dominant clone, a unique clone, or a smaller clone based on relative similarities provided by the similarity matrix. For the purpose of this study, the isolates were designated as either members of the dominant clone or a non-dominant clone. For isolates to be considered as members of a MRPA clone, a threshold of 95% or greater similarity (by the similarity matrix) was used for classification as members or non-members. A group of isolates considered to be 95% similar would indicate that all isolates are at least 95% similar to every other isolate in the group. The dominant clone was first identified by typing a group of 23 MRPA isolates in October 2004. The data showed that roughly 50% of the isolates belonged to a single clone (labeled the dominant clone) exhibiting a highly similar DNA profile. Thorough analysis of four years of MRPA typing data has revealed that isolates within the dominant clone group exhibit greater than 95% similarity, and a recent publication also confirmed

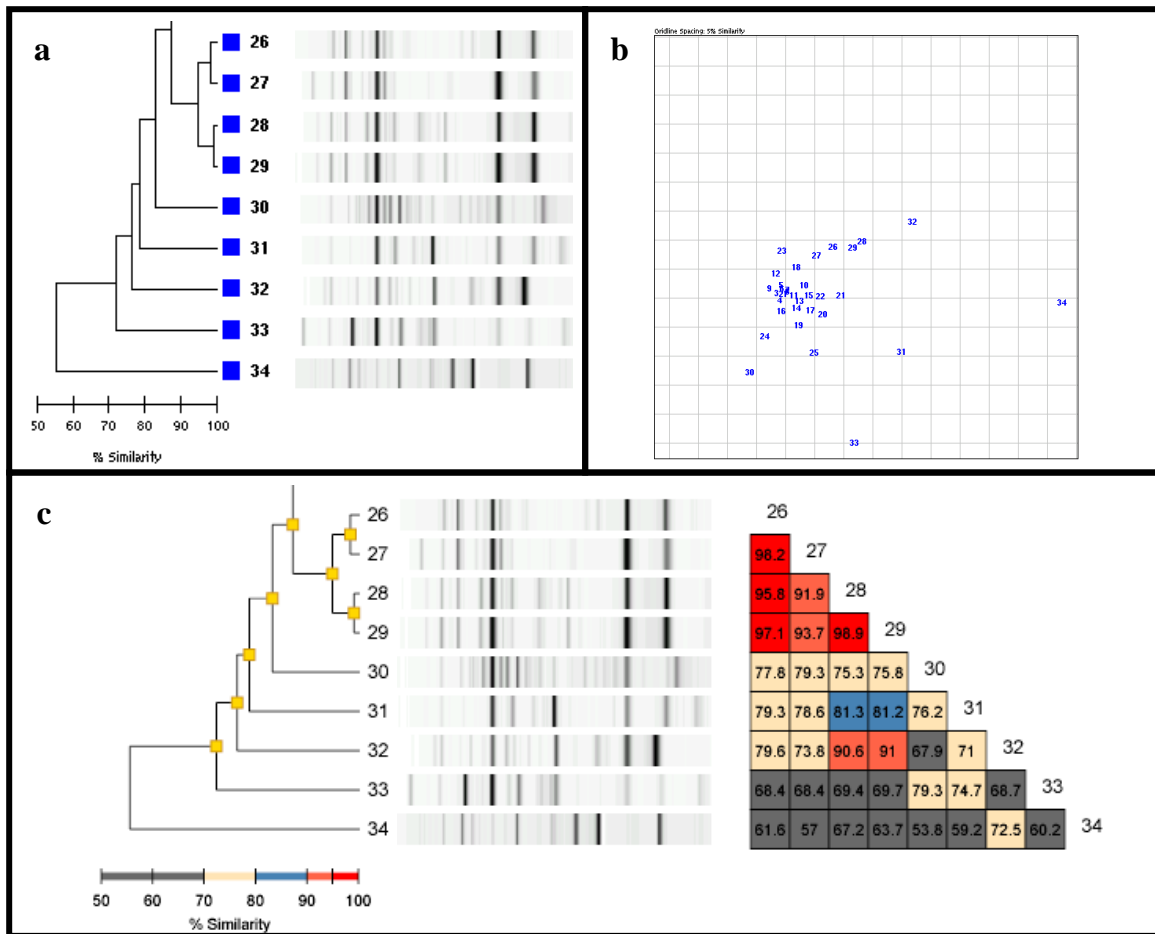


Figure 3. DiversiLab Report Components.

Note: The DiversiLab system provides several visual data interpretations summarizing the DNA typing data including (a) a dendrogram (b) a scatterplot and (c) a similarity matrix.

the use of the 95% similarity cut-off for rep-PCR analysis of *P. aeruginosa* (Doleans-Jordheim et al., 2009). All other clones (referred to as non-dominant clones) are less than 95% similar to the dominant clone group.

Patient Data

The laboratory data generated from patients included in this study spanned the period when the initial typing data was obtained in October 2004 through typing data

obtained in January 2009. Original data generation dates pertaining to patient-related variables, including clinical data, varied based on when the patients were diagnosed and when data was first entered into Port CF. Data for each variable was obtained from the patient's Port CF record and recorded as text in a Microsoft Excel spreadsheet. Categorical variables were coded as described in the Variables section. Once all applicable data was collected for a patient from Port CF, the patient's information was given an anonymous identifier for the remainder of the study, including all data analysis.

Data Analysis

The data collected for this study was analyzed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL). All data, including patient-related variables from Port CF and clone designations generated by DNA typing, were transferred from a Microsoft Excel spreadsheet into SPSS by the primary researcher.

The first research question addressed whether a significant relationship existed between infection with the dominant MRPA clone and patient-related variables prior to MRPA infection. The research hypothesis was that certain characteristics of patients with CF predict whether or not they will be infected with the dominant MRPA clone. The independent variables were multiple patient-related variables including both categorical and quantitative variables. The categorical independent variables include the following: age at time of CF diagnosis (before or after age one), CF genotypes or mutations, mother's education level, gender, days from clinic visit to MRPA infection diagnosis (categorized as ≤ 60 days, 61-120 days, or > 120 days), tobramycin use, culture result, and days from hospitalization to MRPA infection diagnosis (categorized as ≤ 90 days,

91-180 days, 181-365 days, or > 365 days). The quantitative independent variables included the following: age at time of MRPA infection diagnosis, body mass index (BMI), FEV₁, and hospitalization length in the year prior to MRPA diagnosis. The categorical dependent variable was simply the clone infecting that patient, either the dominant MRPA clone or a non-dominant clone. Descriptive statistics were calculated for patients in each clonal group (dominant MRPA clone or non-dominant clone) and included means and medians of quantitative variables for each group as well as distributions for each categorical variable by group. Logistic regression was performed, and a p-value of 0.05 (95% confidence) was considered significant. The Hosmer and Lemeshow goodness-of-fit test was assessed to determine if the model adequately fit the data, resulting in a non-significant p-value ($p > 0.05$). The significance ($p < 0.05$) of the Omnibus test of model coefficients was evaluated to determine if there was overall model significance resulting in at least one significant predictor. Logistic regression provided information (odds ratios) on how much influence each of the predictors (categorical and quantitative independent variables) had on whether the patient became infected by the dominant MRPA clone or a non-dominant clone. The analysis also determined which of the predictors were statistically significant and not due to chance.

The second research question addressed whether infection with the dominant MRPA clone was related to patient outcome. The research hypothesis was that infection with the dominant MRPA clone affects patient outcome. The independent variable was the clone infecting that patient, either the dominant MRPA clone or a non-dominant MRPA clone. The dependent variables for the first patient outcome analysis were

quantitative patient outcome data including change in FEV₁ per month, change in body mass index per month, and hospitalization days per year. The second analysis evaluated long-term patient outcomes including change in FEV₁ per year, change in body mass index per year, and hospitalization days per year. Descriptive statistics were calculated for patient outcomes in each clonal group (dominant MRPA clone or non-dominant clone) and included means and medians of quantitative variables for each group as well as distributions for each categorical variable by group. MANOVA was performed for both the short-term and long-term quantitative patient outcome variables, and a p-value of 0.05 (95% confidence) was considered significant. Several additional tests were run in parallel with the analysis including Box's test, for which a non-significant result upholds the assumption of homoscedasticity of the data, Wilks' lambda, which determines overall model significance ($p < 0.05$), and Levene's test, for which non-significance confirms the assumption of equal group error variances. MANOVA evaluated whether a significant difference existed between the two clonal groups, the dominant clone and the non-dominant clone. Finally, patient mortality was assessed by conducting a Kaplan-Meier Survival Analysis.

Limitations

A major limiting factor of this study was the sample size. The study was limited first by the number of patients with cystic fibrosis treated by the CF Center, and secondly by the number of patients with CF that became infected with MRPA. Limiting the study to only one CF care center added the risk that the dominant clone may have been specific to this one center. The study was also limited by the performance of clinically validated

laboratory assays used to analyze patient samples, specifically due to the prolonged period of clinical evaluation and laboratory testing for this study. Other limitations included possible heterogeneity in terms of clinical evaluation by multiple health care providers (even though the study was based in one center). Individual patients may have been evaluated by different providers during the course of the study (e.g. different respiratory therapists for FEV₁ measurements).

In addition, *P. aeruginosa* isolates may not have been successfully cultured by the laboratory if specimen transport was delayed or compromised in some way. Possible MRPA isolates could have been missed in the clinical microbiology laboratory. MRPA strains may have also mutated or evolved during natural history of the pathogen resulting in altered chromosomal profiles. Finally, only a limited number of bacterial colonies were evaluated so it is possible that the presence of MRPA may have been missed due to colony sampling in the clinical microbiology laboratory.

Patient-related variables and patient outcomes were compared against clone designation to determine which variables exhibited significant relationships. By combining the vast amount of patient data available in Port CF with the molecular typing results, identification of potential risk factors for acquisition of the dominant MRPA clone and implications for patient outcomes once infection with the dominant clone has been confirmed were obtained. Based on potential significant relationships, future prevention and treatment strategies as well as larger studies could be devised.

CHAPTER 4: RESULTS

The study was intended to evaluate the utility of molecular typing in multiply-resistant *Pseudomonas aeruginosa* (MRPA) infections in pediatric patients with cystic fibrosis (CF). The study was designed to retrospectively analyze the relationships between MRPA clone (dominant or non-dominant clone) and patient characteristics, including demographic information, clinical parameters, and patient outcome data in order to determine potential risk factors for acquisition of the dominant MRPA clone and subsequent differences in patient outcome between the two clonal groups.

The following chapter will include a description of the data obtained through the course of the study. Details of the sample set and data preparation will be discussed. Statistical analyses, including logistic regression for potential risk factors related to infection with the dominant MRPA clone and MANOVA for differences between the two clonal groups, will be presented as well as findings related to patient mortality, hospitalization, and statistical power.

Sample Set

Institutional Review Board (IRB) approval was obtained from Baylor College of Medicine (BCM) and Virginia Commonwealth University (VCU). Study participants were pediatric patients of the CF Care Center at Texas Children's Hospital (TCH)/BCM between 2004 and 2009. All patients had a previously confirmed MRPA infection, and

the MRPA isolates had been submitted for molecular typing by the Molecular Microbiology laboratory at TCH. In addition, all patients in the study sample had previously consented to inclusion of their data in the BCM module of the national patient registry maintained by the CF Foundation (Port CF). A total of 71 patients of the 82 patients evaluated met the inclusion criteria for the study.

Eleven patients for which molecular typing data of MRPA infection was available were excluded from the study. These patients were seen at the TCH/BCM CF Care Center for transplant evaluation. All the excluded patients have a confirmed diagnosis of CF and MRPA infection, but as they are visiting patients, their records are maintained by their original CF Care Center. Because consent for this study was based on consent to the BCM module of Port CF, data for these individuals could not be obtained.

Missing Data

In the entire data set, only two individual data points were missing. Both data points involved FEV₁ measurements for the same patient. The patient was too young (5 years old at the time of MRPA infection) to perform accurate lung function testing, so no data was available for FEV₁ measurements surrounding the time of MRPA infection. Because both data points involved a single patient, this patient was omitted from statistical analyses requiring data for FEV₁, yielding a final sample size of 70 for analyses involving lung function.

Data Preparation

Data Collection

Molecular typing data was obtained from the Molecular Microbiology laboratory at TCH, and clone designation was performed as described in the following section. Data pertaining to patient-related variables and patient outcome variables were obtained through Port CF. Each patient record was reviewed in Port CF to obtain demographic information as well as specifics of the original diagnosis (such as age of diagnosis and CF genotype). In addition, the clinic and hospital visits surrounding diagnosis with MRPA were identified, and relevant data was extracted from the records of those visits. Figure 4 displays the timeline for original collection of the data. All relevant patient data was recorded in a Microsoft Excel spreadsheet. Once all necessary data had been captured, each patient was assigned an anonymous identifier (numbered 1-71) and all patient identifiers were removed from the data sheet.

Coding of Data

Once the data set was anonymized, each categorical variable was coded and calculations for quantitative variables, such as changes in lung function or nutritional status and annual hospitalization rates, were performed as described previously.

Clone Designation

Patients were designated as members of the dominant clone or the non-dominant clone group based on previous MRPA molecular typing results generated by the Molecular Microbiology laboratory at Texas Children's Hospital. A separate report was created depicting only the 71 patients included in the study and removing all patient

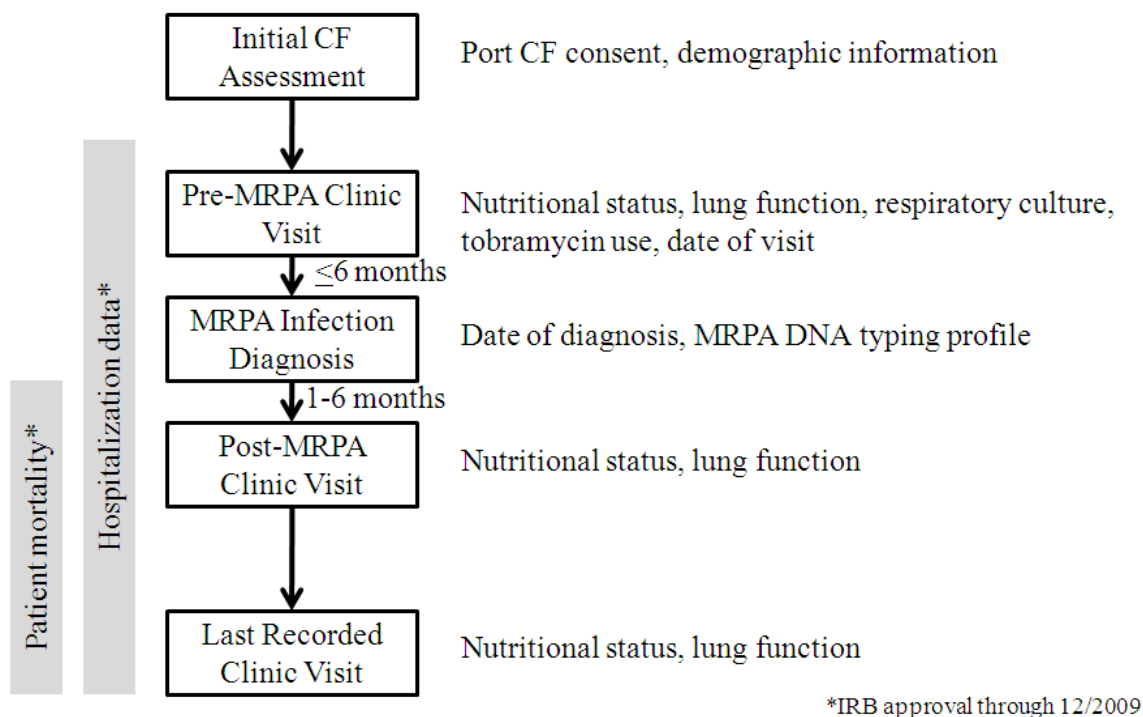
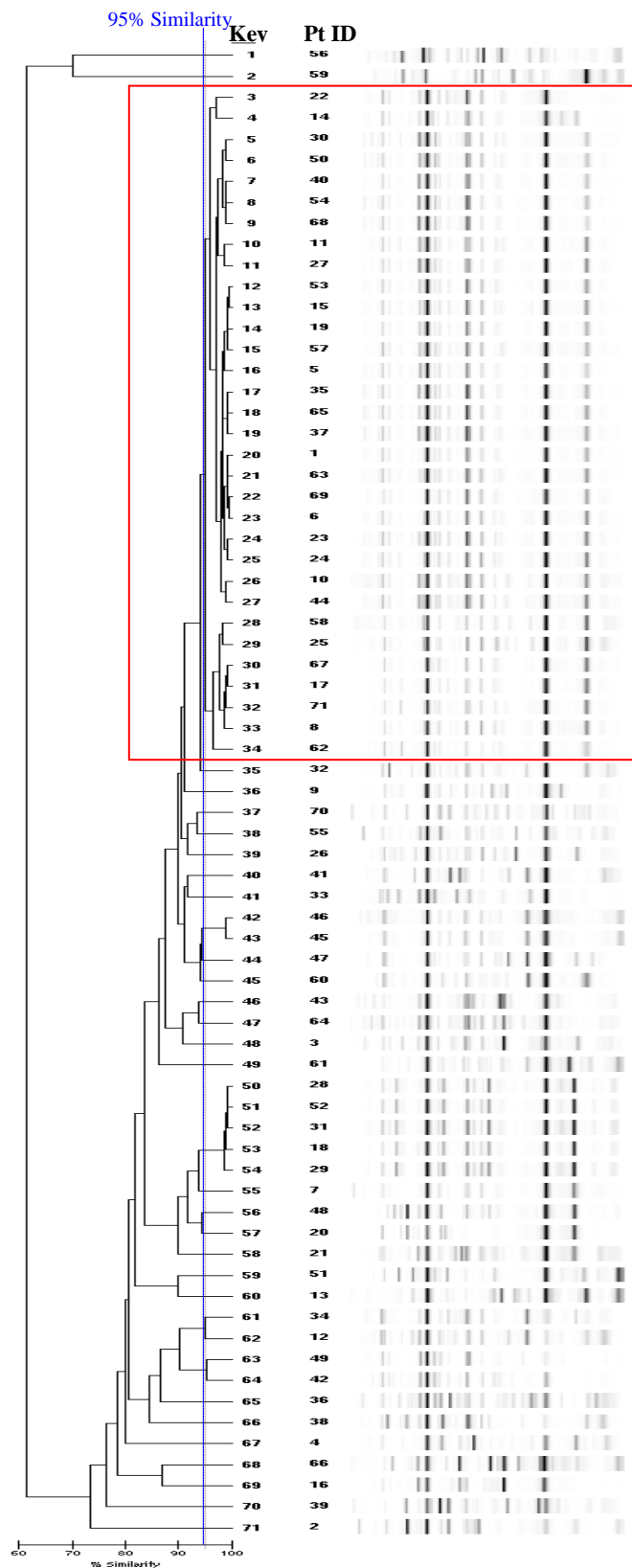


Figure 4. Timeline of Data Collection for Study Participants.

identifiers. Based on the previously described 95% similarity cutoff for group determination, 32 patients (45%) were found to be members of the dominant clone group and the remaining 39 patients (55%) were deemed members of the non-dominant clone group. The molecular profiles and dendrogram are displayed in Figure 5, with the dominant clone group identified by the red box. Similarly, the dominant clone group is highlighted by a red circle in the scatterplot representation in Figure 6. In both graphical depictions of similarity, it is evident that patient 32 (report key number 35) is highly similar to the dominant clone group. However, this patient is below the 95% similarity cutoff as illustrated in the dendrogram, and therefore, was determined to be a member of the non-dominant clone group.



Dominant Clone

Figure 5. Molecular Typing Report Dendrogram.

Note: The similarity of each molecular profile is displayed in a tree-wise hierarchy. Each patient in the report data set is assigned a key number by the online report generator, and the “Pt ID” column represents the anonymous identifier assigned to each patient for the study. The blue line delineates the 95% similarity cutoff, and the red box identifies the dominant clone group.

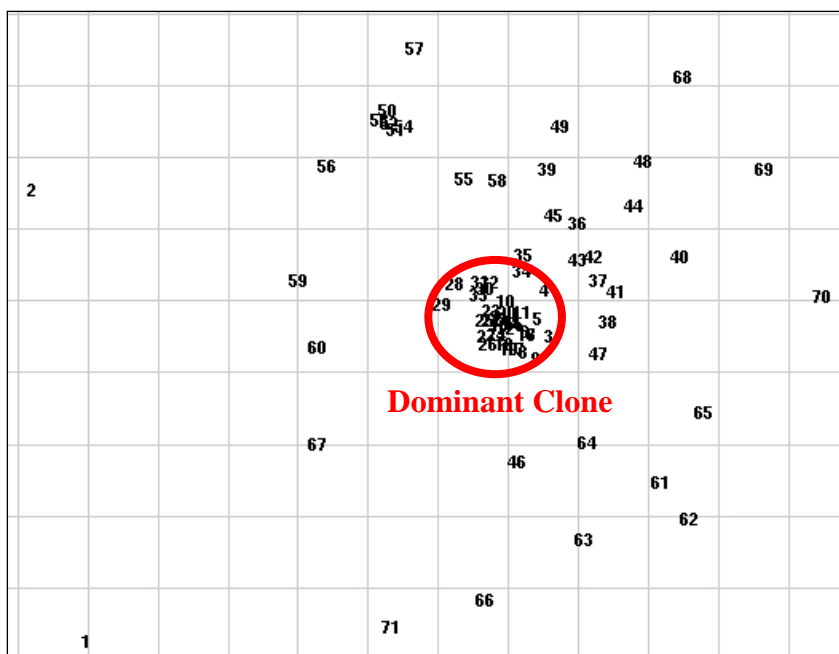


Figure 6. Molecular Typing Report Scatterplot.

Note: The scatterplot representation depicts the relatedness of each molecular profile in the data set. The dominant clone is highlighted by the red circle in the center of the scatterplot grouping.

Statistical Analysis

Patient-related Variables as Risk Factors for Dominant Clone Acquisition

The first question addressed by the study was whether a significant relationship existed between patient-related variables prior to infection and the subsequent clone the patient contracted. Categorical and quantitative variables, as described in Table 2, were selected for analysis. The proposed hypothesis is as follows:

HO1: Patient-related variables prior to MRPA infection are correlated to infection with the dominant MRPA clone.

Descriptive Statistics

Categorical variables. The distribution of all categorical variables included in the analysis of patient-related variables as potential risk factors for dominant MRPA clone

infection is displayed in Tables 4 and 5. Compared to the percentages noted in the overall sample population, there were no noticeable differences in either the dominant or non-dominant clone group in regards to demographic-based variables (gender, age at CF diagnosis, CFTR genotype, and mother's educational level). A similar pattern was seen in the categorical variables related to the time of MRPA infection diagnosis. A slight increase in the number of patients hospitalized less than 90 days prior to MRPA infection diagnosis was noted in the dominant clone group (43.75%) as compared to the non-dominant clone group (25.64%).

Table 4.

Distribution of Demographic Categorical Variables

Variable	Category	Total sample N = 71	Dominant clone N = 32	Non-dominant clone N = 39
Gender				
	Male	41 (57.75%)	20 (62.50%)	21 (53.85%)
	Female	30 (42.25%)	12 (37.50%)	18 (46.15%)
Age at CF diagnosis				
	< Age 1	54 (76.06%)	24 (75.00%)	30 (76.92%)
	> Age 1	17 (23.94%)	8 (25.00%)	9 (23.08%)
CFTR genotype				
	delF508/delF508	42 (59.15%)	18 (56.25%)	24 (61.54%)
	delF508/other	19 (26.76%)	9 (28.13%)	10 (25.64%)
	Other	10 (14.08%)	5 (15.63%)	5 (12.82%)
Mother's educational level				
	Less than HS	10 (14.08%)	4 (12.50%)	6 (15.38%)
	High school/GED	28 (39.44%)	14 (43.75%)	14 (35.90%)
	Some college	10 (14.08%)	6 (18.75%)	4 (10.26%)
	College graduate	14 (19.72%)	4 (12.50%)	10 (25.64%)
	Unknown	9 (12.68%)	4 (12.50%)	5 (12.82%)

Quantitative variables. The means and medians for each of the patient-related quantitative variables included in the analysis of risk factors for dominant MRPA clone acquisition are listed in Table 6. No noticeable difference was observed in age at the time

Table 5.

Distribution of Categorical Variables Prior to MRPA Infection Diagnosis

Variable	Category	Total sample N = 71	Dominant clone N = 32	Non-dominant clone N = 39
Days from clinic visit to MRPA infection				
	≤ 60 days	23 (32.39%)	11 (34.38%)	12 (30.77%)
	61-120 days	33 (46.48%)	13 (40.63%)	20 (51.28%)
	> 120 days	15 (21.13%)	8 (25.00%)	7 (17.95%)
Days from hospitalization to MRPA infection				
	≤ 90 days	24 (33.80%)	14 (43.75%)	10 (25.64%)
	91-180 days	16 (22.54%)	9 (28.13%)	7 (17.95%)
	181-365 days	6 (8.45%)	1 (3.13%)	5 (12.82%)
	> 365 days	25 (35.21%)	8 (25.00%)	17 (43.59%)
Tobramycin use				
	No	47 (66.20%)	23 (71.88%)	24 (61.54%)
	Yes	24 (33.80%)	9 (28.13%)	15 (38.46%)
Culture result				
	No organisms	5 (7.04%)	2 (6.25%)	3 (7.69%)
	<i>P. aeruginosa</i> (PA)	23 (32.39%)	10 (31.25%)	13 (33.33%)
	PA + MSSA	11 (15.49%)	4 (12.50%)	7 (17.95%)
	PA + MRSA	11 (15.49%)	5 (15.63%)	6 (15.38%)
	PA + other	13 (18.31%)	6 (18.75%)	7 (17.95%)
	Other organism(s)	8 (11.27%)	5 (15.63%)	3 (7.69%)

of MRPA infection diagnosis or in BMI or FEV₁ just prior to MRPA infection diagnosis.

A difference was realized in the number of days hospitalized in the year prior to MRPA infection diagnosis between patients in the dominant clone (mean = 26.53 days) and the non-dominant clone (mean = 15.21 days).

Logistic Regression

To determine if a significant relationship exists between clonal group (dominant or non-dominant) and several categorical and quantitative patient-related variables, logistic regression was employed using SPSS Statistics 17.0. Logistic regression is an

Table 6.

Means and Medians of Quantitative Patient-related Variables

Variable	Total sample N = 71	Dominant clone N = 32	Non-dominant clone N = 39
Age at MRPA infection diagnosis (in years)			
Mean	14.30	14.22	14.36
Median	15.00	15.00	15.00
BMI prior to MRPA infection diagnosis (percentile)			
Mean	37.45	37.60	37.33
Median	25.39	19.53	30.83
FEV ₁ prior to MRPA infection diagnosis (percentile)			
Mean	68.50	67.57	69.29
Median	79.54	67.99	73.71
Days hospitalized in the year prior to MRPA infection diagnosis			
Mean	20.31	26.53	15.21
Median	15.00	19.50	4.00

evaluation of the probability of group membership based on a set of predictor variables (Tabachnick & Fidell, 2001). All 12 patient-related variables were entered into the logistic regression model, and then subsets of the patient-related variables pertaining to patient demographics, data collected at the time of MRPA infection, variables related to the chronology of MRPA infection, and variables related to the disease state at the time of MRPA infection were also tested by logistic regression.

The first model included all patient-related variables for the analysis of risk factors of infection with the dominant MRPA clone. The Omnibus tests of model coefficients did not indicate overall model significance ($p = 0.720$). The Hosmer and Lemeshow test did show that the model adequately fit the data ($p = 0.367$). Significance and odds ratios for each predictor included in the model of all twelve patient-related variables are listed in Table 7.

Table 7.

Logistic Regression Output for All Patient-related Variables

Predictor	p	Odds ratio	95% C.I.
Gender	0.284	2.038	0.553-7.505
Age at CF diagnosis	0.585	1.493	0.355-6.285
CFTR genotype (delF508/delF508)	0.928		
delF508/other	0.744	0.706	0.088-5.686
Other	0.930	0.908	0.105-7.866
Mother's education level (less than high school)	0.473		
High school/GED	0.765	0.671	0.049-9.166
Some college	0.504	2.028	0.255-16.131
College graduate	0.344	3.895	0.233-65.223
Unknown	0.656	0.584	0.055-6.221
Age at MRPA infection diagnosis	0.929	0.992	0.830-1.186
Days from clinic visit to MRPA infection (≤ 60 days)	0.473		
61-120 days	0.779	0.751	0.102-5.556
>120 days	0.280	0.350	0.052-2.349
Days from hospitalization to MRPA infection (>365 days)	0.184		
≤ 90 days	0.364	2.912	0.290-29.277
91-180 days	0.105	4.798	0.722-31.879
181-365 days	0.343	0.234	0.012-4.720
BMI prior to MRPA infection	0.909	1.002	0.976-1.028
FEV ₁ prior to MRPA infection	0.967	1.001	0.968-1.035
Tobramycin use	0.761	1.270	0.272-5.918
Culture results prior to MRPA infection (no organisms)	0.681		
<i>P. aeruginosa</i> (PA)	0.981	1.043	0.034-32.412
PA + MSSA	0.628	0.579	0.064-5.258
PA + MRSA	0.178	0.192	0.017-2.114
PA + Other organism	0.525	0.444	0.036-5.446
Other organism(s)	0.906	1.165	0.093-14.668
Days hospitalized in the year prior to MRPA infection	0.453	1.015	0.976-1.056

The second model included only the patient-related variables considered demographic information, specifically age at time of CF diagnosis, CFTR genotype,

mother's educational level, and gender. Again, the model was not found to be significant ($p = 0.846$), and odds ratios were all below 2.0. Results are listed in Table 8.

Table 8.

Logistic Regression Output for Demographic Variables

Predictor	p	Odds ratio	95% C.I.
Gender	0.487	1.423	0.618-3.273
Age at CF diagnosis	0.864	0.904	0.342-2.391
CFTR genotype (delF508/delF508)	0.725		
delF508/other	0.433	0.540	0.148-1.966
Other	0.643	0.669	0.160-2.792
Mother's education level (less than high school)	0.543		
High school/GED	0.717	0.701	0.140-3.522
Some college	0.881	1.127	0.304-4.173
College graduate	0.503	1.884	0.398-8.924
Unknown	0.358	0.422	0.090-1.979

The third model tested by logistic regression included all patient-related variables relevant to the time of MRPA infection (age at time of MRPA infection diagnosis, days from clinic visit to MRPA infection, days from hospitalization to MRPA infection, BMI prior to MRPA infection, FEV₁ prior to MRPA infection, tobramycin use, culture results, and days hospitalized in the year prior to MRPA infection). The model was further divided into two subsets related to chronology of infection (age at time of MRPA infection diagnosis, days from clinic visit to MRPA infection, days from hospitalization to MRPA infection) and disease state (BMI prior to MRPA infection, FEV₁ prior to MRPA infection, tobramycin use, culture results, and days hospitalized in the year prior to MRPA infection).

The model including all patient-related variables at the time just prior to MRPA infection (results shown in Table 9) was non-significant ($p = 0.626$), but the odds ratio

Table 9.

Logistic Regression Output for Variables Related to the Time Just Prior to MRPA Infection Diagnosis

Predictor	p	Odds ratio	95% C.I.
Age at MRPA infection diagnosis	0.653	0.967	0.836-1.119
Days from clinic visit to MRPA infection (≤ 60 days)	0.456		
61-120 days	0.965	1.041	0.175-6.204
>120 days	0.334	0.466	0.099-2.195
Days from hospitalization to MRPA infection (>365 days)	0.135		
≤ 90 days	0.128	4.595	0.644-32.769
91-180 days	0.075	4.880	0.851-27.993
181-365 days	0.437	0.328	0.020-5.456
BMI prior to MRPA infection	0.987	1.000	0.976-1.025
FEV ₁ prior to MRPA infection	0.912	1.002	0.972-1.033
Tobramycin use	0.467	1.637	0.434-6.174
Culture results prior to MRPA infection (no organisms)	0.743		
<i>P. aeruginosa</i> (PA)	0.985	1.028	0.053-19.967
PA + MSSA	0.434	0.447	0.060-3.360
PA + MRSA	0.255	0.281	0.032-2.494
PA + Other organism	0.409	0.400	0.045-3.529
Other organism(s)	0.966	1.051	0.110-10.024
Days hospitalized in the year prior to MRPA infection	0.959	1.001	0.970-1.032

values related to days from hospitalization to MRPA infection were note-worthy. With a reference category of no hospitalization in the year prior to MRPA infection diagnosis, patients who were hospitalized less than 90 days prior to MRPA infection diagnosis were 4.595 times ($p = 0.128$) more likely to be infected with the dominant clone than a non-dominant clone. Similarly, patients who were hospitalized 91-180 days prior to MRPA infection diagnosis were 4.88 times ($p = 0.075$) more likely to be infected with the dominant clone.

Looking more closely at those patient-related variables that were specific to chronology of the MRPA infection (Table 10), the model was non-significant at a p-value of 0.184 but confirmed the findings in relation to hospitalization prior to MRPA infection. The one significant predictor of infection with the dominant MRPA clone versus a non-dominant MRPA clone was hospitalization less than 90 days prior to MRPA infection diagnosis ($p = 0.035$), where infection with the dominant clone was 4.019 times more likely than infection with a non-dominant clone. Patients hospitalized 91-180 days prior to infection were 2.960 times more likely to belong to the dominant clone group ($p = 0.113$).

Table 10.

Logistic Regression Output for Variables Related to Chronology of MRPA Infection

Predictor	p	Odds ratio	95% C.I.
Age at MRPA infection diagnosis	0.678	0.974	0.859-1.104
Days from clinic visit to MRPA infection (≤ 60 days)	0.329		
61-120 days	0.490	0.596	0.137-2.588
>120 days	0.149	0.361	0.091-1.439
Days from hospitalization to MRPA infection (>365 days)	0.076		
≤ 90 days	0.035	4.019	1.100-14.677
91-180 days	0.113	2.960	0.775-11.314
181-365 days	0.509	0.453	0.043-4.755

Because of the significance of recent hospitalization, patients were then divided into two groups based on hospitalization ≤ 180 days prior to MRPA infection diagnosis and > 180 days prior to MRPA infection diagnosis. A chi-square analysis to determine if the observed frequencies were significantly different than the expected frequencies was

performed based on clonal group. The results were statistically significant with a p-value of 0.017.

The subset specific to disease state at the time of MRPA infection (Table 11) was non-significant ($p = 0.731$). The number of days hospitalized in the year prior to MRPA infection was the closest to significant ($p = 0.074$) and is most likely related to the significance of the proximity of hospitalization to MRPA infection with the dominant clone (as compared to no hospitalization in the year prior to infection).

Table 11.

Logistic Regression Output for Variables Related to Disease State Prior to MRPA Infection

Predictor	p	Odds ratio	95% C.I.
BMI prior to MRPA infection	0.951	0.999	0.978-1.021
FEV ₁ prior to MRPA infection	0.723	1.005	0.977-1.033
Tobramycin use	0.473	1.501	0.494-4.561
Culture results prior to MRPA infection (no organisms)	0.890		
<i>P. aeruginosa</i> (PA)	0.769	0.662	0.042-10.360
PA + MSSA	0.280	0.364	0.058-2.277
PA + MRSA	0.295	0.339	0.045-2.564
PA + Other organism	0.362	0.397	0.055-2.895
Other organism(s)	0.527	0.521	0.069-3.933
Days hospitalized in the year prior to MRPA infection	0.074	1.024	0.998-1.052

Differences in Patient Outcome Based on MRPA Clone

The second question posed by the study was whether infection with the dominant clone affected patient outcome. The specific quantitative patient outcome variables enlisted for this analysis included short-term changes in BMI and FEV₁, long-term

changes in BMI and FEV₁, and days hospitalized per year. Patient mortality was also evaluated separately as an outcome. The proposed hypothesis was:

HO2: Infection with the dominant MRPA clone is correlated to patient outcome.

Descriptive Statistics

The mean and median of each of the quantitative patient outcome variables are listed in Table 12. Only minimal differences were noted in changes per month of BMI and FEV₁ percentile, but noticeable differences were seen in the extended time span employed to generate changes per year of BMI and FEV₁ percentile. In addition, patients infected with the dominant MRPA clone (mean = 39.42 days, median = 29.59 days) spent almost 14 more days per year in the hospital than patients infected with a non-dominant clone (mean = 25.78 days, median = 14.81 days). With regard to extended outcomes, patients were followed for a mean of 3.07 years (range = 0.61-6.87 years), with slightly longer follow-up for the dominant clone group (mean = 3.58 years) versus the non-dominant clone group (mean = 2.65 years).

MANOVA

Multivariate analysis of variance was performed to determine if a significant difference existed between the means of quantitative short-term and long-term patient outcomes for the dominant clone and the non-dominant clone. MANOVA tests whether the group mean differences between a set of quantitative dependent variables are likely to have occurred by chance (Tabachnick & Fidell, 2001). Box's test of equality of covariance upheld the assumption of homoscedasticity ($p = 0.096$ for short-term and $p = 0$ for long-term), and Levene's test of equality of error variances confirmed the

Table 12.

Means and Medians of Quantitative Patient Outcome Variables

Variable	Total sample N = 71	Dominant clone N = 32	Non-dominant clone N = 39
Change in BMI per month following MRPA infection (percentile)			
Mean	-0.04	-0.27	0.16
Median	-0.11	0.11	-0.12
Change in BMI per year following MRPA infection (percentile)			
Mean	-1.64	-3.73	0.12
Median	-0.51	-0.60	-0.38
Change in FEV ₁ per month following MRPA infection (percentile)			
Mean	0.33	0.88	-0.12
Median	0.13	0.72	-0.10
Change in FEV ₁ per year following MRPA infection (percentile)			
Mean	-2.10	-3.70	-0.75
Median	-1.92	-2.50	-0.73
Days hospitalized per year following MRPA infection			
Mean	31.92	39.42	25.78
Median	22.85	29.59	14.81

assumption of equal group error variances (p-values ranged from 0.307-0.537). Wilks' Lambda did not determine that the overall model of differences between the two clonal groups was significant (p = 0.337 for short-term and p = 0.212 for long-term). Statistics for each individual variable are listed in Table 13.

Table 13.

MANOVA Results for Differences in Patient Outcome

Outcome	F	p	Partial eta squared	Observed power
Change in BMI/month	0.368	0.546	0.005	0.092
Change in BMI/year	2.473	0.120	0.350	0.341
Change in FEV ₁ /month	1.404	0.240	0.020	0.215
Change in FEV ₁ /year	2.183	0.144	0.310	0.308
Days hospitalized	2.073	0.155	0.030	0.295

Mortality

In the complete data set of 71 patients, only 10 patients died during the course of the study. Patient outcome data was available for a variable length of time due to the design of the study (ranging from three months to six years). Four patients in the non-dominant clone group (10.26%) and six patients in the dominant clone group (18.75%) died. The length of time between MRPA infection diagnosis and death for each patient is listed in Table 14. While the average time to death for the non-dominant clone group (mean = 451 days) was nearly half of that of the dominant clone group (mean = 1062 days), the number of patients who died was too small to determine significance or perform any further analysis.

Table 14.

Patient Mortality by Clonal Group

Clone	Patient ID	Time to death	Average
Non-dominant clone	18	99 days	451 days
	28	456 days	
	32	448 days	
	49	800 days	
Dominant clone	14	380 days	1062 days
	19	1909 days	
	23	1457 days	
	37	1540 days	
	50	763 days	
	53	321 days	

Kaplan-Meier survival analysis was also performed using SPSS Statistics 17.0. The analysis compared the survival rates based on the number of days following MRPA infection diagnosis that follow-up information was available. The number of days varied

depending on when the patient was originally diagnosed with MRPA infection, how long the patient was treated at the specific CF Care center following diagnosis with MRPA, and when the patient died. Survival rates were compared between the dominant clone group and the non-dominant clone group, and the resulting graph is shown in Figure 7. Small vertical lines indicate the last date of follow-up for patients that were alive at the end of the study, and each step-down in the graph indicates a death in the clone group. Survival rates are based on the number of patients in that clone group that were still being monitored at that length of time. Survival was not found to be significant among groups with a p-value of 0.743.

Chronological Analysis of Diagnosis and Hospitalization

MRPA Infection Diagnosis Date

The clustering of the diagnoses of MRPA infections in the sample set provided additional perspective. Figure 8 depicts the date of diagnosis of MRPA infection for every patient in the dataset, with the time from infection to death noted for the 10 patients in the sample set that died. Clusters of MRPA infection diagnosis are evident in early 2004, mid-2005, and late 2006. Interestingly, 75% of new MRPA cases beyond mid-2007 were in the non-dominant clone group. Infection with the dominant MRPA clone as opposed to a non-dominant MRPA clone declined through the timeframe of the study with an incidence of 56% from 2003-2004, 47% from 2005-2006, and 36% from 2007-2008. This finding suggests that the mode of transmission may have been controlled or slowed in some way. However, an additional cluster of the dominant MRPA clone was present in late August 2008, with three patients diagnosed within 20 days of each other.

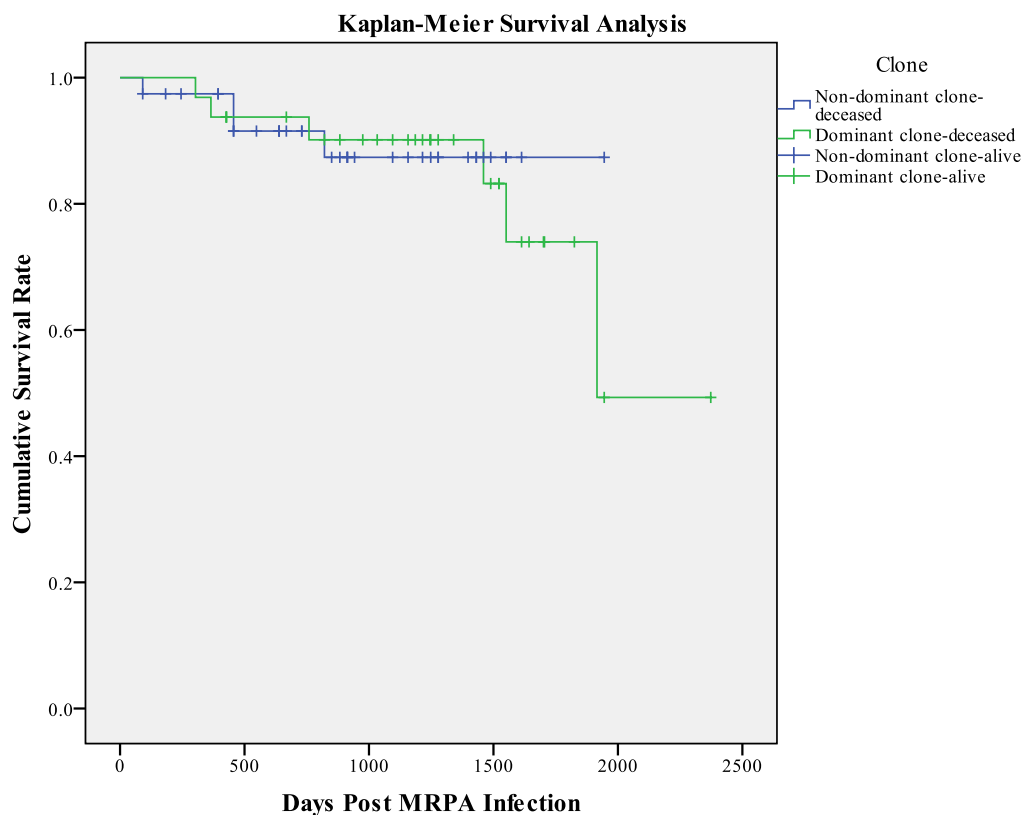


Figure 7. Kaplan-Meier Survival Analysis.

Note: The graph displays the estimated survival rate (in number of days following MRPA infection) between the dominant MRPA clone group and the non-dominant MRPA clone group.

Hospitalization Overlap

The significance of recent hospitalization prior to MRPA infection in the dominant clone group led to a more detailed evaluation of inpatient episodes in the dominant clone group. Based on the hospitalization episode chart in Figure 9, the clustering of patients' in-hospital encounters is evident. The green triangles represent the dominant MRPA clone, and the overlap in patients with that clone can be seen in late 2003 and early to mid-2005.

MRPA Infection Diagnosis Date by Clone

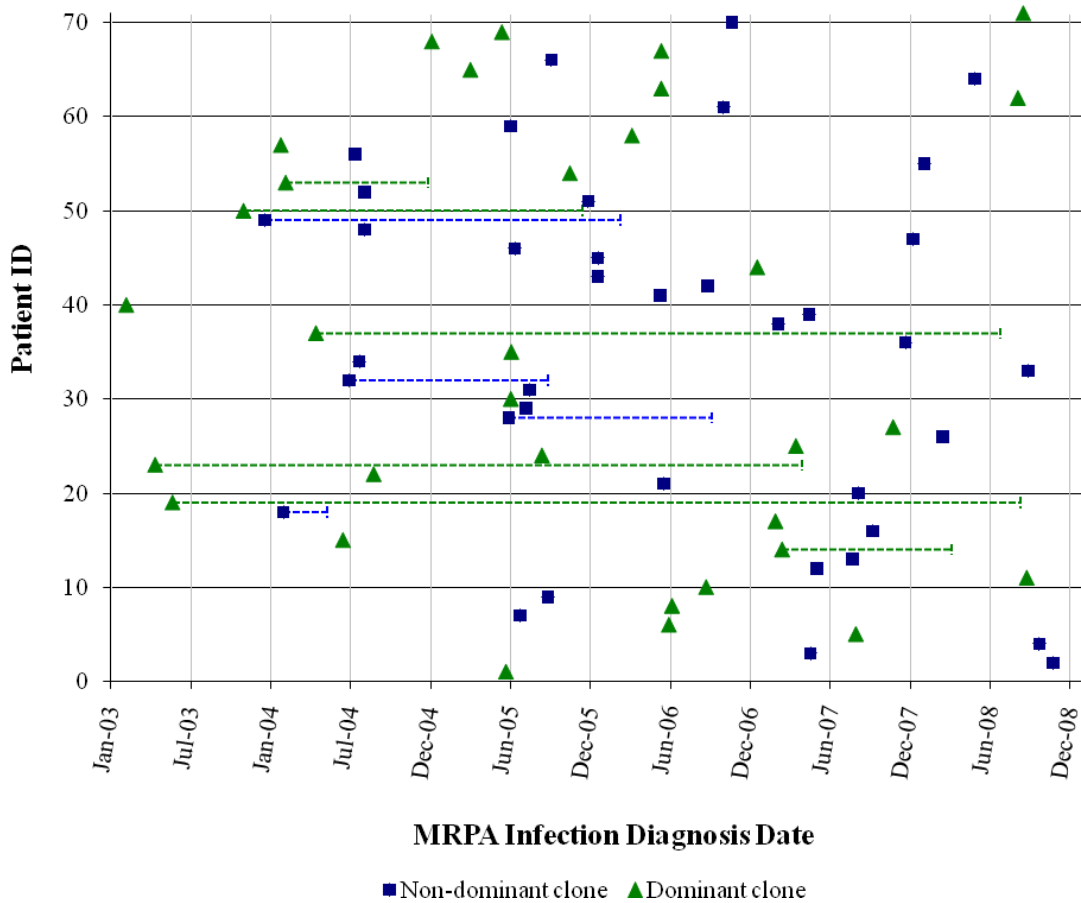


Figure 8. Timeline of MRPA Infection.

Note: MRPA Infection diagnosis dates are noted chronologically by clone, and the extended lines represent time to death for the 10 patients in the data set that died. The y-axis is the anonymized patient identifier, and the x-axis represents the date of MRPA infection diagnosis. Blue squares indicate patients in the non-dominant MRPA clone group and green triangles represent patients in the dominant MRPA clone group.

Pre-MRPA Infection Diagnosis Hospitalization Dates by Clone

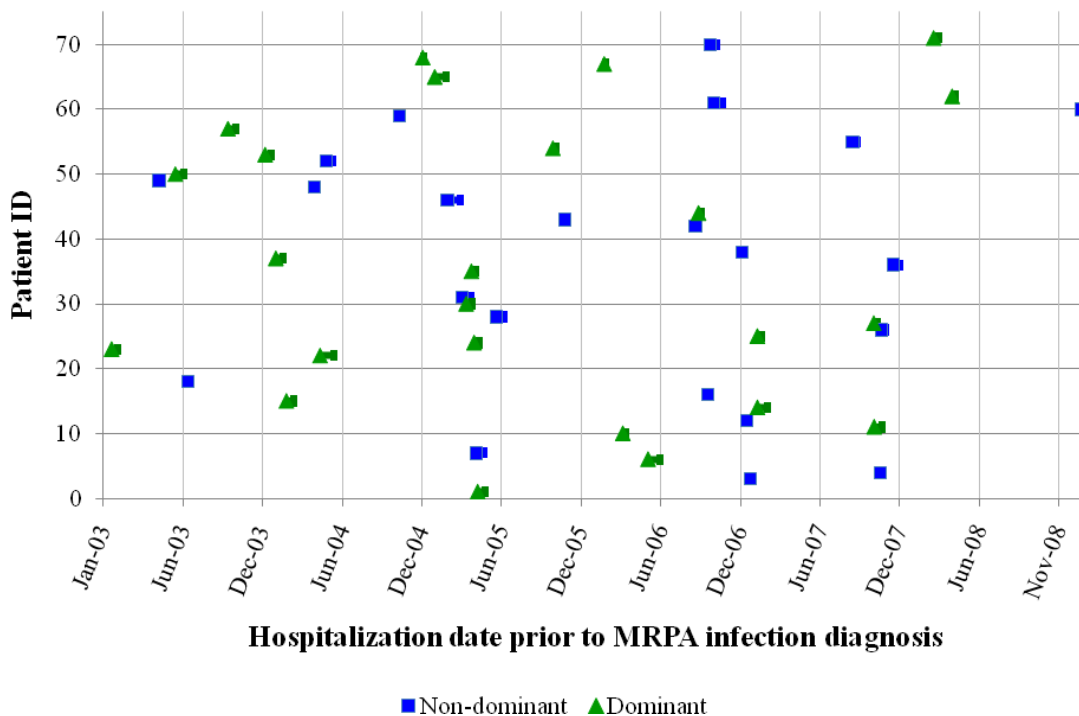


Figure 9. Hospitalization in the Year Prior to MRPA Infection.

Note: The graph displays dates of the hospitalization episode just prior to diagnosis with MRPA infection for each patient in the dominant and non-dominant clone group. The length of the hospital stay is also displayed. The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization just prior to MRPA infection diagnosis. Blue squares and green triangles denote the hospital stay just prior to MRPA infection diagnosis for patients in the non-dominant and dominant MRPA clonal groups respectively.

Taking the analysis one step further, the following figures display all hospitalization episodes, both pre and post-MRPA infection diagnosis, for patients in the dominant clone group. Significant overlap of confirmed dominant MRPA clone patients with pre-MRPA hospitalization episodes was observed. The graphs are divided into two-year intervals to more easily depict the overlap with the pre-MRPA episodes. From 2003-2004 (Figure 10), five patients with no confirmed diagnosis of MRPA were hospitalized

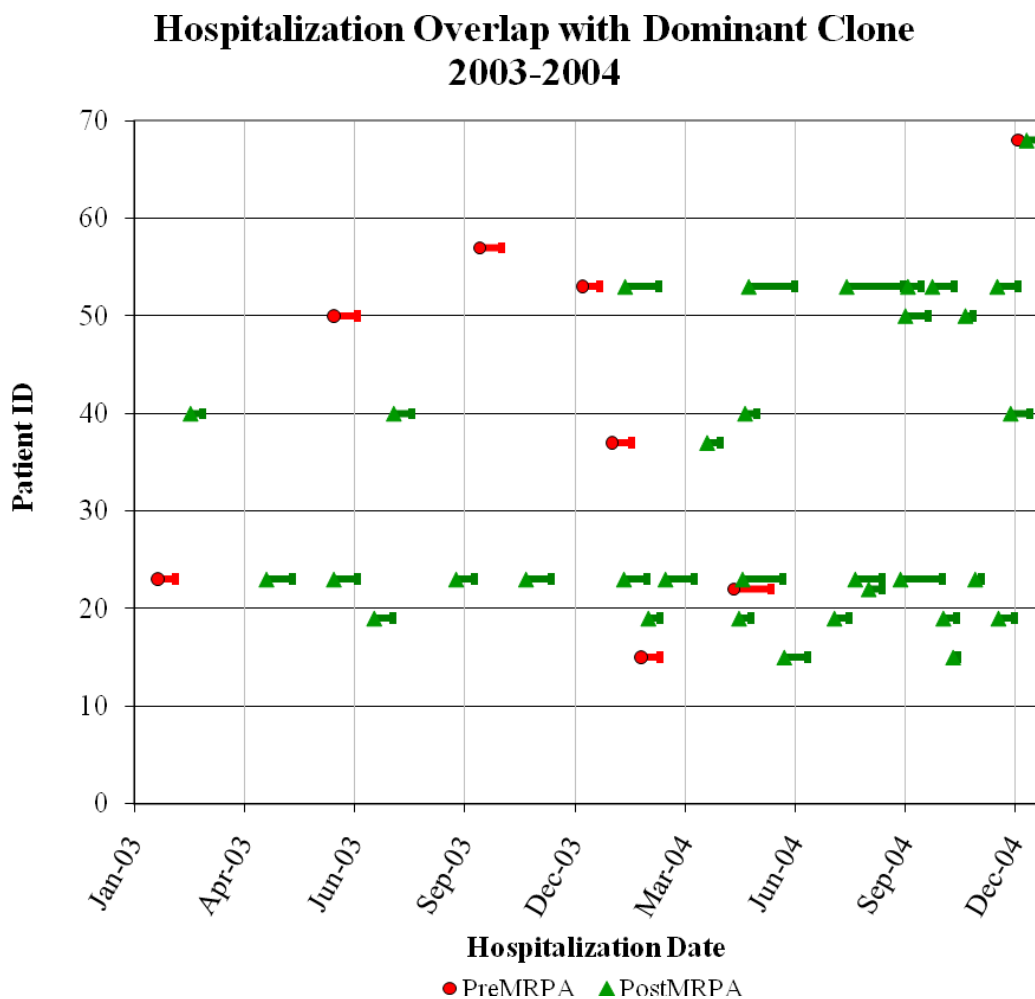


Figure 10. Hospitalization Overlap in the Dominant Clone 2003-2004.

Note: Overlap of inpatient stays for patients in the dominant clone group is compared between the hospitalization episode just prior to MRPA infection (red circles) and all post-MRPA infection diagnosis hospitalization episodes (green triangles) from January 2003 through December 2004. The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization.

simultaneously with patients infected with the dominant MRPA clone and later were diagnosed with the same dominant clone. A total of 21 of the 32 patients (65%) in the dominant MRPA clone had documented simultaneous inpatient hospital stays with previously infected dominant clone patients with ten patients in 2005-2006 (Figure 11)

Hospitalization Overlap with Dominant Clone 2005-2006

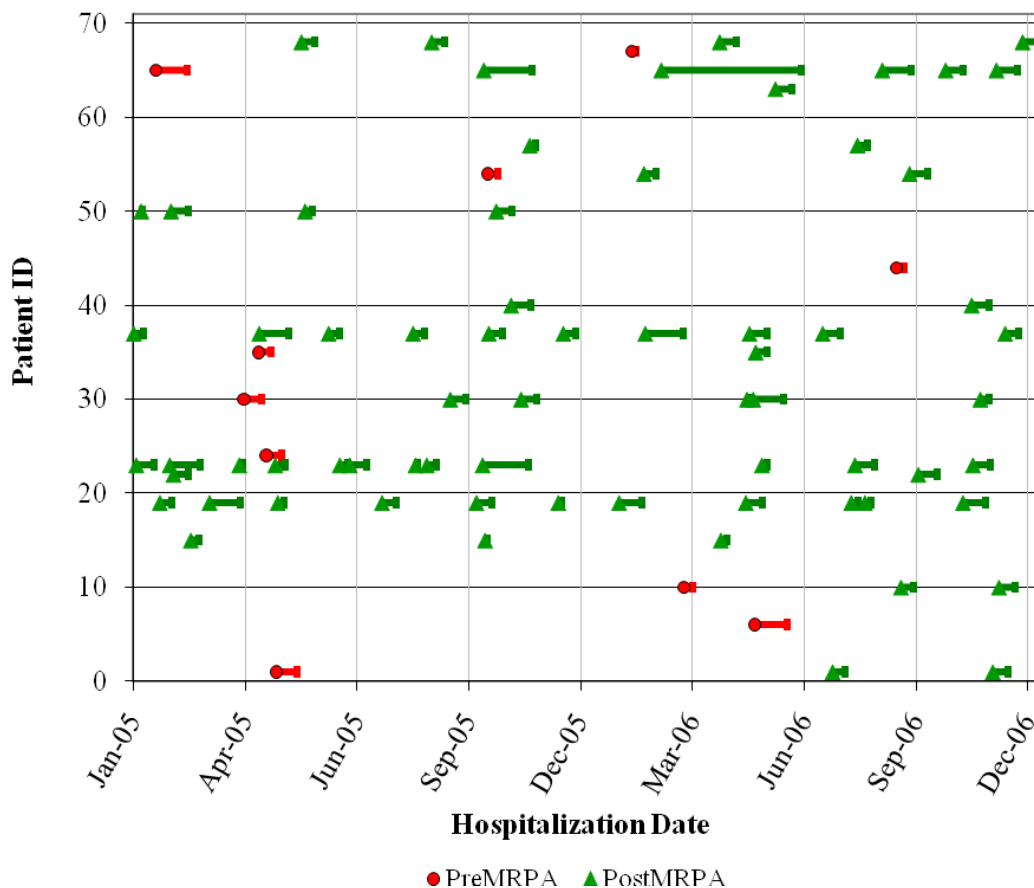


Figure 11. Hospitalization Overlap in the Dominant Clone 2005-2006.

Note: Overlap of inpatient stays for patients in the dominant clone group is compared between the hospitalization episode just prior to MRPA infection (red circles) and all post-MRPA infection diagnosis hospitalization episodes (green triangles) from January 2005 through December 2006. The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization.

and six patients in 2007-2008 (Figure 12) experiencing the same situation. The increased hospitalization rate of patients in the dominant MRPA clone group actually increased the likelihood of exposure to the clone during inpatient episodes for patients with CF.

Hospitalization Overlap with Dominant Clone 2007-2008

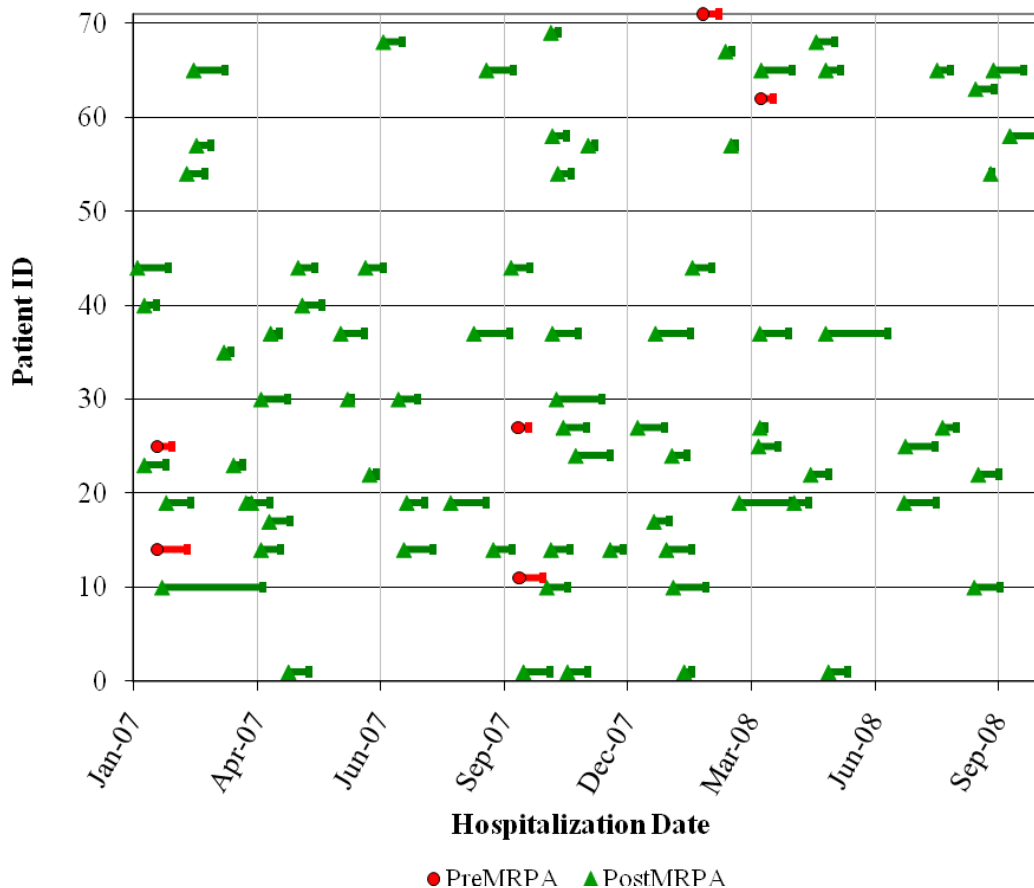


Figure 12. Hospitalization Overlap in the Dominant Clone 2007-2008.

Note: Overlap of inpatient stays for patients in the dominant clone group is compared between the hospitalization episode just prior to MRPA infection (red circles) and all post-MRPA infection diagnosis hospitalization episodes (green triangles) from January 2007 through October 2008. The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization.

A final interesting situation regarding hospitalization is the amount of time from initial respiratory sample collection to culture result. Respiratory cultures are generally obtained upon admission, but it can be several days before final results, especially antibiotic susceptibilities, are available. For nine patients in the dominant MRPA clone

group, the diagnosis of MRPA was made during an inpatient encounter. This means that upon admission, the patient was classified as not having MRPA. However, upon admission, they were in fact infected with the dominant MRPA clone as evidenced but the culture results obtained days later. Prior to September 2005, these patients would not have been treated with additional precautions, such as isolation, because their records did not indicate infection with multiply-resistant *P. aeruginosa*. Therefore, the dominant clone could have been spread throughout the inpatient area even though all standard practices were followed. In September 2005, new infection control practices were adopted in the Pulmonology unit that required all patients be placed under contact isolation until final respiratory culture results confirmed the absence of infections requiring contact isolation. Figure 13 plots the inpatient episodes for the nine patients that were diagnosed with the dominant MRPA clone during hospitalization, six of which were prior to September 2005. Based on this plot, it is possible that as many as five uninfected patients, who later were diagnosed with the dominant clone, may have come into contact with a patient with an undiagnosed dominant MRPA clone infection. Figure 14 depicts the layout of the inpatient area assigned to care for patients with CF. The proximity of the patient rooms as well as central areas for treatment, pulmonary function testing, and patient transport further emphasizes the possibility of inpatient transmission of MRPA between patients with CF.

For comparison, overlap of inpatient stays in the non-dominant clone group was also evaluated. Of the 39 patients infected by a non-dominant MRPA clone, only 14 (36%) exhibited an overlap in hospitalization prior to MRPA infection diagnosis with a

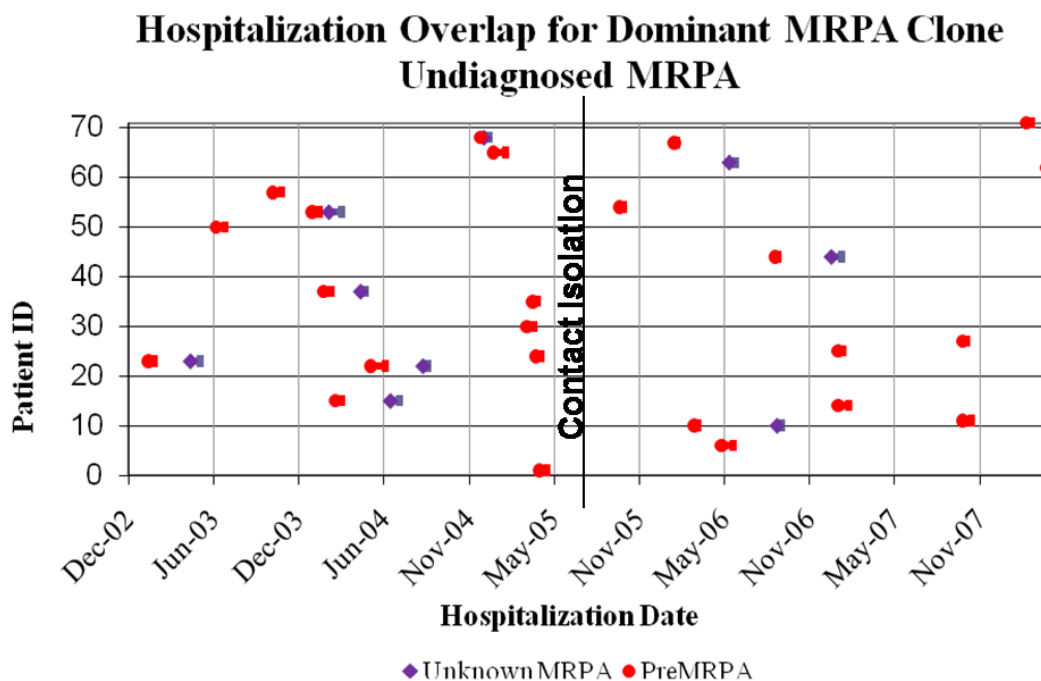


Figure 13. Hospitalization Overlap with Undiagnosed Dominant MRPA Clone.

Note: The overlap between pre-MRPA diagnosis hospital encounters and undiagnosed MRPA inpatient encounters (MRPA diagnosed from the respiratory culture obtained at time of hospital admission) is displayed for the entire study timeframe. The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization. The undiagnosed MRPA instances are marked by a purple diamond while the hospital visits just prior to MRPA infection diagnosis for patients in the dominant clone are shown by red circles. The implementation of contact isolation upon admission in September 2005 is noted by the vertical line.

patient known to be infected with the dominant MRPA clone. This is in contrast to the 65% of patients in the dominant MRPA clone group that had pre-MRPA infection hospitalization episodes coinciding with a confirmed dominant MRPA clone patient. Further analysis evaluated the undiagnosed MRPA during hospitalization factor in the non-dominant clone group. While a total of 10 patients in the non-dominant clone group were diagnosed with a MRPA infection based on their respiratory culture at time of hospital admission (thus not requiring infection control practices specific to MRPA during the first few days of the inpatient episode), no patients in the non-dominant clone

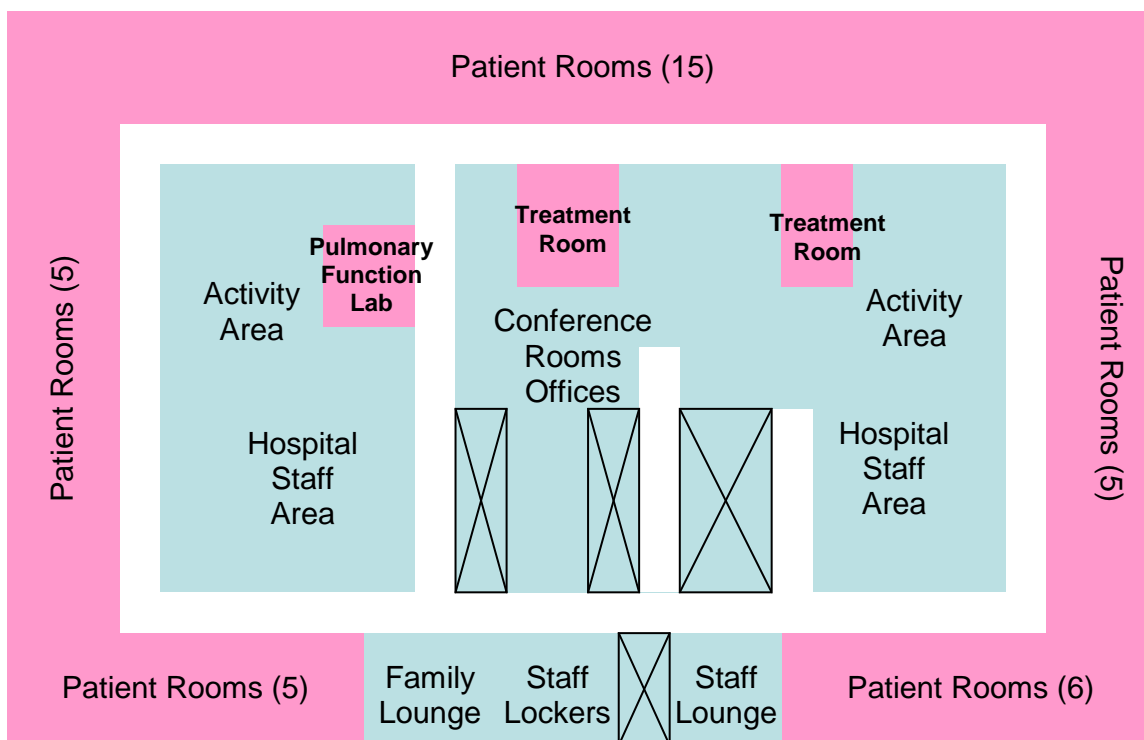


Figure 14. Inpatient Areas Assigned to Patients with CF.

Note: The diagram highlights all patient care areas in pink and hospital staff and family areas in blue. Elevator bays are displayed by the standard "x" marking.

group experienced a coinciding hospitalization prior to MRPA diagnosis before September 2005. This finding, displayed in Figure 15, further highlights the lack of simultaneous hospitalization in the non-dominant MRPA clone group as compared to the dominant MRPA clone group. Also noteworthy in Figure 15 are the coinciding hospital stays of Patients 61 (post-infection hospital stay of a known non-dominant MRPA patient) and 70 (a pre-MRPA hospital stay). The molecular profiles for these two non-dominant MRPA clone patients were unique, with a similarity of only 69.8%.

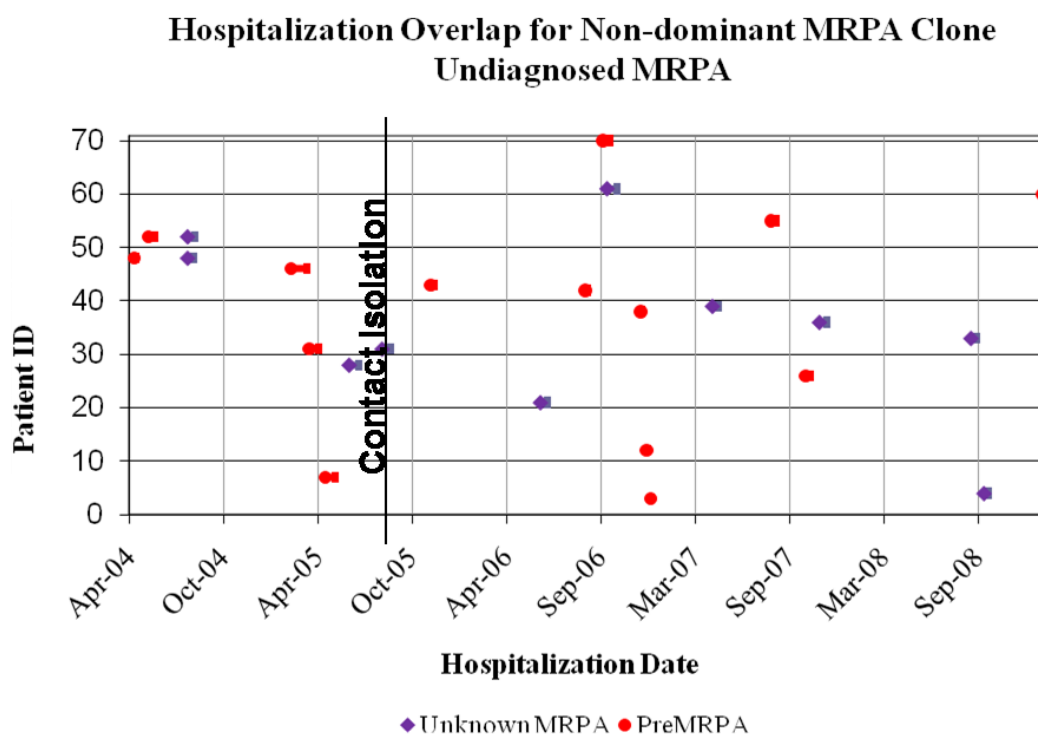


Figure 15. Hospitalization Overlap with Undiagnosed Non-Dominant MRPA Clones.

Note: Several instances of MRPA diagnosis from respiratory culture obtained upon hospital admission were present in the non-dominant clone group, represented by purple diamonds, but no overlap was seen with hospital visits just prior to MRPA diagnosis in the non-dominant clone group (red circles). The y-axis is the anonymized patient identifier, and the x-axis represents the date of hospitalization. The implementation of contact isolation upon admission in September 2005 is noted by the vertical line.

Power Analysis

The most significant limiting factor of this study was the limited sample size. Because the study was restricted to one CF care center, only 71 patients were eligible for the study. The small sample set increases the risk of a Type II error, failing to find a significant relationship when one exists (Polit & Beck, 2004). Power was estimated using nQuery Advisor 7.0 (Statistical Solutions, Saugus, MA) for the identification of risk factors for dominant MRPA clone infection and SPSS Statistics 17.0 (SPSS Inc.,

Chicago, IL) for the analysis of differences between the two clonal groups. Power projections from nQuery Advisor 7.0 assume a normal distribution of variables and were calculated using the resulting R^2 from the logistic regression analysis with an alpha level of 0.05 and a total sample size of 71. Projected power for the overall model for determination of risk factors for infection with the dominant MRPA clone was calculated to be 96%. Further division of variables into demographics and clinical parameters at the time of MRPA infection exhibited an estimated power of 42% and 87% respectively. When analyzing the smaller subsets of variables, the subset related to chronology of MRPA infection ($p = 0.184$) was the most statistically relevant and had an estimated power of 85%. The subset specific to disease state prior to MRPA infection had an estimated power of 57%. Power output from MANOVA analysis of differences in patient outcome in SPSS Statistics 17.0 is listed in Table 13. The quantitative patient outcome variables all had an estimated power below 35%.

Summary

Seventy-one patients with CF infected with MRPA were included in the study. The first research question was designed to establish if a significant relationship existed between infection with the dominant MRPA clone and patient-related variables prior to MRPA infection. The second research question evaluated if infection with the dominant MRPA clone affected quantitative patient outcome variables. Descriptive statistics were calculated for all variables, logistic regression was performed for the patient-related variables, and MANOVA was performed for the patient outcome variables. A survival analysis was performed to evaluate the significance of patient mortality, and statistical

power for each analysis was estimated. Hospitalization prior to MRPA infection diagnosis was found to be a significant factor in subsequent infection with the dominant MRPA clone. Evidence of overlapping hospitalization episodes was present in a majority of patients in the dominant MRPA clone group. These findings suggest inpatient stays were a source of transmission of the endemic MRPA strain in this specific CF population.

CHAPTER 5: DISCUSSION AND CONCLUSION

Multiply-resistant *Pseudomonas aeruginosa* is a critically important pathogen in patients with cystic fibrosis in regards to prognosis. The vast majority of patients with CF acquire *P. aeruginosa* at some point in their lives, but the antibiotic resistant strains prove the most difficult to manage and most detrimental to quality of life. The thick mucus build-up characteristic of patients with CF, due to a defective chloride transport channel, provides an ideal environment for the proliferation of respiratory pathogens such as *P. aeruginosa*. While many antibiotics have been selected to target the infection, *P. aeruginosa* eventually develops into a chronic infection due to the adaptation of the organism into a mucoid phenotype that is difficult if not impossible to eradicate in the lungs of patients with CF. Compounding the difficulty in treating patients with CF are the multiply-resistant *Pseudomonas aeruginosa* strains, organisms that are resistant to all antibiotics in two of three antibiotics classes (aminoglycosides, fluoroquinolones, and beta-lactams).

Multiple studies have been published highlighting the utility of molecular typing to determine relatedness of clones and common sources of transmission. In addition, many studies have focused on epidemiological surveillance of *P. aeruginosa* in patients with CF. Researchers in the United Kingdom and Australia have performed extensive studies across multiple CF clinics and hospitals and have documented shared *P.*

aeruginosa clones (D. Armstrong et al., 2003; Scott & Pitt, 2004). Several studies further evaluated patients with CF infected with an epidemic strain of *P. aeruginosa*, and the data showed that those infected with the clone of interest suffered worse clinical outcomes such as more rapidly declining lung function, poorer nutritional status, and increased need for hospitalization (Al-Aloul et al., 2004; Edenborough et al., 2004; Nixon et al., 2001; O'Carroll et al., 2004). However, few studies have focused solely on identifying risk factors and patient outcomes specifically in MRPA infections in patients with CF. The results of this study provide insight for the future prevention and treatment of highly antibiotic resistant *P. aeruginosa* infections in patients with CF.

Summary of the Study

The CF Center at Texas Children's Hospital has had a higher than average MRPA infection rate since the CF Foundation began listing MRPA as a separate pathogen. In 2004, the MRPA infection rate was 25% at TCH and 16.9% nationally. The latest data available from 2008 shows the infection rate at TCH to be 28% and nationally to be 17.9%. A pilot study was conducted in 2004 that evaluated the use of molecular typing for determination of relatedness in MRPA clones in patients with CF at TCH. At that time, a dominant MRPA clone was identified in the patient population. Since 2004, molecular typing of MRPA isolates has been performed quarterly, and additional patients have clustered with the dominant clone group.

Data from a total of 71 patients of the TCH CF Care Center were obtained through the BCM module of Port CF, the national data repository of the CF Foundation. Molecular typing reports regarding the MRPA isolates of all 71 patients were obtained

from the Molecular Microbiology laboratory at TCH. Patients were categorized as being infected with the dominant MRPA clone or with a non-dominant MRPA clone based on their molecular profiles. Figure 16 describes the workflow of the study including data collection, analysis, and interpretation.

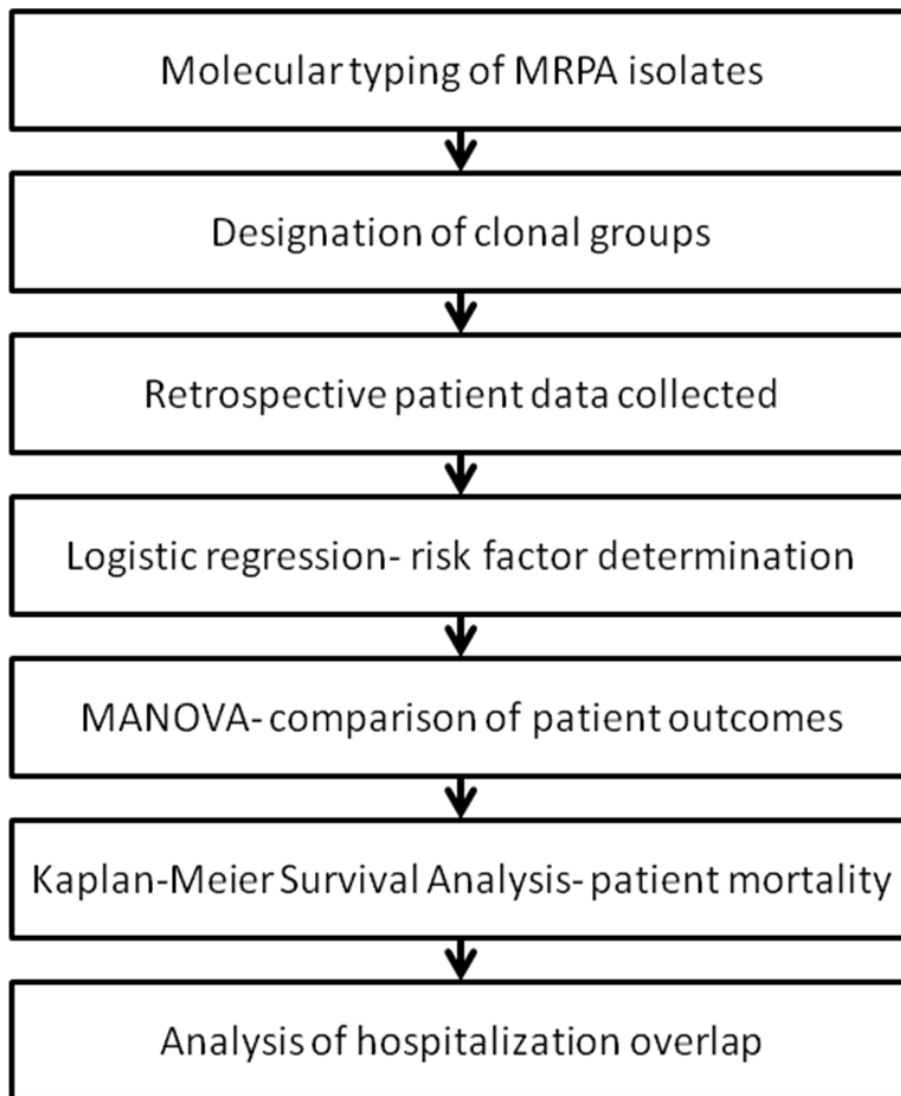


Figure 16. Workflow of Data Collection and Analysis.

Note: Molecular typing of known MRPA isolates from patients with CF provided the necessary data for designation of clonal groups. Once retrospective patient data had been collected, statistical analyses such as logistic regression, MANOVA, and Kaplan-Meier Survival Analysis could be performed. The results then led to further analysis of hospitalization overlap.

Discussion of Findings

Risk Factors for Infection with the Dominant MRPA Clone

A review of descriptive statistics for the patient-related variables in Table 2 did not uncover noticeable differences in demographic factors. While previous studies suggested these variables may have an impact on acquisition of *P. aeruginosa* infections (Demko et al., 1995; Kosorok et al., 1998; McKone et al., 2006; Rodman et al., 2005), similar distributions were observed between the two groups for gender, age at CF diagnosis, CFTR genotype, and mother's educational level. Most other categorical variables specific to the time just prior to MRPA infection also exhibited no difference in distribution, specifically days from clinic visit to MRPA, tobramycin use, and culture result. Days from last hospitalization to MRPA infection did show a noticeable difference between the two groups, and that finding will be discussed in the following section.

The quantitative patient-related variables also displayed agreement between the dominant MRPA clone group and the non-dominant MRPA clone group. The mean age at time of MRPA infection was 14 years for both groups as well as a median of 15 years for both groups. BMI and FEV₁ mean percentiles were within 1% and 2% respectively between the two groups. A difference was realized in days hospitalized in the year prior to MRPA infection diagnosis based on membership in the dominant or non-dominant MRPA clone group.

Logistic regression was performed with five different groups of patient-related variables. While no overall model was found to be significant, this could be attributed to the limited sample size available to the study. The only difference between the two

groups of patients found to be significant was hospitalization less than 90 days prior to MRPA infection diagnosis ($p = 0.035$), and the odds ratios for patients hospitalized less than 90 days and 91-180 days prior to MRPA infection were noteworthy in several of the analyses.

Hospitalization Prior to MRPA Infection Diagnosis

Several different statistical analyses highlighted the differences in hospitalization prior to MRPA infection in the two groups. While 43.75% of the patients in the dominant clone group were hospitalized less than 90 days prior to MRPA infection diagnosis, only 25.64% of patients in the non-dominant clone were hospitalized in that timeframe. Patients hospitalized within 180 days of MRPA infection diagnosis were more likely to be infected with the dominant clone than with a non-dominant clone (71.88% versus 43.59%). The model which predicted that hospitalization less than 90 days prior to MRPA infection was a significant risk factor for acquisition of the dominant MRPA clone also estimated that patients hospitalized less than 90 days prior to MRPA infection were four times more likely to acquire the dominant clone rather than a non-dominant clone. Similarly, patients hospitalized 91-180 days prior to MRPA infection diagnosis were three times more likely to acquire the dominant MRPA clone.

Further supporting the evidence of hospitalization as a risk factor for acquiring the dominant MRPA clone was the data regarding hospitalization rate in the year prior to MRPA infection diagnosis. Patients in the dominant clone group spent an average of 11 more days in the hospital in the year prior to MRPA infection diagnosis compared to patients in the non-dominant clone group. Figure 9 shows the hospitalization episode just

prior to MRPA infection diagnosis for each patient in each group. A total of 8 patients in the dominant clone group and 17 patients in the non-dominant clone group were not hospitalized in the year prior to MRPA infection and thus are not listed in this chart.

Differences in Patient Outcome Based on MRPA Clone

There was no statistically significant difference between the two clonal groups based on quantitative patient outcomes: change in BMI, change in FEV₁, and annual hospitalization rate. The patients in the dominant clone group did spend more time in the hospital with an average of 39.42 days per year (median = 29.59 days) compared to an average of 25.78 days per year (median = 14.81 days) for the non-dominant clone group. Although the dominant clone group had a higher hospitalization rate prior to MRPA infection, they exhibited a post-infection increase in hospitalization rate that was 27% greater than the non-dominant clone group resulting in almost 14 more days per year in the hospital for those infected with the dominant MRPA clone. MANOVA found none of the quantitative patient outcome variables significant, but this analysis was highly impacted by a low statistical power, which increases the risk of a Type II error.

Short-term differences in BMI and FEV₁ were not evident between the two clonal groups. Overall, there was very little short-term change in BMI or FEV₁ for any patients in the data set. Ninety-two percent of patients exhibited a less than 5% change in BMI percentile within six months of MRPA infection diagnosis. The case was similar for short-term FEV₁ changes with 86% of patients in the dataset having a less than 5% change in FEV₁ percentile within six months of MRPA infection diagnosis.

Extended outcomes began to show a separation between the two groups in terms of both nutritional status and lung function, with the dominant MRPA clone group exhibiting poorer outcomes in both areas. Data collected from the last recorded clinic visit, with a mean follow-up time of three years, showed a 4% decrease per year in both BMI and FEV₁ for patients in the dominant clone group while patients in the non-dominant clone group experienced a less than 1% change. BMI changes are difficult to interpret in this sample set due to the age range (mean age = 14 years), most patients are experiencing growth spurts so the rates of change in BMI percentile can vary greatly. However, the fact that both clonal groups had identical mean/median ages makes the finding of a greater rate of decline in the dominant MRPA clone group more noteworthy. The Cystic Fibrosis Foundation has also shown a positive association between BMI and FEV₁ percentiles (Patient Registry 2008 Annual Report, 2009; Milla, 2007).

A decrease in lung function of 1-2% per year is expected in patients with CF as they age, especially during adolescence when the frequency of lung infections increases (Patient Registry 2008 Annual Report, 2009). Recent studies have also suggested that this generation of patients with CF may have an even slower rate of decline in FEV₁ (Que, Cullinan, & Geddes, 2006). The increased rate of decline in lung function for patients infected with the dominant MRPA clone translates into a shorter life expectancy (Schunemann, Dorn, Grant, Winkelstein, & Trevisan, 2000). The previous studies documenting decreased lung function in the Liverpool epidemic *P. aeruginosa* strain and the Australian epidemic *P. aeruginosa* strain as compared to non-epidemic strains found differences in FEV₁ decline of -4.4% and -1.3%, respectively, comparable to the

difference of -2.95% documented in this study (Al-Aloul et al., 2004; O'Carroll et al., 2004). The previous studies involved both antibiotic-resistant and susceptible strains of *P. aeruginosa*, and neither study reached statistical significance due to the small number of patients meeting their inclusion criteria, similar to the limitations of this study.

With the current rate of decline, patients in the dominant clone would reach severe lung disease status ($FEV_1 < 40\%$) and qualify for lung transplantation ($FEV_1 < 30\%$) in less than 10 years (Belkin et al., 2006). To contrast, the median FEV_1 for a 30 year old patient with CF is 60% (Patient Registry 2008 Annual Report, 2009), compared to the median FEV_1 of 51% (mean = 59%) in our dominant clone population with an estimated age of 18 years (mean age at time of MRPA diagnosis = 14.22 years, mean length of follow-up = 3.58 years) at the conclusion of the study. Considering that the expected lifespan of a patient with CF was 37.4 years in 2008, it can easily be hypothesized that infection with the dominant MRPA clone will ultimately be associated with decreased life expectancy. Early studies of lung function concluded that patients with an FEV_1 value $< 30\%$ had a 50% chance of dying within two years (Corey et al., 1997; Kerem et al., 1992).

From this data, it is reasonable to conclude that the dominant MRPA clone does contribute to increased patient morbidity in terms of lung function and nutritional status in the long-term (mean = 3.07 years post-MRPA infection diagnosis). The additional hospitalization time experienced by patients in the dominant clone is also clinically relevant. In the dominant clone group, 25 patients (78%) spent more than 10 days per year in the hospital post-MRPA infection compared with 22 patients (56%) in the non-

dominant clone group. A much higher hospitalization rate was also observed in the 10 patients who died (average of 94 days per year).

Chronological Evaluation of MRPA Diagnosis and Hospitalization

The clustering of diagnosis of MRPA infection in patients with CF was evident in Figure 8. Groups of patients with CF infected with the dominant MRPA clone were present from 2004-2006 with an additional cluster in mid-2008. Beyond 2007, the majority of new MRPA infections were members of the non-dominant clone group. While the rate of infection with the dominant clone appeared to be declining, the continued incidence of new cases is further proof that a common source or mode of transmission is present at the care center.

The chronological grouping recognized in MRPA infection diagnosis was further confirmed by the evaluation of recent hospitalization episodes prior to MRPA diagnosis in Figure 9. The clusters of dominant MRPA clone acquisition could be due to a shared encounter with a patient previously infected with the dominant clone. It is difficult to determine how the clone may be spread, but due to the significance of recent hospitalization and the noticeable difference in hospitalization rates prior to MRPA infection, nosocomial transmission of the dominant MRPA clone is suspected. When comparing overlap of hospitalization in the non-dominant MRPA clone group, far fewer patients had potential inpatient exposure to patients in the dominant MRPA clone group or patients with undiagnosed MRPA.

Significance of the Results

While no complete model comparing the dominant and non-dominant MRPA clonal groups proved statistically significant by logistic regression or MANOVA, the practical significance of the findings is useful and consistent with results of similar studies. The lack of significance of demographic characteristics (gender, age at time of CF diagnosis, and mother's educational level) as risk factors for acquisition of a particular clone can help narrow the variables that should be monitored. CFTR genotype, and in turn phenotype, did not predict susceptibility to infection with the dominant MRPA clone. Likewise, the data suggests that the patient's BMI, FEV₁, tobramycin use, and culture results did not contribute to infection with the dominant clone. The most clinically significant finding is the clustering of hospitalization with patients infected with the dominant clone. With recent hospitalization as a statistically significant risk factor for acquisition of the dominant clone and increased hospitalization rates in the year prior to MRPA infection, the results do not rule out the possibility of a common source of infection within the hospital. Significant overlap in hospitalization episodes between patients infected with the dominant MRPA clone and patients who became infected with the same clone a short time later further supports this theory.

The Hopital Erasme CF clinic in Brussels, Belgium was implicated as a common source of *P. aeruginosa* infection in one study (Kersulyte et al., 1995), but inpatient hospital stays were also documented as a risk factor for infection with the epidemic *P. aeruginosa* clone. Inpatient encounters within two years of infection and overlapping hospital stays were implicated in two studies as the reason for emergence of a dominant

clone in patients with CF (Denton et al., 2002; Jones et al., 2001). Other studies went on to document the presence of epidemic strains in environmental samples collected in the clinic and hospital such as the water, sinks, creams, and counters (Bosshammer et al., 1995; Festini et al., 2007). Further research into the Liverpool epidemic strain of *P. aeruginosa* identified no clear environmental source of infection and determined that airborne dissemination was the most likely culprit in patient-to-patient transmission (Panagea, Winstanley, Walshaw, Ledson, & Hart, 2005).

The results confirm prior findings of increased virulence of an epidemic strain as documented in the Liverpool, Sheffield, and Australian epidemic strains (Al-Aloul et al., 2004; Edenborough et al., 2004; O'Carroll et al., 2004). Further investigation regarding hospitalization also suggests a difference may exist in terms of transmissibility. Patients infected with a non-dominant clone spent a substantial amount of time in the hospital post-MRPA infection diagnosis, but no extensive outbreaks of other clones have been discovered. The proliferation of the dominant clone during a period of several years speaks to the potential increased transmissibility of this epidemic strain in this particular patient care environment. Previous analysis of two epidemic *P. aeruginosa* strains in the Australian CF clinics also found increased transmissibility, and additional studies evaluating phenotypic characteristics have suggested that protease activity may play a role in the increased infectivity of the strains (Tingpej et al., 2007). Subsequently, a recent microarray analysis of the Australian Epidemic Strain-2 indicated that differential gene expression in *P. aeruginosa* may increase transmissibility (Manos et al., 2009).

Limitations

The largest limitation of the study is the sample size. This study was restricted to patients with CF at one CF Center, and inclusion criteria required a confirmed diagnosis of MRPA and prior consent to participate in the Port CF database. The small sample size resulted in potentially insufficient statistical power and increased risk of a Type II error. A significant relationship was realized between infection with the dominant clone and recent hospitalization, but it is plausible that additional risk factors could exist. Similarly, the low statistical power due to small sample size contributed to the inability of the study to identify statistically significant differences in patient outcome. While clinically significant differences were realized in terms of nutritional status and lung function, a much larger sample size was necessary to reach statistical significance. For instance, a published study regarding changes in lung function stated that for differences of 5% in FEV₁ rate of change a sample size of > 250 patients per group was necessary (Corey, 2007). As proof of that concept, an extensive study, comparing lung function of patients with CF chronically infected with MRSA compared to those who are not, using data from the national patient registry found that a -0.62% difference in rate of decline of FEV₁ was statistically significant ($p < 0.001$) (Dasenbrook, Merlo, Diener-West, Lechtzin, & Boyle, 2008). For comparison to this current study of 71 patients, the MRSA study included over 17,000 patients, of which 1732 patients were included in the MRSA infected group.

Further limiting the study was the inclusion of only one CF center. Because consent for Port CF was required for a patient's inclusion in the study and consent was only obtained for this CF care center, it was not possible to obtain data from other

centers. Therefore, it remains feasible that this dominant clone could be a geographic anomaly specific to the TCH CF Care Center. Without molecular typing data from patients across the country and access to clinical data for those patients, it is impossible to determine whether the identification of this clone is a widespread problem or a single instance of an endemic strain in one institution.

Finally, the minimal amount of information regarding details of hospitalization episodes available in Port CF was a limitation to further analysis. Port CF simply documents the dates and length of each encounter along with routine clinical data such as lung function, nutritional status, medication regimen, and microbiology results along with demographic information. Specific data regarding inpatient episodes such as which practitioners were seen, timing and frequency of procedures performed, and even hospital room number were not available. By obtaining access to this type of detailed information, more specific studies could be designed to determine the potential sources of transmission.

Implications for Clinical Practice

The significant relationship between recent hospitalization and acquisition of the dominant MRPA clone is relevant to clinical practice for patients with CF. While patients are expected to become chronically colonized with *P. aeruginosa* at some point during the course of the disease, delaying the infection as well as preventing the acquisition of multiply-resistant strains is of great importance to patient prognosis. The findings of this study point to inpatient stays as a potential source of transmission of the endemic strain, and a careful review of infection control processes is warranted. In addition, the results

highlight the potential for increased transmissibility of the dominant MRPA clone, which further reinforces the need for rigorous infection control standards. The CF Foundation has issued many recommendations regarding infection control practices specific to clinics and hospitals that care for patients with CF, but the extent to which those recommendations are adhered can vary between and within care centers (Saiman & Siegel, 2003). Following the confirmation of the existence of the dominant MRPA clone group in 2006, TCH has undergone multiple improvements to the infection control practices in the areas of the hospital that care for patients with CF. This appears to have led to a decrease in the incidence of patients becoming infected with the dominant MRPA clone as depicted in Figure 17. Discussion has also begun regarding implementation of contact precautions for all patients during inpatient episodes.

Upon discovery of an endemic or epidemic strain within an institution, it would be recommended to review infection control practices in all areas potentially caring for patients with CF based on the results of this study. In addition, once the strain has been identified, patients should be monitored closely for new infections, possibly by more frequent respiratory cultures, following a recent hospitalization. Isolation precautions are currently recommended for patients with MRPA, but review of how those procedures are handled, in patients with CF at that particular institution, with hospital staff may be prudent each time a patient currently infected with MRPA is admitted for inpatient treatment. While TCH has staff dedicated to the CF Clinic and the Pulmonology floor, education of interim personnel, such as float staff and fellows, as well as ancillary staff, such as environmental or food service personnel, is vital. In addition, a methodical re-

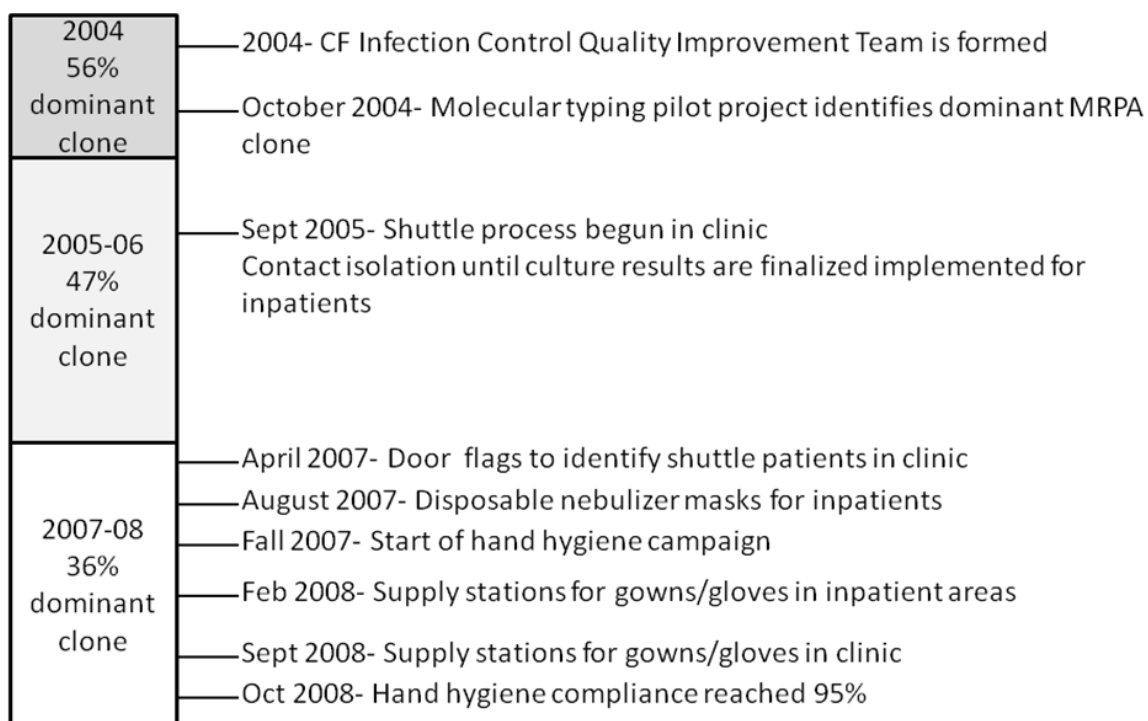


Figure 17. Infection Control Process Improvements in the Care of Patients with CF at TCH.

Note: Multiple process improvements including the implementation of contact isolation upon admission and renewed availability of proper equipment have coincided with a decrease in the number of MRPA patients infected with the dominant MRPA clone.

education of each hospital section and specific personnel involved in the care of patients with CF may be an additional resource in the prevention of transmission of an endemic or epidemic strain of MRPA.

The results of this research further confirm the clinical utility of molecular typing as a tool for epidemiological surveillance in cystic fibrosis. By identifying an endemic or epidemic MRPA strain in the CF population, molecular typing enables care providers to investigate possible sources of transmission and evaluate transmissibility of the identified strain. In addition, the stratification of patients provided by molecular typing results allows for the determination of differences in patient morbidity associated with infection

with particular strains, which in turn provides information on potential virulence of the strain.

Recommendations for Future Research

The most obvious recommendation for future studies is to expand the application of molecular typing beyond one CF center. Inclusion of multiple CF centers in various areas of the country would reveal if the dominant MRPA clone identified in this study is a geographic anomaly specific to the TCH CF Care Center. Evaluating the MRPA infection patterns at other institutions would determine if inpatient hospitalization plays a role in acquisition of MRPA globally. In addition, the larger sample size would increase the statistical power of the study. It is possible that factors that were not significant in this study could become significant in an analysis of a much larger sample set.

In terms of the CF Care Center at Texas Children's Hospital, new MRPA infections should continue to be monitored by molecular typing to determine if the isolates are members of the dominant MRPA clone group. If consent can be obtained from the original CF care centers for the transplant consultation patients infected with MRPA, then those patients should be added to the dataset in the future. Molecular typing and comparison of clinical data for transplant patients will provide further information on acquisition of the dominant clone, as well as determine if the dominant MRPA clone was present in these patients prior to their first visit to Texas Children's Hospital. Inclusion of transplant patients would also enable the use of variables related to lung transplant evaluations and procedures as potential patient outcome parameters.

In addition, patient outcome data for patients infected with MRPA should be continually reviewed to track patient morbidity associated with infection with the dominant MRPA clone. Additional years of patient outcome data for the two clonal groups may yet produce statistically significant differences that were not evident in the span of time during which this study was conducted. It is also worth noting that if improved infection control processes continue to be effective in preventing the spread of the dominant MRPA clone, then as patients originally infected by the endemic strain either transfer to adult CF centers or die, the prevalence of MRPA infection in the TCH CF Care Center should decrease and approach the national average.

An evaluation of the cost of molecular typing and the cost of treatment of patients infected with the dominant MRPA clone is another avenue to consider. While molecular typing costs are not charged to the individual patient, the cost to the hospital in terms of financial support of molecular epidemiological surveillance and increased treatment of the patients infected with the endemic MRPA strain would be useful information to both health care providers and administrators.

Future studies should also focus on the overlap of hospitalization between previously infected patients and new infections identified by molecular typing. While increasing all infection control processes to curtail further spread of the organism is ideal, determination of the original source of proliferation of the clone would provide valuable information to CF centers worldwide. Studies designed to collect highly specific information related to hospitalization episodes, such as each nurse, physician, or other care provider that enters the room, every procedure performed including time and

location, and even visitors to every patient, would enable centers to pinpoint the most likely sources of infection.

Adding to the more detailed study of inpatient hospitalization parameters would be an environmental sampling of the entire CF care center including the clinic and dedicated areas of the hospital. While patients are routinely cared for in areas designated for patients with CF, shared areas and equipment for physical therapy, patient transport, and even meal delivery could harbor the endemic strain. A thorough microbiological analysis of all sources of patient contact would be a difficult but medically valuable study to undertake. Molecular typing would enable researchers to determine if strains isolated from environmental samples were related to strains isolated from patients with CF.

Finally, it may prove beneficial to evaluate the degree of antibiotic resistance in an endemic/epidemic strain population. While classification of a strain as multiply-resistant *P. aeruginosa* is based on resistance to all antibiotics in two of the three recognized classes, incomplete resistance in the third class or which of the two classes for which complete resistance was present could vary between clones as well as within clonal groups. More thorough analysis of the different resistance patterns and how or if they relate to molecular typing profiles may reveal additional data relevant to treatment plans.

Summary and Conclusions

An endemic strain of multiply-resistant *Pseudomonas aeruginosa* was identified in the patients of the CF Care Center at Texas Children's Hospital. Molecular typing of the patient isolates confirmed the presence of a dominant clone that persists in the

institution through the conclusion of this study. Data concerning patient-related variables prior to infection with MRPA, such as demographic information and factors related to the time of MRPA acquisition and disease state just prior to MRPA infection diagnosis, were compared between the dominant MRPA clone group and the non-dominant MRPA clone group. Recent hospitalization, within 180 days of MRPA infection diagnosis, was found to be a clinically and statistically significant factor in acquisition of the dominant MRPA clone. Hospitalization rate in the year prior to MRPA infection was also higher for the dominant clone group compared to the non-dominant clone group. The majority of patients in the dominant clone group experienced a pre-MRPA diagnosis inpatient episode concurrently with a patient previously diagnosed with the dominant MRPA clone, and this extent of hospitalization overlap was not observed in the non-dominant MRPA clone group. Patient outcome variables were compared between the two groups, and clinically significant differences were noted in regard to nutritional status, lung function, and hospitalization rate. The results suggest the potential for increased virulence and transmissibility of the endemic MRPA strain.

The CF Care Center at Texas Children's Hospital has had a higher than average MRPA infection rate among patients for several years. The discovery of an endemic strain in the center has led to improved infection control practices concerning both clinic processes and inpatient protocols. A decrease in the number of patients infected with the endemic strain was realized at the end of the data collection period for this study. Continued monitoring by molecular typing will enable us to determine if this trend continues, and further diligence in infection control based on the data provided by this

study should allow for the elimination of common sources of multiply-resistant *Pseudomonas aeruginosa* infections in patients with cystic fibrosis.

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