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2016

Emerging Pathogens in Cystic Fibrosis Patients at Virginia Commonwealth University Medical Center (VCUMC)

Emily M. Hill
Virginia Commonwealth University

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Emerging Pathogens in Cystic Fibrosis Patients at Virginia Commonwealth University Medical

Center (VCUMC)

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of

Philosophy at Virginia Commonwealth University

By

Emily Marie Hill

Bachelor of Science in Clinical Laboratory Sciences, Virginia Commonwealth University, 2003

Master of Science in Clinical Laboratory Sciences, Virginia Commonwealth University, 2007

Advisor: Teresa S. Nadder, Ph.D.

Chairman and Associate Professor

Department of Clinical Laboratory Sciences

Virginia Commonwealth University

Richmond, Virginia

December 9, 2016

Acknowledgments

I would like to take this opportunity to express my gratitude to everyone who supported me throughout the course of this project. I wish to offer my most heartfelt thanks to the following people:

To my committee, Dr. Nadder, Dr. Dodd-McCue, Dr. Forbes, Dr. Korzun and Dr. Schmidt: You have all been tremendous mentors by encouraging my research and allowing me to grow as a scientist. Without your support, guidance, and encouragement, this dissertation would not have been possible.

To the clinical laboratory scientists in the clinical microbiology laboratory: Thank you for allowing me to work alongside you and lending your technical expertise!

To my family: Thank you for all the sacrifices you have made on my behalf.

To my parents, Larry and Donna: Thank you for fostering an inquisitive and curious nature, instilling a strong work-ethic, and tirelessly supporting me throughout my academic career.

To my husband, Aaron: Thank you for your unconditional love, words of encouragement, and support throughout this long journey.

To my children, Brayden and Chloe: Thank you for making every aspect of my life interesting and exciting. You both continue to inspire me each and every day.

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Glossary of Abbreviations and Definitions of Terms

a.a.	Amino acid
ARMS	Amplification Refractory Mutation System
ASL	Airway surface liquid
BA	Blood agar
BAL	Bronchoalveolar lavage
BBE	Bacteroides Bile Esculin agar
BCC	*Burkholderia cepacia* Complex
BCSA	*Burkholderia cepacia* Selective agar
BHI	Brain Heart Infusion agar
°C	Degrees Celsius
CHOC	Chocolate agar
CF	Cystic Fibrosis
CFF	Cystic Fibrosis Foundation
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CNA	Columbia Nalidixic Acid Agar
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
IRT	Immunoreactive trypsinogen
LIS	Laboratory information system
LKV	Laked Kanamycin/Vancomycin agar
MAC	MacConkey agar
MALDI-TOF	Matrix-assisted Laser Desorption Ionization Time of Flight
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NF GNR	Non-fermenting Gram-negative rod
NGS	Next Generation Sequencing
OF	Oropharyngeal flora
PCR	Polymerase Chain Reaction
PE	Pulmonary exacerbation
QC	Quality control
SAG	*Streptococcus anginosus* Group
TSA	Tryptic Soy agar
US	United States
VA	Virginia
VCUMC	Virginia Commonwealth University Medical Center

Abstract

EMERGING PATHOGENS IN CYSTIC FIBROSIS PATIENTS AT VIRGINIA COMMONWEALTH UNIVERSITY MEDICAL CENTER (VCUMC)

By: Emily M. Hill, 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, 2016.

Advisor: Teresa S. Nadder, Ph.D., Chairman and Associate Professor, Department of Clinical Laboratory Sciences

Cystic fibrosis (CF) is an autosomal recessive disorder affecting 70,000 individuals worldwide. This disease is characterized by the buildup of mucus in the airways leading to chronic lung infections resulting in pulmonary failure and death in 95% of CF patients. Routine surveillance of CF pathogens using traditional microbiology culture guides management and treatment of CF patients. Molecular profiling studies have revealed emerging pathogens that may play a role in CF lung disease by either directly causing infection or upregulating the virulence factors of classic CF pathogens, such as *P. aeruginosa*; however, routine CF culture protocols have not been modified to detect these organisms. The goal of this study was to expand the data relevant to the use of microbiology cultures for the management and treatment of CF patients at Virginia Commonwealth University Medical Center (VCUMC) by directly selecting for emerging CF pathogens in culture. This was accomplished by developing,

optimizing, and implementing an agar to select for colistin-resistant non-fermenting Gram-negative rods (NF GNRS). In addition, McKay agar and anaerobic media were utilized to recover members of the *Streptococcus anginosus* group (SAG) and anaerobes in CF respiratory samples. The prevalences of SAG, anaerobes, and colistin-resistant NF GNRs recovered on study media from 75 adult and pediatric CF patients at VCUMC were 17.33%, 41.33%, and 4% respectively. Approximately 62% of patients culture-positive for SAG were also infected with *P. aeruginosa* and 53.8% of SAG recovered in culture were from CF patients experiencing PE. These findings further support the claim that interspecies interactions among emerging and classic CF pathogens may result in periods of clinical instability or PE. Twenty-eight of the 75 patients were culture-positive for *Veillonella* species, with the majority of samples collected during a period of surveillance. Four colistin-resistant NF GNRs were isolated on the study media alone. The selective nature of the study media prevented the mixed respiratory flora and classic CF pathogens from overgrowing and obscuring the growth of these colistin-resistant NF GNRs.   The presence and role of emerging pathogens in the CF patient population at VCUMC warrants further investigation; therefore, the routine culture protocol needs to be revised to recover and select for those organisms thought to play a role in PE and lung function decline.

Chapter 1: Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder that affects the lungs and digestive system of approximately 70,000 individuals worldwide. Thirty-thousand of those diagnosed with CF reside in the United States (US) and more than 10 million Americans are carriers of one mutation of the CF gene. The number of people who carry a CF gene mutation based on race is as follows: 1 in 29 Caucasian Americans, 1 in 46 Hispanic Americans, 1 in 65 African Americans, and 1 in 90 Asian Americans. Based on this data and the autosomal recessive inheritance pattern, approximately one in every 3,000 Caucasian newborns have CF (CF Foundation, www.cff.org). Virginia Commonwealth University Medical Center (VCUMC) houses adult and pediatric CF programs, both of which are accredited by the CF Foundation (CFF). Combined, these programs follow 63 pediatric and 90 adult CF patients in Central Virginia (VA) and neighboring areas.

To date more than 1,800 CF gene mutations have been described (CF Foundation, www.cff.org). Mutations in the CF transmembrane conductance regulator (CFTR) gene result in abnormal transport of chloride and sodium across epithelium, leading to the production of thick, sticky mucus that may clog the lungs and obstruct the pancreas (Simon, 2009). Decreased mucociliary clearance leads to chronic pulmonary infections. Throughout the course of the disease, CF patients experience episodes of acute pulmonary exacerbations (PE) which eventually lead to irreversible lung damage. A universal definition of PE does not exist

among clinicians; however, it is agreed that PE is characterized by increased respiratory symptoms and worsening lung function (Zemanick et al., 2013; Sibley et al., 2008).

Progressive lung function decline is the primary cause of morbidity and mortality in the CF population. The median predicted survival of CF patients is 41 years. Treatment approaches consist of chronic suppressive antimicrobial therapy in addition to aggressive antibiotic regimens during periods of acute PE (LiPuma, 2010; CF Foundation, www.cff.org). Respiratory cultures from CF patients indicate early colonization of the lungs most commonly with *Staphylococcus aureus*, typically followed by *Pseudomonas aeruginosa*. Due to phenotypic changes within the lung, *P. aeruginosa* often transitions to a mucoid strain, which is common in CF patients. Mucoid *P. aeruginosa* strains are very drug resistant and almost impossible to eradicate (Waters, 2012). Later, patients can be colonized with members of the *Burkholderia cepacia* Complex (BCC). This is associated with advanced disease and poor patient outcomes (LiPuma, 2010). All of the above listed organisms are considered to be the classic CF pathogens.

As a result of molecular profiling studies, emerging pathogens have been increasingly recognized in the CF population (Zhao et al., 2012; Armougom et al., 2009; Rabin and Surrette, 2012; Sibley et al., 2011). These are defined as organisms found in CF respiratory samples that are not part of the canonical or classic list of pathogens. Several possible reasons exist for the increase in emerging pathogens including increased use of antibiotics, identification of pathogens with selective media and molecular methods, and increased surveillance. In the past 10 years, the life span of CF patients has risen which, in turn, is associated with an increased use of antibiotics. Incorporation of selective media for BCC isolates has caused phenotypically similar organisms such as *Ralstonia* and *Pandoraea* to be recovered in culture. Molecular methods, such as multilocus sequence typing (MLST) and pyrosequencing, are often used to characterize

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the microbial epidemiology of CF respiratory samples and have the ability to detect a wide variety of organisms that are uncultivable or simply not selected for using the current CF microbiology protocol. Frequent surveillance is also associated with an increase in emerging pathogens (Miller and Gilligan, 2003); CF patients are typically monitored on a quarterly basis. The role of emerging pathogens in CF lung disease is poorly understood. Investigating the presence of these potential pathogens in culture is the first step in trying to determine their association in CF lung disease.

Molecular profiling studies of CF patient samples revealed the presence of oral flora and anaerobes in the lung suggesting that they may play a significant role in CF lung disease. Historically, these organisms have not been considered pathogens; however, they are present in equal or greater quantities than the principal or classic pathogens. Members of the *Streptococcus anginosus* Group (SAG), formerly known to as the *Streptococcus milleri* group (SMG), have been associated with PE and lung function decline. SAG consists of *Streptococcus constellatus*, *Streptococcus anginosus*, and *Streptococcus intermedius*, which exist as normal flora in the oropharynx and gastrointestinal (GI) tract in approximately 15-30% of the population (Parkins et al., 2008). Their presence in CF respiratory samples has been overlooked and attributed to oropharyngeal contamination acquired through the specimen collection process.

Current CF microbiology protocols do not select for SAG. Using the prescribed battery of media, SAG are often overgrown by principal pathogens such as *P. aeruginosa*. In addition, they are phenotypically indistinguishable from other nonpathogenic *Streptococcus* species. Sibley et al. (2010) developed and incorporated a semi-selective solid medium, McKay agar, to select for SAG. A six-month surveillance study revealed that SAG had a prevalence of approximately 40% in the population studied. In another study conducted by Sibley et al.

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(2008), molecular methods identified SAG as the numerically dominant organism in a group of hospitalized adult CF patients experiencing acute PE, strongly suggesting association of this organism group with clinical deterioration. Notably, when this group of organisms was targeted for therapy, resolution of symptoms was observed and baseline lung function restored even in the absence of anti-pseudomonals. Waite et al. (2012) reported on the ability of SAG to proliferate within the lung prior to and during the onset of PE, indicating that this group could potentially represent a microbiologic marker of exacerbation. The increasing recognition of SAG as a key player in the microbial community of the CF lung as well as its reported ability to cause acute exacerbations in this specified population of patients warrants the need for routine isolation and identification in culture (Waite et al., 2012; Filkins et al., 2012).

Hypoxic conditions in the CF lung provide a favorable environment for anaerobes, though their role in the disease process is unclear. Animal lung models have shown that certain anaerobes have the ability to become cytotoxic and immunogenic in high quantities (Zemanick et al., 2013). Others have reported the ability of anaerobic bacteria to enhance the virulence of classic CF pathogens such as *P. aeruginosa* (Field et al., 2010). Currently, CF respiratory culture protocols do not include anaerobic media or incubation conditions. Specifically targeting anaerobes in culture could aid in determining their role in CF lung disease.

Selecting for glucose non-fermenting Gram-negative rods (NF GNRs) in culture by incorporating additional media has led to the emergence of other, previously unrecognized NF GNRs, which often exhibit multidrug resistance. For example, selective media for members of the BCC, is part of the routine battery of culture media. Phenotypically similar NF GNRs, with multidrug resistant susceptibility profiles, grow on this media as well. This can lead to misidentifying organisms such as *Ralstonia* or *Pandoraea* as BCC, a confirmed and widely

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recognized CF pathogen, which has great clinical and psychosocial implications for the patient. Because members of the BCC are considered epidemic strains, infection control measures of culture-positive patients must be followed. BCC culture-positive CF patients are often isolated or physically separated from those who are BCC-negative. In addition, acquisition of BCC is often associated with more severe disease and worsening lung function. Before selective BCC media was added to routine CF culture protocols, the presence of emerging NF GNRs in clinical samples was not detected. Due to extremely resistant susceptibility patterns, particularly to colistin, their role in CF lung disease needs to be determined. Colistin is frequently used as the last line of defense when treating multidrug resistant *P. aeruginosa* infections and also in efforts to help prevent the establishment of chronic infections in the lung. As a result, Kiska and Riddell (2012) suggested fully identifying any colistin-resistant NF GNRs isolated in CF samples. Therefore, development and incorporation of media selecting for colistin-resistant NF GNRs is warranted to further recognize their presence in respiratory samples and determine their role in CF lung disease.

  Inquilinus limosus is a NF GNR associated with CF lung function decline. This organism has a mucoid phenotype and either grows poorly or fails to grow on MacConkey agar (MAC). *I. limosus* can grow on Blood agar (BA) and Chocolate agar (CHOC); however, the colony morphology is identical to that of mucoid *P. aeruginosa*. Since many CF patients harbor strains of mucoid *P. aeruginosa*, *I. limosus* is not recognized as an additional NF GNR in culture, and could potentially be overlooked. Distinguishing between the two organisms is imperative for treatment purposes. Most strains of mucoid *P. aeruginosa* are susceptible to colistin, whereas *I. limosus* are resistant (Kiska and Riddell, 2012).

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I. limosus has been cultivated on all *Burkholderia cepacia* selective media except *Burkholderia cepacia* selective agar (BCSA) due to incorporation of gentamicin. The clinical microbiology laboratory at Virginia Commonwealth University Medical Center (VCUMC) uses BCSA in the primary battery of media for CF respiratory cultures. Consequently, if *I. limosus* is present in patient samples, the BCSA will not support its growth. Development and incorporation of media selecting for this organism is needed to determine the presence of *I. limosus* in CF patients at VCUMC (Coenye et al., 2002; Hayes et al., 2009).

Over the past 10 years, much attention has been devoted to developing and implementing molecular techniques, such as MLST, Sanger sequencing, and pyrosequencing to characterize the microbial epidemiology of CF airways. As a result, little effort has been spent on revisiting and revising CF microbiology protocols and the classic list of CF pathogens. Although molecular methods provide a vast amount of information regarding the CF lung microbiome, it is not currently feasible to adopt in the clinical setting due to cost and labor intensiveness. With the growing body of literature reporting on the presence of emerging pathogens in CF and their potential role in disease progression, culture protocols must be reviewed and optimized in order to provide clinicians with the data needed to manage and treat CF patients (Miller & Gilligan, 2003; Armougom et al., 2009).

Purpose and Specific Aims

The purpose of this study was to expand data relevant to the use of microbiology cultures for the management and treatment of CF patients at VCUMC. Three specific aims guided this study. Refer to Table 1 for an overview.

Table 1. *Overview of Specific Aims*

Specific Aims	Steps to Accomplish Aims
SA1: Develop culture-dependent screening protocols to detect emerging pathogens in the CF population at VCUMC.	Incorporate previously developed agar to select for SAG and anaerobes and develop agar to select for colistin-resistant NF GNRs in CF respiratory cultures.
SA2: Determine the prevalence of emerging CF pathogens in the patient population at VCUMC.	Calculate the prevalence of targeted emerging pathogens recovered in culture from CF patients at VCUMC.
SA3: Prospectively evaluate current culture techniques in conjunction with newly developed and implemented protocols.	Compare culture results from the routine and research protocols and document discrepancies.

Specific aim 1. Develop culture-dependent screening protocols to detect emerging

pathogens in the CF population at VCUMC. Members of the SAG, anaerobes, and colistin-

resistant NF GNR, which have been recognized as emerging CF pathogens that may play a

significant role in the onset of PE, were targeted. McKay agar, a semi-selective solid medium

for SAG was added to the routine battery of media. Anaerobic media and anaerobic incubation

conditions were utilized to detect this select group of organisms. Development and

implementation of an agar-based medium supplemented with colistin sulfate was used to select

for colistin-resistant NF GNRs. Culture conditions were optimized in order to detect the

presence of targeted or emerging pathogens in the CF population at VCUMC.

Specific aim 2. Determine the prevalence of emerging CF pathogens in the patient

population at VCUMC. The routine CF culture protocol selects for and cultivates the classic CF

pathogens known to play a role in CF lung disease. Molecular profiling studies have revealed

the growing list of emerging pathogens thought to play a role in lung function decline. The first

step needed to investigate the potential role of emerging CF pathogens in a population is to

determine the prevalence. Therefore, prevalences of SAG, anaerobes, and colistin-resistant NF GNR were calculated.

Specific aim 3. Prospectively evaluate current culture techniques in conjunction with newly developed and implemented protocols. This aim was accomplished by comparing culture results from both the routine and research microbiology culture protocols. Any organisms that were cultivated and identified in the research protocol only were recorded. Any discrepant results between the routine and research protocols were evaluated.

Significance of Study

CF affects approximately 70,000 individuals worldwide (CF Foundation, www.cff.org). Routine surveillance of CF pathogens at individual CF centers are used to track and trend the incidence and prevalence of specific organisms and remains the method of choice to effectively manage CF patients. There has been an increased interest in developing molecular methods to uncover members of the CF lung microbiome. These molecular profiling studies have been very useful in revealing the growing list of pathogens that may play a role in CF lung function decline; however, CF culture protocols have not been modified to detect these organisms. Traditional cultures succeed in recovering only the same well-characterized species and provide an incomplete picture of bacteria in the airway that could either directly or indirectly contribute to lung function decline in this population. Therefore, the current CF protocol for culturing, isolating, and identifying CF pathogens needs to be reviewed, revised, and updated to include emerging pathogens strongly associated with worsening clinical status. The presence of emerging pathogens and their role in CF lung disease cannot be ignored. If present in clinical specimens, emerging pathogens must be reported in order for clinicians to make informed decisions and provide optimal care when managing and treating CF patients.

Chapter 2: Literature Review

Introduction

The following chapter will present background information relevant to CF. First, CF will be described in general terms to include the following: inheritance patterns, incidence, CFTR protein and function, CFTR mutations, carrier screening and detection, treatment and infection control, pulmonary exacerbations and classic CF pathogens. Next, information related to the lung microbiome will be presented highlighting emerging pathogens, oral flora, SAG, anaerobes, and NF GNRs. Finally, molecular profiling methods and culture-dependent methods used to isolate both classic and emerging CF pathogens will be summarized.

Cystic Fibrosis

CF is an autosomal recessive disorder that affects approximately 30,000 children and adults in the United States (US) and over 70,000 individuals worldwide (CF Foundation, www.cff.org). The incidence in the US for Caucasians, Hispanics, Asians, and African Americans is 1 in 2,500 to 3,500, 1 in 4,000 to 10,000, 1 in 100,000, and 1 in 15,000 to 20,000, respectively. Approximately 1,000 new cases of CF are diagnosed each year with a median predicted survival of 41 years (CF Foundation, www.cff.org).

This chronic disease, affecting the lungs and digestive system, is caused by mutations in a single gene located on the long arm of chromosome 7 (7q.31.2) that encodes the cystic fibrosis

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transmembrane conductance regulator (CFTR) protein. More than 10 million Americans are symptomless carriers of the defective genes which make CF the most common life-shortening disorder in Caucasians (CF Foundation, www.cff.org). Based on the autosomal recessive inheritance pattern, each time two carriers of a defective CF gene have a child, there is a 25% chance the child will have CF, a 50% chance the child will be a symptomless carrier of a defective CF gene, and a 25% chance the child will neither carry a defective CF gene nor have CF.

In 2001, CF carrier screening was incorporated into routine obstetric practice. Molecular methods, such as the Amplification Refractory Mutation System (ARMS), multiplex PCR, and Next-Generation Sequencing (NGS) are employed for prenatal or carrier screening. It is estimated that as many as 1 in 29 Caucasians are carriers of a defective CF gene with approximately 1,800 mutations documented to date. In 2004, the American College of Medical Genetics recommended that a standard 23 mutation panel be used for prenatal or carrier screening. This pan-ethnic panel consists of mutations present in at least 0.1% of those diagnosed with classic CF. The spectrum of mutations is heterogeneous among ethnic groups; therefore, the sensitivity of carrier screening is as follows: 94% in Ashkenazi Jews to 88% in non-Ashkenazi North American Caucasians, about 72% in Hispanic Americans, 64% in African Americans, and 49% in Asians (American Congress of Obstetricians and Gynecologists, www.acog.org).

Newborn screening for CF began in March 2006, and by 2010 was conducted in all 50 states as well as the District of Columbia. CF screening methods employed in state laboratories may vary; however, increased immunoreactive trypsinogen (IRT) levels in blood suggest CF and warrant additional testing in the newborn. In some states, such as Virginia (VA), screens with

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increased IRT levels reflex to DNA testing. If CF gene mutation(s) are detected, confirmatory

testing is performed. The sweat chloride test is considered the standard diagnostic test with a

result greater than or equal to 60 mmol/L confirming the disease. Once definitively diagnosed,

the CF genotype is determined by molecular methods. Most children are diagnosed by the age of

two and a small number of CF patients with a milder form of the disease are not diagnosed until

approximately 18 years of age. Symptoms in newborns include delayed growth, failure to gain

weight during childhood, absence of bowel movement in the first 24-48 hours of life, and salty

tasting skin. (CF Foundation, www.cff.org).

The CF gene spans approximately 230 kb and is composed of 27 exons. This gene is

transcribed into a 6.5 kb messenger RNA (mRNA) that encodes a 1,480 amino acid (a.a.)

protein. More than 1,800 mutations of the gene have been identified to date; however, only 23

mutations have been identified with a frequency of at least 0.1% of known alleles (Salvatore et

al., 2011; Gibson et al., 2003; American Congress of Obstetrics and Gynecologists,

www.acog.org). The 1,800 mutations can be classified as CF causing mutations, mutations of

unknown significance, mutations of varying clinical consequence, and non-CF causing mutations

(CF Foundation, www.cff.org). Delta F508 accounts for 70% of CF alleles in the Caucasian

population. This mutation is characterized by a three base pair deletion that codes for

phenylalanine at position 508 of the CFTR protein (Gibson et al, 2003).

The CFTR protein regulates the transepithelial ion flow of airway surface liquid (ASL).

Mutations in the CFTR protein affect sodium and chloride ion transport, disrupting the ionic

composition and volume of ASL. Under normal circumstances, ASL is thin in consistency

allowing clearance of inhaled microorganisms via ciliary action. When CFTR mutations are

present, ASL increases in viscosity which clogs the airways. This alteration impedes the

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clearance of microorganisms; and as a result, mucosal surfaces are heavily colonized by microbes that contribute to progressive lung function decline over the lifetime of the patient. The neutrophilic inflammatory response is chronically activated in the presence of persistent microbes which ultimately lead to pulmonary failure and death in 95% of patients (Kiska & Riddell, 2012; LiPuma, 2010; Lynch & Bruce, 2013; Tunney et al., 2008).

Treatment and infection control. CFTR is expressed on epithelial cells found in the airway, paranasal sinuses, pancreas, gut, biliary tree, vas deferens, and sweat ducts. This protein is responsible for regulating the flow of salt and fluids in and out of cells. Mutations in the gene that codes for the CFTR protein cause CF, which is truly a multisystem disease. Pulmonary manifestations include sinusitis and persistent lung infections, characterized by chronic cough and sputum production, chronic wheezing, bronchiecstasis, and atelectasis. In addition, pancreatic insufficiency and CF-related diabetes is also seen in this population (Spoonhower and Davis, 2016; Flume, 2009). Over the years, improved clinical outcomes are attributed to more effective formulations of pancreatic enzymes, mucolytic agents, airway clearance techniques, and antibiotic treatment regimens. Despite the many advances, greater than 90% of CF mortality and morbidity are due to suppurative lung disease (Bell & Robinson, 2007). Various treatment strategies exist to treat and manage this multisystem disorder. For example, mucolytic agents, hypotonic saline, physiotherapy, and antibiotics are chronically prescribed to prevent PEs. Pancreatic enzymes and nutritional supplementation are used to provide and maintain nutritional support. Periods of acute PE often involve hospitalization where additional antibiotics are administered intravenously.

Infection control among the CF population is imperative. Pathogens, such as members of the BCC, are referred to as epidemic strains; that is, they may be acquired from another CF

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patient or from contaminated environments. This may occur through direct or indirect contact with infected respiratory secretions. Infection control guidelines are issued by the American CF Foundation (CFF). Recommendations include obtaining quarterly surveillance cultures, discouraging socialization among hospitalized CF patients, educating patients and families about hand hygiene and cleaning, and disinfecting nebulizers (Garber et al., 2008). In addition, it is also recommended that CF patients wear hospital masks in healthcare settings, maintain a six foot separation from other CF patients, and limit the time spent in waiting areas (Saimanet al., 2013).

Pulmonary exacerbations. PEs are the leading cause of morbidity in CF patients. Overt immunological responses in patients during periods of PE lead to irreversible lung damage (Sibley & Surette, 2011). Clinical features of PE include the following: increased cough, increased sputum production, shortness of breath, chest pain, loss of appetite, weight loss, and lung function decline. Recurrent episodes of PE in CF patients are associated with long term decline in lung function and shortened survival (Zemanick et al., 2013). Due to the chronic nature of this disease, PEs are difficult to treat. The goal is not to clear the pulmonary infection but rather restore the patient back to baseline lung function. Currently, there are no clinical or microbiological predictors of PE. However, recent research has revealed the importance of changes in the dynamics of the microbial community in the lungs, such as microbial density and diversity, in relation to acute PEs (Jones, 2011; Sibley and Surette, 2011; Waite et al., 2012).

Microorganisms contributing to PEs vary in CF cohorts, and their specific role in the disease process remains unclear. Microorganisms may directly produce virulence factors or upregulate virulence factors by interacting with other members of the microbial community in the lung which could lead to PE. Antimicrobial therapy is usually guided by the presence of

classic CF pathogens grown in respiratory culture; however, their activity is broad and often effective against many other organisms. Since the microbiome of the lung is so diverse, it is difficult to determine if antibiotics are targeting an unknown or emerging pathogen inadvertently which could possibly play a significant role in PE (Sibley & Surette, 2011). Approximately 25% of patients fail to fully respond to culture-guided treatment of PE; therefore, investigating the presence and role of other possible contributors in the lung microbiome could have an effect on predicting and treating PE, as well as improving patient outcomes in this population (Zemanick et al., 2013).

Classic CF pathogens. Based on the natural history of CF, classic pathogens contributing to morbidity and mortality have been identified. Early on, CF patients are colonized by *S. aureus* and *H. influenzae*. This is followed by acquisition of *P. aeruginosa*, which over time, undergo phenotypic changes in the CF lung causing them to become mucoid, multi-drug resistant, and difficult to treat (Spoonhower and Davis, 2016; Waters, 2012). In addition, other NF GNRs such as *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* are frequently isolated. Patients with more advanced disease are often infected with members of the BCC which is associated with a progressive decline in lung function (Mahenthiralingam, 2014; Parkins, Sibley, Surette & Rabin, 2008). Organisms thought to play a secondary role in CF lung disease include respiratory viruses and fungi, such as *Aspergillus fumigatus* (Miller and Gilligan, 2003). Microbiological and molecular laboratory methods have described CF lower airways as polymicrobial; therefore, the list of classic pathogens and their role in CF should be revisited and possibly expanded to improve management and treatment of patients (Mahenthiralingam, 2014; Sibley et al., 2008).

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Lung Microbiome

Molecular methods have demonstrated the dynamic community of microorganisms within the CF lung. Many have not been grown in culture using standard CF protocols. Research suggests that microbiome composition and perturbations in these communities may impact pulmonary status. Understanding the impact of this polymicrobial community on airway health is essential to improve patient care (Lynch & Bruce, 2013; Armougom et al., 2009).

Molecular methods, such as MLST, Sanger sequencing, and pyrosequencing, are based upon analysis of the genetic content of bacteria. These techniques are more reproducible than phenotypic methods and provide greater discriminatory power when differentiating between epidemic and non-epidemic strains. MLST is a popular method for bacterial genotyping. DNA sequence variations in genes known to be present in all strains of a given species are measured and characterized by unique allelic profiles (Saiman et al., 2013). Pyrosequencing, a next generation sequencing method, detects pyrophosphate release on nucleotide incorporation, rather than dideoxynucleotides used in Sanger sequencing. A complimentary strand of single stranded DNA is synthesized enzymatically hence the associated name, "sequencing by synthesis". Thousands of 100 base pair sequences are generated without the need for cloning. Theoretically, the number of sequences is proportional to the number of genomic copies present in the sample. Therefore, pyrosequencing evaluates the relative amounts of different species present. The molecular methods described above are examples of massive sequencing techniques frequently employed by researchers conducting molecular profiling studies (Armougom et al., 2009).

The CF lungs are colonized by a diverse and dynamic community of bacteria, viruses, and fungi. On average, the lungs of CF patients harbor 22 metabolically active microbial species. These include the classic CF pathogens in addition to other species, such as members of

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the oropharyngeal flora (OF) that have not been previously identified as significant in this population (Parkins et al., 2008; Olson et al., 2010). The role and interspecies interactions of this complex community on patient outcomes remains poorly understood (Filkins et al., 2012). Rabin and Surette (2012) demonstrated through animal coinfection models that organisms in the CF lung considered to be nonpathogenic were capable of acting synergistically with the classic pathogen, *P. aeruginosa*, to dramatically increase disease. The dynamic microbiome of the CF lung and bacterial community interactions could lead to enhanced pathogenesis and, therefore, must be considered to effectively guide and optimize the treatment strategies of CF pulmonary infections (Sherrard et al., 2013; Sibley et al., 2008; Sibley et al., 2010).

Emerging Pathogens

Less prevalent or emerging organisms isolated from CF respiratory cultures could potentially affect microbial community dynamics in the lungs. However, the role of these emerging pathogens in CF disease progression is unknown (Kiska & Riddell, 2012). Typically, emerging pathogens are organisms not considered pathogens under normal circumstances. However, their ability to alter pathogenesis of the community through interspecies interaction may make them relevant. For example, OF could serve as a reservoir of unrecognized pathogens in CF. When these organisms travel to the lower airways, they may have the ability to cause disease within a polymicrobial context. It has been determined that treatment of PE does not frequently impact pathogen load. In many cases, the microbiology culture results remain unchanged before, during, and after treatment. Research suggests that detecting the presence and investigating the role of emerging pathogens in CF patients may correlate with periods of clinical stability and PE (Sibley & Surette, 2011).

The presence of emerging pathogens in the CF population is due to many factors. Frequent surveillance of CF patients is associated with an increased incidence and prevalence of specific organisms such as *Ralstonia*, *Pandoraea*, and other NF GNRs (Saiman et al., 2013). The predicted survival rate for CF patients in the US increased from 28 years in 1990 to approximately 40 years today. This provides a greater opportunity for previously considered nonpathogenic microbial species to colonize and infect the lower airways. CF patients are commonly prescribed chronic suppressive antimicrobial therapy to manage their disease. In addition, when classic CF pathogens are initially isolated in culture, eradication is attempted with aggressive therapy. Exposure to multiple antibiotics in high doses over long periods of time places greater selective pressure on organisms which could contribute to infection. Further, within the dynamic microbiome of the lung, microbial species may acquire genes encoding virulence factors. This could result in an increased capacity to cause infection (LiPuma, 2010).

The use of selective media in the clinical microbiology laboratory has revealed emerging pathogens with potential impact on the lung's microbial community. An example is BCSA. Phenotypically similar organisms, such as *Ralstonia* and *Pandoraea* are able to grow on this media as well. Resemblance to *B. cepacia*, a classic pathogen associated with lung function decline and poor outcomes, brings into question their role in the CF lung. As development and use of improved laboratory methods continues, emerging pathogens will be isolated with increased frequency (Miller & Gilligan, 2003).

The classic CF pathogens have been documented to play a role in progression of lung disease. The increased presence of emerging pathogens in this population warrants an investigation of their prevalence in the CF community as well as their impact on lung function in

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this unique ecological niche. It is conceivable that they, too, belong on the classic CF pathogen

list (Vrankrijker, Wolfs & van der Ent, 2010.)

Oral flora. Based on molecular profiling studies, the CF airways are colonized by many

polymicrobial communities consisting of organisms referred to as normal OF (Field et al., 2010).

The oral cavity is colonized with hundreds of species of bacteria such as *Streptococcus*,

Veillonella, *Prevotella* and *Gemella* which have all been identified as community members of

the lung microbiome. Historically, when members of the OF were found in sputum cultures,

their presence was attributed to specimens contaminated by bacteria in the oral cavity. In fact,

the classic list of CF pathogens do not contain any OF flora, and specific culture conditions are

not utilized to target this group of organisms. Research has confirmed the true presence of OF

flora in the lower airways of CF patients rather than sample contamination as it passes through

the oral cavity. It has also been well documented that members of the OF flora act as synergens

in the CF lung by enhancing the virulence of *P. aeruginosa* (Field et al., 2010; Sibley et al.,

2008; Waite et al., 2012).

Streptococcus anginosus **group.** The presence of SAG in the CF lung and their role in

acute PE has been highlighted. There are three members of the SAG: *Streptococcus*

constellatus, *Streptococcus intermedius*, and *Streptococcus anginosus*. SAG colonize the

oropharynx, gastrointestinal (GI) tract, and vagina in approximately 15-40% of the general

population (Parkins et al., 2008; Sibley et al., 2010). Members of this group have great

pathogenic potential and are responsible for a wide variety of infections including brain and liver

abscesses, empyema, as well as other purulent infections throughout the body. In fact, when

specifically sought, SAG can be identified in up to 25% of culture-negative pneumonia samples.

Antibiotic therapy for SAG infections normally includes pencillin G, ceftriaxone, cefotaxime,

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imepenem, and meropenem. However, antimicrobial susceptibility patterns of CF SAG isolates may differ due to chronic antibiotic exposure (Parkins et al., 2010).

SAG are phenotypically diverse within species making isolation and identification a difficult task for the clinical laboratory. Often times, they are indistinguishable from other streptococci that have little or no known pathogenic potential. SAG typically exhibit slow growth rates, a distinctive caramel smell, and the inability to ferment sorbitol. Various patterns of hemolysis including alpha, beta, and gamma are observed in culture. Lancefield grouping is inconsistent among this group: A, C, F, G, and non-typeable results have been obtained (Olson et al., 2010).

The presence of SAG is often underrepresented in culture due to the battery of media and incubation conditions used to target only classic CF pathogens even when it is the numerically dominant pathogen (Grinwis et al., 2010). In addition, these organisms could have fastidious growth requirements that are not adequately provided in standard culture media (Parkins et al., 2008). As a result, the clinical significance of SAG in CF lung disease has been overlooked (Sibley et al., 2008). Until recently, SAG have not been considered to play a role in CF lung disease. Due to their strong correlation with clinical status, propensity to cause pyogenic infections, and antibiotic resistance in this population, SAG is now being considered a CF pathogen (Sibley et al., 2008; Sibley and Surette, 2010).

Grinwis et al. (2010) characterized 128 SAG isolates from the sputum of adults with CF. Forty-five SAG isolates from non-CF patients with invasive disease were used for comparison. The following tests were performed on both sets of isolates: Lancefield grouping, tests for hemolysis, tests for production of hyaluronidase, chondroitin sulfatase, DNase, proteases, hydrogen peroxide, and PCR detection of the intermedilysin gene. Upon comparison of group

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strains (CF vs. non-CF), *S. intermedius* isolates were similar while *S. constellatus* and *S. anginosus* yielded several biotypes. Approximately 75% of *S. constellatus* CF isolates were beta- hemolytic Lancefield group C compared to 13.3% of invasive isolates from non-CF patients. Another key difference between groups included hyaluronidase-positive *S. anginosus* strains found only in CF isolates, indicating increased virulence in this population. Cluster analysis revealed both strain groups clustering together which suggests that CF isolates and invasive isolates are equally pathogenic (Grinwis et al., 2010). These data supports the need to further investigate the role of SAG in CF lung disease.

Due to the pathogenic potential of SAG and possible contribution to PE, Sibley et al. (2010) conducted a six-month surveillance study on a Canadian CF cohort using McKay agar. McKay agar is a semi-selective solid medium used to isolate SAG from nonsterile body sites, such as the CF airways. A total of 108 patients were screened in the surveillance study. From those, a total of 226 samples were collected (180 surveillance samples and 46 exacerbation samples). During the six-month period, SAG was isolated from eight of the 108 specimens on at least three separate occasions at levels associated with chronic colonization. SAG was common in this adult CF cohort with a specimen prevalence rate of greater than 40%. Further, SAG in concentrations greater than 10^7 cfu/mL was associated with a decline in clinical status. This supports the idea that SAG play a role in the progression of CF lung disease (Sibley et al., 2010).

Parkins et al. (2008) reported on six cases of SAG-related infection over four years in a Canadian adult CF cohort. SAG was found in patients co-colonized with *P. aeruginosa*. Previous studies have shown that pathogenicity of SAG and other viridians streptococci are enhanced within a polymicrobial context supporting a microbial interaction between SAG and *P. aeruginosa*. The presence of SAG in the lower airways colonized with *P. aeruginosa* may prove

to be a significant finding. Therefore, detecting the presence of SAG in CF lower airways is warranted (Parkins et al., 2008).

Studies characterizing the microbial epidemiology of CF patients report SAG as the numerically dominant organism at the onset of PE (Grinwis et al., 2010; Sibley et al., 2010; Parkins et al., 2008; Sibley et al., 2008). Established CF protocols do not specify this organism as a pathogen; therefore, specialized culture conditions are not employed to seek SAG. In a study by Sibley et al. (2008), a sputum sample was collected from a patient experiencing an acute PE before the initiation of antibiotics. Using McKay agar, culture revealed SAG as the numerically dominant organism. Using the culture to guide therapy, IV ceftriaxone was selected for treatment. Ceftriaxone has no anti-pseudomonal activity which is usually one of the main targets when selecting an antimicrobial regimen. Remarkably, *S. constellatus* responded to the ceftriaxone which resolved the clinical symptoms of PE as well as restoring baseline lung function. Subsequent cultures (six days post treatment) did not grow *S. constellatus*. Without the addition of McKay agar, *S. constellatus* would not have been detected. The presence of SAG is not evident on routine CF media due to overgrowth of classic CF pathogens such as *P. aeruginosa* (Sibley et al., 2008). During this study period, the same patient experienced another PE. The identical culture picture was observed which prompted the previously described treatment strategy. Again, targeting *S. constellatus* resolved symptoms (Sibley et al., 2008).

The importance of SAG has also been recognized by Zhao et al. (2012) who reported that SAG were persistent and abundant in all patients included in their longitudinal study (nine years, n=6). Based on these findings, acknowledging the role of SAG in PE as well as modifying culture conditions to detect this group in CF pulmonary samples is necessary to provide optimal patient care.

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Anaerobes. Recent attention has been directed toward anaerobes and their possible role in CF lung disease. Steep oxygen gradients and regions of hypoxia in the CF lung make a hospitable environment for anaerobes. Molecular analysis of CF airway samples has revealed obligate and facultative anaerobes in high quantities during periods of PE. At this time, their specific role in this process is not defined (Zhao et al., 2013). Sherrard et al. (2016) suggest that fluctuation in the abundance of anaerobes could possibly be used as a marker of clinical status. When specifically sought, anaerobes has also been isolated and identified in culture (Field et al., 2010; LiPuma, 2010). However, it is difficult to obtain a pure culture which could lead to underestimating the importance of anaerobes in CF lung disease.

Tunney et al. (2008) reported *Prevotella, Veillonella, Propionibacterium*, and *Actinomyces* in high quantities, defined as $10^4 \le n \le 9 \times 10^7$ cfu/g of sputum, from 42 out of 66 adult CF sputum samples. Similar findings were described in the pediatric population. Interestingly, different species of anaerobes in much lower quantities (10^2 to 10^5 cfu/g of sputum) were detected in respiratory samples from healthy volunteers. These data suggests high quantities of species specific anaerobes may be unique to CF patients and may play a role in lung disease. Colonization with *P. aeruginosa* significantly increased the likelihood that anaerobic bacteria would be detected in the patient samples (Tunney et al., 2008). It is possible that mucoid strains of *P. aeruginosa* render the airways anaerobic, which promotes the growth of this specific group of organisms.

Virulence factors of anaerobes isolated in CF specimens include the following: secretion of extracellular enzymes such as proteases and B-lactamases, capsule production, biofilm formation, neutrophil chemotaxis, and resistance to phagocytosis. These factors could all contribute to periods of acute PE and lung damage. The presence of anaerobes in CF sputum

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samples in equal or greater quantities than classic pathogens such as *P. aeruginosa* should be considered when managing patients (Tunney et al., 2008).

Rabin and Surette (2012) observed a correlation between bacterial load in CF clinical specimens in which the anaerobe *Gemella* species was detected and bacteriologic response in patients suffering exacerbations. This raises awareness and suspicion of *Gemella's* role in CF lung disease. A 10-year longitudinal study following six CF patients reaffirmed that anaerobes are common and abundant in the CF lung. *Veillonella* species and *Prevotella* species were most frequently seen (Zhao et al., 2012).

Prevotella species are obligate anaerobic bacteria that have recently been detected in CF respiratory samples. Normally, *Prevotella* are isolated as normal members of oral, intestinal, and urogenital flora. *Prevotella* has been associated with a wide variety of infections ranging from periodontal disease and lower respiratory tract infections to bacteremia. Field et al. (2012) reported that *Prevotella* was isolated from the airways of 80% of CF patients examined in greater or equal quantities than that of classic aerobic pathogens. A mouse coinfection pneumonia model demonstrated the synergistic effect of *Prevotella intermedia* and *S. constellatus* on the severity of symptoms and mortality. These findings support the theory of polymicrobial interactions enhancing virulence of emerging CF pathogens (Field et al., 2010). Mahenthiralingam (2014) cites *Prevotella* species as an emerging pathogen and novel CF microbiome player. *Prevotella* species persist in the lungs of CF patients receiving chronic antimicrobial therapy; however, their association with CF lung disease is not understood.

Sherrard et al. (2016) propose several mechanisms in which anaerobes may contribute to lung disease. Interspecies interactions among anaerobes and classic CF pathogens are hypothesized to enhance pathogenicity and colonizing ability of *P. aeruginosa*. Anaerobic

bacteria such as *P. melaninogenica*, *V. parvula*, and *F. nucelatum* secrete various short chain fatty acids which can lead to neutrophil recruitment and activation, ultimately causing inflammation in the lung.

The exact role of anaerobes in CF lung disease is unknown. Anaerobes isolated from CF patients exhibit more resistant antimicrobial susceptibility patterns when compared to healthy controls, which can complicate treatment regimens if not specifically targeted. (Sherrard et al., 2016). In addition, the presence of anaerobes in significant quantities may contribute to inflammation and CF lung infection; therefore, their presence in clinical specimens and possible role in disease progression should be considered when managing CF patients (Sherrard et al., 2013).

Glucose non-fermenting Gram-negative rods. The isolation frequency of glucose NF GNRs other than *P. aeruginosa* and BCC is increasing in CF respiratory samples. The majority of these bacteria are environmental, multi-drug resistant organisms that persist in the CF lung even after aggressive antibiotic therapy. The emergence of these organisms in the CF lung is due to several factors. The CF population is aging. An increased life span creates an opportunity for emerging organisms to colonize the CF lung. In addition, chronic suppressive therapy in this population could select for multi-drug resistant organisms able to persist in the lower airways (Bittar & Rolain, 2010; Kiska & Riddell, 2012).

The pathogenic role of NF GNRs in the CF lower airways is unclear. Multi-drug resistance patterns, in addition to biofilm formation, are cause for concern because few effective antibiotics are available for treatment. The emergence of this distinct group has caused problems for the clinical microbiology laboratory as well. Commercial identification systems routinely employed in clinical laboratories are unable to correctly identify many of these emerging

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organisms. Due to similarities among NF GNR phenotypic characteristics, they are often misidentified as BCC. Colonization with BCC is associated with advanced lung disease and has severe psychosocial consequences for the patient (Kiska & Riddell, 2012).

Kiska and Riddell (2012) made many recommendations in regard to the presence of NF GNR in CF samples. First, any glucose NF GNR that grows on *B. cepacia* selective agar should be identified. Since limitations with current commercial identification systems exist, these isolates should be sent to reference laboratories for full identification. This is considered good laboratory practice for all clinical microbiology laboratories. Secondly, great consideration and attention should be given to any NF GNR resistant to colistin. Since colistin is usually reserved as the last line of defense for multi-drug resistant *P. aeruginosa* isolates, other NF GNRs exhibiting resistance to this particular antimicrobial is concerning and its presence warrants investigation.

The emergence of NF GNRs in the CF population has been highlighted in several case reports. *Inquilinus limosus*, a nonsporulating NF GNR exhibits multi-drug resistant patterns and is associated with poor clinical outcomes. Acquisition of *I. limosus* by a 20-year-old male with CF resulted in clinical, spirometric, and radiographic lung function decline. This isolate had a mucoid phenotype displaying multi-drug resistant susceptibility patterns. Forced expiratory volume (FEV1) six months before isolating *I. limosus* was 3.12 L. Chronic colonization with this organism resulted in a decreased FEV1 (2.65 L). This particular case supports the pathogenic potential of *I. limosus* in the CF population (Hayes et al., 2009).

Chiron et al. (2005) reported on five CF patients with *I. limosus*-positive respiratory cultures. In four of the five patients, *I. limosus* was isolated from at least seven sputum specimens indicating chronic colonization. Susceptibility testing indicated all isolates were

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resistant to penicillins, cephalosporins, kanamycin, tobramycin, fosfomycin, colistin, doxycycline, and co-trimoxazole. *I. limosus* was susceptible to imipenem and ciprofloxacin. Similar to previous reports, lung function decline was observed in one patient following acquisition of *I. limosus* despite treatment targeting this organism. The clinical impact of this organism remains unknown; however, its pathogenic potential and association with lung function decline should be recognized (Chiron et al., 2005).

Pandoraea species have also been isolated from the sputum of CF patients. Like many of the other emerging NF GNRs, their role in CF lung disease is unclear. These isolates are problematic for several reasons. In the clinical microbiology laboratory they are often misidentified as members of the BCC or *Ralstonia* species. In addition, antimicrobial susceptibility in these species is generally limited to tetracycline, imipenem, and trimethoprim-sulfamethoxazole. Misidentification and multi-drug resistant susceptibility patterns have a direct impact on patient outcomes (Costello, 2010).

Laboratory Identification Methods

Molecular methods. Over the past decade, molecular methods, such as MLST, Sanger sequencing, and, pyrosequencing, have been performed in research settings, and have revealed the dynamic and diverse microbial communities occupying the CF lung. Based on these data, there has been a renewed interest in organisms typically considered clinically insignificant in this population, such as anaerobes and other members of the OF. Many of the organisms identified by molecular profiling studies are not grown in culture with standard media and incubation conditions, and/or are overgrown by those dominating the community. Although molecular methods provide a wealth of information, they are not a component of routine CF care in the US. Clinically, the molecular methods listed above are only performed when epidemiologically

indicated, such as strain typing BCC, and other organisms such as *P. aeruginosa* and NTM (Saiman et al., 2013). Organism growth in culture is essential to functionally characterize, isolate, and determine susceptibility patterns (Bittar & Rolain, 2010; Sibley et al., 2008; Zhao et al., 2012). Therefore, this study used information gleaned from molecular profiling analyses to develop culture-dependent screening methods for emerging pathogens suspected to play a role in CF lung disease. Again, once grown in culture, emerging pathogens can be functionally characterized.

Microbiology culture. Antimicrobial treatment regimens and patient management are dependent on the isolation and identification of CF pathogens. Classic pathogen identification protocols involve Gram stain, growth at different temperatures on various routine and selective media, and antibiotic susceptibility testing (Bittar & Rolain, 2010). CF respiratory cultures are used to detect new pathogens, guide therapy, monitor the success of eradication strategies, and distinguish transient from persistent colonization (Saiman et al., 2013).

CF respiratory cultures remain the most accurate method available to obtain data on classic pathogens known to play a role in disease progression (Rabin & Surette, 2012). In fact, active surveillance using molecular methods is not a component of routine CF care in the US (Saiman et al., 2013).   The routine battery of media used for CF specimens consist of BA, CHOC, CNA, MAC, MS, and BCSA. However, there are disadvantages to these media that pose challenges to the laboratorian. Mucoid strains of *P. aeruginosa* often invade the agar, and obscure the growth of Gram-positive organisms and slower growing Gram-negative organisms. In addition, this organism can produce substances that inhibit the growth of other bacteria (Bittar & Rolain, 2010; Gibson, Burn & Ramsey, 2003). Table 2 describes the purpose of each media type included in routine CF culture protocols.

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Table 2. *Purpose of Routine CF Culture Media*

Media Type	Purpose
Blood Agar (BA)	Isolation and detection of hemolytic activity of streptococci and other fastidious microorganisms.
Chocolate Agar (CHOC)	Isolation and cultivation of a variety of fastidious microorganisms.
Columbia Nalidixic Acid (CNA)	Selective isolation, cultivation, and differentiation of Gram-positive cocci from clinical specimens.
MacConkey Agar (MAC)	Selective isolation and differentiation of coliforms and enteric pathogens based on the ability to ferment lactose.
Mannitol Salt (MS)	Selective isolation, cultivation, and enumeration of staphylococci from clinical specimens.
Burkholderia cepacia Selective Agar (BCSA)	Selective isolation of *B. cepacia* from respiratory secretions of patients with CF.

Adapted from Manual of Clinical Microbiology, 2011.

Selective media, such as BCSA, is used to detect members of the BCC. Phenotypically similar organisms, however, have the ability to grow on such media which could lead to misidentification. For example, other glucose NF GNRs such as *Ralstonia, Pandoraea, Inquilinus*, and *Herbaspirillium* have the ability to grow on selective *Burkholderia* agar (Kiska & Riddell, 2012; Bittar & Rolain, 2010). Although microbiology culture underestimates the diversity of organisms in the CF airway, it is routinely used in the majority of clinical laboratories to monitor and guide treatment of patients.

Over the last decade, there has been an increased interest in developing molecular methods to uncover members of the CF lung microbiome. As a result, little or no effort has been placed on revisiting and revising traditional microbiologic practices for CF. This is problematic for several reasons. It is not currently feasible for clinical microbiology laboratories to adopt expensive molecular methods for routine surveillance cultures of CF patients. Molecular profiling studies have been very useful in revealing a growing list of emerging potential

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pathogens that may play a significant role in CF lung function decline. However, routine CF culture protocols have not yet been modified to detect these organisms. In fact, laboratories often fail to detect a bacteriologic response of isolated principal pathogens to antibiotic treatment suggesting the presence of additional bacteria responsible for clinical symptoms. Molecular profiling studies have indicated that if particular organisms are sought, selective media and conditions can be optimized to recover specific targets. Review of the current CF culture protocol is warranted (Sibley et al., 2008; Sibley & Surrette, 2011).

Culture results obtained utilizing current microbiology protocols do not accurately reflect all organisms in the CF lung. Emerging pathogens, and even numerically dominant organisms, can be overlooked due to the battery of media employed and culture incubation conditions. Due to cost and labor intensiveness, molecular methods such as MLST and pyrosequencing are not adopted by routine clinical laboratories. Therefore, it is desirable to closely evaluate traditional CF culture protocols and their ability to isolate emerging pathogens thought to play a role in CF disease progression as well as all classic pathogens.

Conclusion

Approximately 30,000 people in the US are diagnosed with CF and it is estimated that one in every 31 Americans are symptomless carriers of the defective CF gene. In the last decade, more effective treatment strategies have improved patient outcomes. Based on the 2012 CF Foundation National Patient Registry Annual Data Report, the median predicted survival of an individual diagnosed with CF is approximately 40 years of age. Despite these advancements, 95% of deaths are due to respiratory failure. Chronic bacterial lung infections eventually lead to lung function decline. Culture-dependent methods are currently used in the US to monitor CF patients during both periods of clinical stability and PE. Data from molecular profiling studies

reveal that there is a diverse microbial community in the CF lung that is not being captured using the routine battery of media for culture. The classic CF pathogens are routinely cultivated using current microbiology protocols; however, the presence of emerging pathogens thought to play a direct or indirect role in CF lung function decline are often overlooked or missed due to limitations of media and incubation conditions. In order to determine the role of emerging pathogens in this population, they need to be directly sought in culture.   This study proposed to expand microbiology culture strategies for management and treatment of CF patients at VCUHS.

Chapter 3: Materials and Methods

Introduction

Current CF culture protocols only select for classic CF pathogens known to play a role in lung disease. There is a growing list of emerging pathogens associated with PE and lung function decline. Culture-dependent screening protocols were utilized to select for emerging pathogens in CF respiratory specimens in order to determine their prevalence in the population at VCUMC. This chapter covers the research design of the study which includes population and sample collection, specimen transport, specimen processing, as well as methods used to accomplish the goal of expand the use of microbiology cultures strategies to improve management and treatment of CF patients at VCUMC. The following specific aims contributed to accomplishing this goal:

Specific aim 1. Develop culture-dependent screening protocols to detect emerging pathogens in the CF population at VCUMC.

Specific aim 2. Determine the prevalence of emerging CF pathogens in the patient population at VCUMC.

Specific aim 3. Prospectively evaluate current culture techniques in conjunction with newly developed or proposed protocol.

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Research Design

Population and sample. The population for this study consisted of adult and pediatric CF patients treated at VCUMC. A convenience sample was used, which consisted of all residual respiratory cultures collected from CF patients during routine clinic visits or hospitalizations that were submitted to the clinical microbiology laboratory at VCUMC for diagnostic purposes from July to September 2016. Inclusion criteria consisted of all specimen types collected from CF patients including Eswabs and sputum. Respiratory samples were excluded if the quantity was insufficient to inoculate the proposed study media.

Typically, CF patients are monitored on a quarterly basis during periods of clinical stability; therefore, four encounters are estimated per patient per year. Signs of PE require additional encounters which often times result in hospitalization. A respiratory sample is collected at each visit and hospitalization to guide treatment. There are two separate CF clinics operated by VCUMC. One clinic serves the pediatric CF population, and the other serves the adult CF population. The pediatric and adult populations are distinguished by their 18th birthday.

During 2011-2012, 142 patients were seen in the CF clinic. This number may reflect multiple encounters for some patients but not all due to non-compliance with recommended follow-up visits. Based on these numbers, it was estimated that between 50 and 70 patients would be seen at either the adult or pediatric CF clinics at VCUMC for surveillance or be hospitalized for PE over the study time frame with at least one respiratory sample, in the form of Eswab or sputum, collected. The rationale for sample size was determined based on collecting at least one data point (respiratory culture), either from routine surveillance or episodes of PE, from approximately half of CF patients seen at VCUMC. The study time frame began upon IRB

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approval (June 2016) and ended when 75 CF respiratory samples, were prospectively evaluated (September 2016).

Recruitment and IRB. This study presented no greater than minimal risk to the sample population, as residual CF patient respiratory samples ordered for diagnostic purposes were used. To protect patient confidentiality, coding was used when labeling the study media, recording data, and reporting results. In addition, the research took place in the clinical microbiology laboratory at VCUMC, located in the Clinical Support Center, 5th floor which is not a patient care facility.

A waiver of authorization was requested and granted for this study. The second aim was to determine the prevalence (see methods for Specific Aim 2) of emerging CF pathogens in the patient population at VCUMC. In order for the true prevalence of emerging pathogens in the CF population at VCUMC to be determined over the study period, all residual CF respiratory samples were included in the study. If only a portion of these samples were included, based on patient consent, the sample would have been biased; therefore, true prevalence could not have been determined. As a result, the waiver of authorization was needed to fully and accurately accomplish the second aim. IRB approval for this expedited study (**HM20001275**), which included a waiver of authorization to allow all CF respiratory samples collected during the study time frame to be included, was granted in June 2016.

Specimen collection and transport. Respiratory samples in the form of expectorated sputum or Eswabs were collected from each CF patient by clinicians or nurses according to standard operating procedure during all routine clinic visits, and termed "surveillance cultures". In addition, respiratory samples, which included Eswabs or sputum, were also collected by health care providers from patients during periods of hospitalization due to PE.

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If a patient was able to expectorate, a sputum sample was obtained in a sterile collection container. For patients unable to produce sputum, an ESwab was used for collection. Copan ESwab is a liquid-based multipurpose collection and transport system that maintains viability of aerobic, anaerobic, and fastidious bacteria. The health care provider removes the flocked swab from the package. Immediately after swabbing the tonsillar pillars or cough secretions of the patient, the swab is placed in the sterile transport system containing one milliliter of modified liquid Amies medium. This allows the entire sample to elute into the medium providing up to 10 identical 100 microliter aliquots of liquid sample suspension. Specimen collection using Eswabs or expectorated sputum constituted no more than minimal risk; any possible adverse effects (e.g., gagging, discomfort) were overshadowed by the potential benefit to the CF patients.

All respiratory specimens, whether in the form of sputum or ESwabs, were properly labeled in the CF clinic or hospital floor by health care providers collecting the sample and sent to the clinical microbiology laboratory at VCUMC for culture.   Figure 1 depicts routing of patient specimens for routine CF culture and the research protocol. Refer to Appendix A for the routine CF culture protocol utilized in the clinical microbiology laboratory at VCUMC.

Specimen processing. Upon receipt in the clinical microbiology laboratory, respiratory specimens were accessioned by laboratory personnel which prompted culture-specific labels to print. CF cultures generated six labels with the following information: patient name, medical record number, accession number, collection and receipt date and time, culture type, and respective culture media (BA, CNA, CHOC, MAC, BCSA, MS). The sputum, Eswab, or BAL submitted for culture was processed according to standard operating procedure for diagnostic testing in the clinical microbiology laboratory upon receipt. CF sputum samples were

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┌─────────────────────────────────────────────────────────────────────┐
│                      CF Respiratory Specimens                       │
│                                                                     │
│                        Sputum or ESwabs                             │
│                                                                     │
│                               │                                     │
│                               │                                     │
│                               ▼                                     │
│                                                                     │
│                     Routine CF Culture Analysis                     │
│                                                                     │
│        Blood agar, CNA, Chocolate agar, MacConkey agar, BCSA, MS    │
│                                                                     │
│                               │                                     │
│                               │                                     │
│                               ▼                                     │
│                                                                     │
│               Residual CF Sample Used for Research Protocol         │
│                                                                     │
│  Blood agar, TSA w/ colistin and vancomycin, MAC w/ colistin (35°C, │
│                             ambient air)                            │
│    McKay Agar (SAG), Brucella, PEA, LKV/BBE (35°C, anaerobic        │
│                             conditions)                             │
└─────────────────────────────────────────────────────────────────────┘
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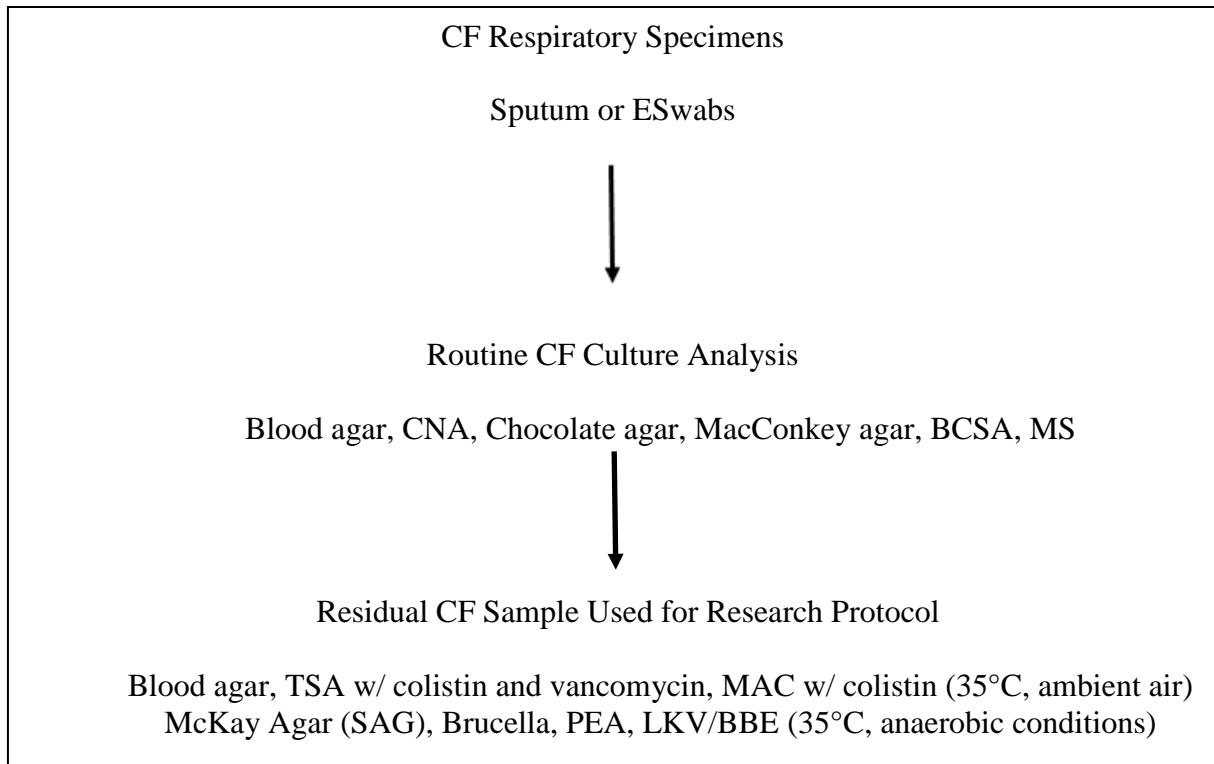
Figure 1. Routing of CF Respiratory Specimens for Routine and Research Culture Protocols

treated with Copan SLsolution. This solution contains Dithiotreitol (DTT), a mucolytic agent,

which emulsified the sputum and mucus. The resulting homogenous suspension was vortexed

and used to inoculate the battery of culture media. ESwabs collected for CF respiratory cultures

were vortexed for five seconds, and the liquid-based medium was used to inoculate agar plates

for culture. All media were then placed in the appropriate incubation conditions. Residual

samples were immediately stored in refrigerated temperatures for seven days.

All residual CF respiratory samples were retrieved for the study protocol, which was

accomplished by requesting a pending log queried through the laboratory information system

(LIS), Cerner. Pending logs were generated each day during the study period to determine if CF

respiratory samples were submitted to the clinical microbiology laboratory for culture. If

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cultures were submitted, residual samples were retrieved from their designated storage space in the refrigerator. These samples were coded and inoculated to the study media which included one plate each of the following media: BA, McKay agar, MAC, MAC with colistin, and TSA with colistin and vancomycin. All study media were placed in the appropriate incubation conditions.

For each CF respiratory specimen included in the study, the following information was recorded: patient name, medical record number, date of birth, age, sex, accession number, location, and diagnosis. To protect patient confidentiality, a three-part study number was assigned to each specimen and all study media were labeled only with study numbers. The first represented the patient, the second the date, and the third the sample number. The first number was unique to each patient and not repeated for respiratory specimens submitted by different CF patients. This ensured that the desired number of respiratory samples from individual CF patients were included in the study. For example, if there were four residual samples from four different patients on a given day, they were coded as follows: 1.5.9.16.01, 2.5.9.16.01, 3.5.9.16.01, and 4.5.9.16.01. If a residual sample from the same patient was repeated throughout the study period, the first digit of the culture number remained the same, however, the date and sample number changed (Example: 1.6.1.16.02).

Specific Aim 1

Based on their potential pathogenicity and association with PEs and lung function decline, the following emerging pathogens were sought in culture: SAG, anaerobes, and colistin-resistant NF GNRs. A protocol to identify these targeted emerging pathogens in CF respiratory cultures was developed by evaluating the study media for over a seven-day incubation period and

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determining a work-flow for presumptive and definitive identification when suspicious colonies were observed.

Culture identification of SAG. McKay agar, which selects for members of the SAG, was used to culture this group of emerging pathogens in the respiratory samples of CF patients at VCUMC. Routine CF microbiology protocols are inadequate to detect SAG in patient specimens. Incorporation of a semi-selective media to separate and isolate these potential pathogens in this patient population is warranted (Parkins et al., 2008). Sibley et al. (2008) developed a semi-selective solid medium, McKay agar, for detecting SAG from non-sterile body sites such as CF airways. The following are needed to make 1 liter of McKay agar: 13.3g nutrient broth, 5g glucose, 10g yeast extract, 5g tryptone, 2g K_2HPO_4, 40 ml salt solution, 1 mL Tween 80, 1 mg crystal violet, 60 mg bromcresol purple, 10 ug vitamin K, 0.005 g haemin, and 15 g Bacto agar. The pH is adjusted to 7.2 before autoclaving. The sterilized medium is then supplemented with 20 mL L-arginine (2.5% w/v) and filtered antibiotic stock solutions of: sulfadiazine, colistin sulfate and oxolinic acid to final concentrations of 500, 10, and 5 ug/mL, respectively. Colistin and oxolinic acid are added to inhibit the growth of two classic CF pathogens: *S. aureus* and *P. aeruginosa*. Sulfadiazine is incorporated to select for SAG against other viridians streptococci. SAG isolates produce an acid environment on McKay agar which is detected by the colorimetric pH indicator, bromcresol purple. The above formula was provided to Teknova Inc., a manufacturing company that prepared the custom McKay agar for use in this study.

Quality control. Quality control testing was conducted weekly throughout the study period to verify that McKay agar selectively cultivated SAG. This was accomplished using 0.5 McFarland standard suspensions of the following American Type Culture Collection (ATCC)

strains: *S. anginosus* (ATCC 33397), *S. intermedius* (ATCC 27335), *S. constellatus* (ATCC 27823), and *S. aureus* (ATCC 29213). Each strain was inoculated to a different McKay agar plate. Plates were incubated at 35ºC in CO_2 for 18-24 hours and observed for growth. In addition, a sterility control, which consisted of an uninoculated McKay agar plate, was incorporated with weekly QC to ensure that the McKay agar itself was not contaminated with bacteria or fungi during production or upon storage in the laboratory after it was received. Weekly QC was deemed acceptable when the prepared McKay agar supported the growth of all SAG ATCC strains, inhibited the growth of *S. aureus* (ATCC 29213), and exhibited no growth on the sterility plate. All QC data was recorded in a laboratory notebook.

   Recovery and optimization of McKay agar. Recovery studies involve inoculating known amounts of reference ATCC strains to agar and determining the actual quantity detected after incubation. This parameter is essential when optimizing media to determine which atmospheric incubation condition yields the highest recovery rate of viable organisms. The three SAG ATCC isolates specified above were suspended in saline to a density equivalent to a 0.5 McFarland standard and 10-fold serially diluted. For each of the three ATCC strains, the 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated to six McKay agar plates and two blood agar plates. The blood agar plates were incubated at 35°C with CO_2. Two McKay agar plates for each dilution specified above were incubated at 35ºC with CO_2, 35°C in ambient air, and 35ºC in strict anaerobic (oxygen-free) conditions, respectively. *Streptococcus* species are facultative anaerobes and often times grow better in anaerobic conditions. In a study performed by Sibley et al. (2010), anaerobic incubation conditions of McKay agar was not assessed. Further, Grinwis et al. (2010) conducted a six-month surveillance study in which 99 strains of SAG were isolated on McKay

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agar. Though aerobic and anaerobic incubation conditions were assessed, the authors did not comment on which was the optimal environment for growth.

At both 18-24 hours and 48 hours of incubation under each condition, growth on the BA and McKay agar plates were assessed; and colonies were counted and recorded for each ATCC strain. Inoculum preparations of the ATCC strains visually comparable to the density of a 0.5 McFarland standard should contain approximately 1.5×10^8 cfu/mL. If the inoculum was prepared and serially diluted correctly, the 10^{-6} dilution should contain 10^2 cfu/mL. This verifies that the expected amount of organisms present remain viable and are recovered on the study media under varying incubation conditions.

Ideally, McKay agar should selectively cultivate SAG and inhibit other *Streptococcal* species and classic CF pathogens. Media verification studies were conducted to assess the selective nature of McKay agar. SAG ATCC reference strains, several *Streptococcal* species, and classic CF pathogens were inoculated to McKay agar, incubated under varying conditions, and observed for growth.

Optimized incubation conditions were determined by comparing the number of organisms recovered to expected results, observing the appearance of the SAG colonies on each set of plates, and evaluating the selectivity of McKay agar. Members of the SAG are small colony forming bacteria (colony size, less than or equal to 0.5 mm) on BA. These factors were considered when determining the optimized incubation conditions of SAG in the study protocol.

Anaerobes. Brucella blood agar, LKV/BBE (biplate), and PEA were used to cultivate anaerobes from CF respiratory samples. The anaerobe protocol currently in place for deep wounds, tissues, and sinus aspirates was used for the research samples in this study (Appendix B). This purchased media is prepared, stored, and dispensed under oxygen-free conditions by

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the manufacturer which prevents formation of oxidized products. An enzyme system, known as Oxyrase, is utilized by the manufacturer to specifically remove oxygen from liquids and semisolids. This further ensures the media will cultivate obligate anaerobes.

Brucella blood agar, LKV/BBE (biplate), and PEA were plated and incubated in an anaerobic chamber for five days. Brucella blood agar is an enriched nonselective agar used to isolate obligate anaerobes from clinical specimens and contains Vitamin K and hemin in the medium for recovery and pigment production of *Prevotella melaninogenica*. The addition of sheep blood in the agar allows observation of hemolytic patterns if present.

LKV agar was used to isolate obligately anaerobic GNR. Vancomycin is incorporated in this media to inhibit the growth of Gram-positive organisms, and kanamycin offers inhibition of facultative GNRs in clinical specimens. Further, laked sheep blood and vitamin K_1 are present in the agar to aid in the recovery of pigment producing anaerobes. BBE is an enriched selective and differential medium used for isolating Gram-negative bacilli. This agar contains enough gentamicin to inhibit the growth of most anaerobes except those belonging to the *Bacteriodes fragilis* group. Incorporation of esculin in BBE allows esculin hydrolysis of organisms to be recognized by a brown to black coloration around the colonies.

The third selective medium utilized for cultivating anaerobes was PEA. Phenylethyl alcohol in the media inhibits the growth of facultative GNRs and prevents swarming of organisms (PRAS package insert). Tunney et al. (2008) detected anaerobes in CF respiratory cultures by adding anaerobic blood agar, phenylethyl alcohol agar (PEA), and kanamycin-vancomycin laked agar (LKV) to the routine battery of media and incubating in strict anaerobic conditions.

Culture identification of colistin-resistant non-fermenting Gram-negative rods.

MAC and BHI agar with colistin sulfate were prepared and used in this study to select for emerging colistin-resistant NF GNRs in CF respiratory samples. MAC agar is routinely used in clinical microbiology laboratories to select for Gram-negative rods. This media contains bile salts and crystal violet which inhibits the growth of Gram-positive organisms. There are strains of NF GNRs unable to grow on MAC agar; therefore, BHI agar base was also used. Since BHI agar supports the growth of a wide variety of Gram-positive and Gram-negative bacteria, an additional antibiotic, oxalinic acid, was added to suppress the growth of normal oropharyngeal flora and classic CF pathogens, such as *S. aureus* and *P. aeruginosa*. Colistin originates from *Bacillus polymxya*. Interaction of this cationic polypeptide with the outer membrane of Gram-negative bacteria displaces calcium and magnesium which compromises the integrity of the cytoplasmic membrane resulting in cell lysis and death. In addition, colistin has the ability to bind and neutralize endotoxins released by GNRs (Waters, 2012).

Kiska and Riddell (2012) cautioned laboratorians to pay close attention to any colistin-resistant NF GNRs isolated in CF respiratory samples, as colistin is often the last line of therapy used to treat multi-drug resistant strains of *P. aeruginosa*. Phenotypically similar profiles among this group can lead to misidentification of organisms; therefore, it is recommended that any colistin-resistant NF GNR be identified with methods such as Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) or partial DNA sequencing. MALDI-TOF is a robust identification system that speciates organisms based on a unique spectral protein fingerprint. This method allows reliable identification of colistin-resistant NF GNRs despite their phenotypically similar profiles. Molecular methods, such as DNA sequencing, allows organisms to be identified based upon genotypic rather than phenotypic characteristics.

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Utilization of these techniques allows discrimination among the NF GNRs resulting in more accurate and timely identification of potential pathogens.

A culture enriched molecular profiling study performed by Sibley et al. (2011) revealed the complexity of microbial species recovered in the CF lung. By incorporating specific media and altering incubation conditions, this group was able to recover almost all organisms identified by molecular methods in culture. The current CF culture protocol does not support the growth of all organisms identified by molecular analysis, such as obligate anaerobes and those obscured by classic CF pathogens, such as mucoid *P. aeruginosa*. Sibley and colleagues used media containing colistin sulfate to inhibit the growth of mucoid *P. aeruginosa* which commonly overgrows other organisms in routine CF culture. BHI agar was used as a base with the addition of 10 mg/L of colistin sulfate and 5 mg/L of oxalinic acid. Oxalinic acid is a quinolone antibiotic that has the capability to suppress the growth of Gram-positive and Gram-negative bacteria. This media, supplemented with colistin and oxalinic acid prevented the growth of colistin-susceptible GNRs as well as Gram-positive organisms that are found in clinical samples.

The BHI base with colistin should support the growth of *I. limosus*, an emerging CF pathogen that has yet to be recovered from CF patients at VCUMC. BHI agar contains digests of gelatin and animal tissue, glucose, and brain heart infusion. This media is particularly useful in cultivating fastidious aerobic and anaerobic bacteria. The colistin and oxalinic acid concentrations described above were used as a starting point to prepare BHI with colisitin. In addition, a MAC base, which supports the growth of Gram-negative organisms, with colistin only was prepared. MAC is a selective medium used to differentiate organisms based on their ability to ferment lactose. Lactose-fermenting Gram-negative rods appear pink; whereas, non-lactose fermenters appear colorless or transparent. MAC agar contains gelatin, lactose, bile salts,

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casein, animal tissue, neutral red, and crystal violet (Versalovic, J., 2011). Performance of each media base containing colistin was evaluated. Both prepared media types were used in the study protocol.

Quality control. Before assessing performance characteristics of the prepared colistin-containing media, quality control (QC) was performed to ensure that the media suppressed the growth of colistin-sensitive isolates and supported the growth of colistin-resistant organisms. Five bacterial isolates were used: *Escherichia coli* (ATCC 25922), a colistin-susceptible NF GNR (patient derived strain of *P. aeruginosa*), colistin-resistant GNR (patient derived strain of *Serratia marcescens*), colistin-resistant NF GNR (patient derived strain of *B. cepacia*), *S. aureus* (ATCC 29213), and *E. faecalis* (ATCC 29212). One plate of each agar base (MAC and BHI) containing colistin was divided into six quadrants and labeled appropriately. A suspension of the above specified bacterial isolates were prepared at a density equivalent to that of a 0.5 McFarland standard and 10 µL inoculated to each respective quadrant. One quadrant was left uninoculated as a sterility control. Plates were incubated at 35ºC in ambient air for 18-24 hours and observed for growth. Expected QC results are as follows: the MAC and BHI with colistin should inhibit the growth of *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212) and the colistin-sensitive NF GNR (patient derived strain of mucoid *P. aeruginosa*) and support the growth of the colistin-resistant GNR (patient derived strain of *S. marcescens*) and colistin-resistant NF GNR (*B. cepacia*). No growth should be observed in the sterility quadrant. If these expected growth patterns were observed, the prepared media passed QC. In the event of a QC failure, the composition of the media was reevaluated. Troubleshooting failed QC results involved adjusting the type of media and antibiotics used and the concentration of colistin sulfate

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and oxolinic acid based on the observed growth patterns. Optimization continued until expected QC results were observed.

Performance characteristics of prepared media. Performance of each colistin containing prepared medium was assessed using frozen isolates of a colistin-resistant NF GNR (patient derived strain of *B. cepacia)* and a colistin-resistant GNR (patient derived strain of *S. marcescens*). Each of the above specified isolates were thawed, subcultured twice to BA, and serially diluted (ten-fold) and plated to two BHI with colistin and two MAC with colistin plates. One set of plates was incubated in ambient air and the other set at 35ºC with CO_2. Characteristic morphology, phenotypic characteristics, and limit of detection were recorded for each agar base and incubation condition. Based on this data, an incubation condition for each agar base with colistin was selected.

Recovery in clinical background. Media developed to screen for colistin-resistant NF GNR must provide visible growth of the desired organism in the presence of other organisms. De-identified patient sputum negative for NF GNRs was pooled for use as a diluent when performing serial dilutions. This matrix contained a plethora of organisms which allowed specificity to be assessed. The pool was divided and treated with a mucolytic agent, Copan SLsolution. Dithiotrietal (DTT) is the active ingredient in SLsolution that liquefies sputum without affecting the morphology, growth, or staining of organisms. Emulsification results in a homogeneous, liquid sample which allows more consistent distribution of organisms and reproducible planting and streaking of specimens (SLsolution Package Insert). The processed, pooled aliquots were spiked with known numbers of colistin-resistant NF GNR (patient derived strains of *A. xylosoxidans* and *S. marcescens*) and plated to the colistin-containing media to assure recovery of the desired organism and inhibition of colistin-susceptible and Gram-positive

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organisms. The optimal incubation conditions were determined by performance characteristics and used in the CF research protocol.

Specific Aim 2

The prevalence of emerging pathogens in the CF population at VCUMC was determined using optimized and verified media and incubation conditions. Cultures were evaluated for the presence of emerging pathogens. If colonies resembling targeted emerging pathogens were observed, they were definitively identified and recorded. Patients culture-positive for emerging pathogens recovered on the study media were divided by the total number of patients during the study period (n=75) and multiplied by 100, which defined the prevalence of emerging pathogens in the CF population at VCUMC. Upon receipt in the clinical microbiology laboratory at VCUMC, the respiratory samples were logged into the LIS. Once all physician-ordered tests were processed, inoculated, and placed in the appropriate incubation conditions, the remaining residual sample was stored in the refrigerator. Residual samples were retrieved from the refrigerator and used in the study protocol by inoculating to the following media: BA, MAC, McKay agar, TSA with colistin and vancomycin, MAC with colistin, and anaerobic plates to include Brucella, PEA, and LKV/BBE agar. The three anaerobic plates were immediately placed in an anaerobic pouch and incubated at 35ºC. The BA and MAC agar were be placed in a 35°C ambient air incubator. McKay agar, TSA with colistin and vancomycin, and MAC with colistin were incubated in optimized conditions according to Specific Aim 1 results (Figure 1).

Isolation of emerging pathogens. All plates were examined on a daily basis for up to seven days. Characteristic morphology and phenotypic traits of the targeted emerging pathogens were documented for each type of study media. The following guidelines were used when determining the possible presence of suspected emerging pathogens on study media: yellow

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colonies growing on McKay agar were subcultured and identified; any organism growing on colistin-containing media was subcultured, investigated, and identified; and anaerobes growing in greater than or equal to quantities than the classic CF pathogens were tested for aerotolerance (Appendix B) and identified when indicated. Following examination, all plates were returned to appropriate conditions for incubation throughout the seven-day period.

A work card, located in a laboratory notebook, was kept for each specimen documenting quantity (rare, growth of one to two colonies in the first quadrant; few, growth of several colonies in the first quadrant; moderate, growth in the second quadrant; or many, growth in the third quadrant), characteristic morphology, phenotypic traits, subcultures for isolation, as well as Gram stain and rapid test results for the presumptive identification of possible emerging pathogens. Rapid biochemical tests included the following: catalase, oxidase, indole, and PYR. Pure colony isolates were definitively identified by laboratorians in the clinical microbiology laboratory using the MALDI-TOF. If unreliable results were achieved with this method, the isolate was identified by partial sequencing of its 16S rDNA. Final organism identification was recorded.

Definitive identification of emerging pathogens. Several identification techniques are employed in microbiology laboratories to identify organisms. Many of the emerging pathogens sought in this study share similar phenotypic profiles, which can lead to the misidentification of organisms when using biochemical and carbohydrate utilization testing platforms. Alternative methods, such as MALDI-TOF and 16S rDNA sequencing overcome this challenge by employing different principles that reliably and accurately identify phenotypically similar organisms.

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MALDI-TOF. The clinical microbiology laboratory at VCUHS uses the MALDI-TOF method to identify bacteria isolated from clinical specimens (Appendix C). MALDI-TOF provides a protein spectrum fingerprint of cultured organisms by separating and detecting ionized proteins by their mass/charge ratio. Each ion detected is characterized by the following: molecular mass, charge, mass/charge ratio, and signal intensity. From these data, a unique profile of the bacterial components is constructed. Comparison of the spectrum of proteins generated during laser desorption is made against a database library of reference strains to identify the organism. Reproducibility of this method is attributed to the measurement of high abundance ribosomal proteins which is unaffected by variability in environmental or growth conditions. Identification of cultured organisms can be obtained in minutes at a low cost (Carbonnell et al., 2011; Fernandez-Olmos et al., 2012). Kiska and Riddell (2012) described a study in which MALDI-TOF was used to identify 558 strains of glucose NF GNRs. This method correctly identified 98% of those isolates which consisted of 58 separate species and approximately 52 BCC isolates. Many microbiologists have suggested that MALDI-TOF be used as the gold standard for CF isolate identification with confirmation by partial 16S rRNA sequencing when necessary (Bittar & Rolain, 2010).

Sequencing. Partial 16S rDNA sequencing is used in the clinical microbiology laboratory to identify rare bacteria, slow-growing bacteria, organisms that do not match recognized phenotypic profiles, and organisms that yield a poor or no identification by commercial systems or MALDI-TOF. Universal primers anneal to conserved regions of the bacterial rRNA gene. During dye terminator cycle sequencing, variable regions generate unique nucleotide base sequences that are species-specific. Capillary electrophoresis, carried out in an automated DNA sequencer, is conducted for size separation, detection and recording of dye

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fluorescence, and data output as fluorescent peak trace electropherograms. These are downloaded to SmartGene software, manually analyzed, and compared to reference sequences in a curated database to determine organism identification.

Prevalence. Prevalence is defined as the proportion of a population found to have a condition. For the purpose of this study, the population consisted of 75 CF patients in which respiratory specimens were submitted to the clinical microbiology laboratory at VCUHS, and the condition is the presence of emerging pathogens isolated in culture. Because there are no means to indicate whether a particular emerging pathogen was present in previous cultures, prevalence, which includes both new and old cases, is preferable to incidence, for this study. Prevalence was calculated as follows:

Prevalence = Patients culture-positive for emerging pathogens   x 100
                     Total number of patients during study period

The prevalences of each group of targeted emerging pathogens (SAG, anaerobes, and colistin-resistant NF GNRS) were calculated separately. A specimen was classified as positive for emerging pathogens if a single colony of SAG or colistin-resistant NF GNRs was recovered in culture. Specimens were classified positive if anaerobes were present in equal or greater quantities than the principal pathogens. In addition to prevalence, organisms falling into each category of emerging pathogens were identified to the species level.

Access to all routine microbiology culture data results through Cerner, the laboratory information system, was requested for residual specimens used in the study. This allowed comparison of the routine and research culture protocol (Specific Aim 3). The routine CF culture protocol (Appendix A) detected the classic CF pathogens known to play a role in CF lung disease. The research protocol utilized McKay agar, anaerobic media, and the colistin-containing agar to select for targeted emerging pathogens. In addition, access to Cerner enabled

the researcher to match respiratory samples with patient identifiers. This was necessary in order to ensure that at least 50 separate patients were sampled during the study period. In addition, if a patient was sampled more than once during the study period, culture review allowed matching multiple culture results to the appropriate patient. This was accomplished by tracking the accession number generated when the culture was ordered and logged into the clinical microbiology laboratory.

Specific Aim 3

Specific aim 3 was accomplished by prospectively evaluating the current CF culture procedure with the research protocol to identify discrepant results. Traditional CF cultures were evaluated and reported by clinical laboratory scientists in the clinical microbiology laboratory at VCUMC. These results were entered into Cerner, the laboratory information system. Culture results included pathogens or potential pathogens isolated in clinical specimens and their corresponding antimicrobial susceptibility profiles. The final culture results were obtained for each residual respiratory sample included in the study and compared to the results of the research study including organisms subcultured for purity, results of rapid tests, and unique phenotypic or morphologic traits. A final organism ID, obtained by MALDI-TOF, was provided for any targeted emerging pathogen isolated on the additional media incorporated for this study protocol. Any culture that was positive for the presence of emerging pathogens using the research protocol was earmarked. If more than one culture was submitted for an individual patient, culture results were compared over more than one data point.

Conclusion

In summary, Chapter 3 presented the materials and methods employed to accomplish the goal and specific aims of the study. The validation and implementation of McKay agar targeting

members of the SAG was described. The development, optimization and implementation of a culture-dependent screening method for colistin-resistant GNRs was discussed, and protocols used to cultivate anaerobes in the respiratory samples of CF patients was presented. In addition, analyses used to determine prevalence of emerging pathogens in CF patients at VCUMC and methods to prospectively evaluate routine and research protocol culture results were delineated.

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Chapter 4: Results

Introduction

The purpose of this study was to expand the use of microbiology culture strategies to improve management and treatment of CF patients at VCUMC. Results obtained are described and reported based on each of the accomplished specific aims. Specific Aim 1 results focus on the development, optimization, and implementation of culture-dependent screening protocols used to detect SAG, anaerobes, and colistin-resistant NF GNR in the CF population at VCUMC. Results of Specific Aim 2 provide a summary of the CF population studied as well as the respiratory samples used to determine the prevalence of emerging pathogens. Specifically, patient demographics and sample characteristics are described. In addition, the prevalences of emerging pathogens in the CF population at VCUMC is reported. Lastly, results from the study protocol are highlighted and compared to those obtained using CF Foundation recommended culture techniques.

Specific Aim 1

Specific Aim 1 was accomplished by developing, optimizing, and implementing or incorporating previously-developed, culture-dependent screening protocols to detect the following emerging CF pathogens: members of the SAG (McKay agar), anaerobes (Brucella,

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PEA, BBE/LKV), and colistin-resistant NF GNR (MAC with colistin and TSA with colistin and

vancomycin). Table 3 provides a summary of how this aim was accomplished.

Table 3. *Development, Optimization and Validation of Study Media*

Targeted Emerging Pathogens	Media	Quality Control Organisms/Expected Results	Media Studies for Optimization/Validation
SAG	McKay agar	*S. anginosus* (ATCC 33397) (+) *S. intermedius* (ATCC 27335) (+) *S. constellatus* (ATCC 27823) (+) *S. aureus* (ATCC 29213) (-) Sterility (-)	Recovery Media Verification
Anaerobes	Brucella PEA BBE/LKV	CLSI-exempt from QC	N/A
Colistin-resistant NF GNR	Agar bases • BHI/TSA with colistin/ vancomycin • MAC with colistin	*E. coli* (ATCC 25922) (-) *P. aeruginosa* (-) *S. marcescens* (+) *B. cepacia* (+) *S. aureus* (ATCC 29213) (-) Sterility (-)	Recovery • *B. cepacia* and *S. marcescens* Recovery in Clinical Background • *A. xylosoxidans S. marcescens*

+=growth, -=no growth

Identification of SAG with McKay agar. In this study, SAG were specifically targeted

as emerging CF pathogens; therefore, McKay agar (Sibley et al., 2008) was incorporated into the

research protocol. Using the previously published formula for McKay agar, custom agar plates

were prepared by Teknova Inc. and shipped to the clinical microbiology laboratory at VCUMC.

Quality control testing, limit of detection, performance characteristics, and media verification were performed on McKay agar to ensure suitability for use in the study protocol.

   Quality control. Upon arrival, and weekly thereafter, throughout the duration of the study period, quality control testing was performed on McKay agar with the following organisms: *S. anginosus* (ATCC 33397), *S. intermedius* (ATCC 27335), *S. constellatus* (ATCC 27823), and *S. aureus* (ATCC 29213). A sterility plate was also included each time quality control was performed. The agar plates were deemed acceptable if all of the following were observed: growth of *S. anginosus* (ATCC 33397), *S. intermedius* (ATCC 27335), and *S. constellatus* (ATCC 27823) on McKay agar, which was indicated by the formation of yellow colonies on the media; and absence of growth of *S. aureus* (ATCC 29213) on McKay agar. The sterility control, which consists of an uninoculated McKay agar plate, was incubated and expected to exhibit no growth. The McKay agar plates passed QC each time, throughout the duration of the study period.

   Recovery and performance characteristics of McKay agar. To determine the optimized incubation condition for McKay agar in this study, recovery and performance characteristics were assessed and compared to those on BA under the following incubation conditions: 35°C ambient air, 35°C CO_2, and 35°C anaerobic conditions. When cultivating SAG isolates in a clinical laboratory, optimal growth is achieved when organisms are subcultured to BA and incubated at 35°C with CO_2; therefore, this was considered the standard to which all McKay agar data, with regard to recovery and performance characteristics, were compared. In addition, members of the SAG grow as pinpoint colonies on BA, produce a buttery or caramel smell, and vary in regard to hemolytic patterns. These documented growth characteristics were recorded

and used to compare and contrast those observed on McKay agar for each of the three SAG strains.

Recovery was determined by preparing 0.5 McFarland turbidity standard suspensions of the following: *S. anginosus* (ATCC 33397), *S. intermedius* (ATCC 27335), and *S. constellatus* (ATCC 27823).   The prepared 0.5 McFarland standard suspensions of each respective SAG isolate were 10-fold serially diluted, subcultured to solid media in duplicate, and incubated for 48 hours in the following: 35°C ambient air, 35°C CO_2, and 35°C anaerobic conditions. Colonies observed on solid media at each dilution were counted after 24 and 48 hours of incubation, averaged, and recorded. Based on results of the recovery study (Table 4), it was determined that anaerobic incubation conditions were optimal for recovering all three members of SAG. An increased number of SAG colonies were recovered on McKay agar under anaerobic conditions when compared with aerobic or aerobic with CO_2 atmospheric incubation conditions. As indicated by the recovery study, aerobic conditions (35°C) should not be used to cultivate SAG. *S. intermedius* (ATCC 27335) and *S. constellatus* (ATCC 27823) did not yield any observable colonies on McKay agar after 48 hours of incubation under aerobic atmospheric conditions. In addition, after 48 hours, SAG colonies were larger in size under anaerobic conditions than the typical pinpoint size colonies seen on BA, and in this case, McKay agar at 35°C in CO_2. The characteristic buttery or caramel smell of SAG isolates on BA was not noted with McKay agar. Also, hemolytic patterns could not be assessed on McKay agar.

In order to ensure that the prepared 0.5 McFarland standard suspensions of each organism fell within the acceptable range (1-2 x 10^8 cfu/mL), final colony counts from the original inoculum were calculated from the BA plates incubated for 48 hours at 35°C in CO_2 and

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Table 4. *Average Colony Counts of SAG on McKay Agar*

Incubation Conditions	Colony Counts (cfu/mL) Aerobic CO_2 $35°C$			Colony Counts (cfu/mL) Aerobic $35°C$			Colony Counts (cfu/mL) Anaerobic $35°C$		
Dilution Factors	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
S. intermedius	64	20	1	0	0	0	134	21	2
S. constellatus	59	12	2	0	0	0	112	19	2
S. anginosus	132	9	2	112	8	2	158	12	2

compared to expected results. Based on these data (Table 5), it was confirmed that the 0.5

McFarland standard suspensions of each organism were prepared and serially diluted correctly.

Table 5. *Average Colony Counts of SAG Isolates on BA*

Incubation Conditions	Average Colony Counts Aerobic CO_2 $35°C$			Organisms (cfu/mL)
Dilution Factors	10^{-6}	10^{-7}	10^{-8}	
S. intermedius	61	19	2	1.9×10^8
S. constellatus	75	18	2	1.8×10^8
S. anginosus	141	10	2	1.0×10^8

Media verification. The specificity of McKay agar for SAG in the presence of other

organisms was determined. Ten de-identified respiratory samples, consisting of four Eswabs and

six sputa, were plated to McKay agar and incubated for 24 hours at 35°C under anaerobic

conditions. After 24 hours of incubation, the plates were observed for the presence of possible

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SAG, which produce yellow colonies on McKay agar. Scant growth was observed; therefore, the plates were reincubated for an additional 24 hours. After a total of 48 hours of incubation, growth on McKay agar revealed several different morphotypes of yellow colonies, varying in size and texture. All colony types were subcultured to BA, incubated for 24 to 48 hours at 35°C in CO_2, and identified using MALDI-TOF. Members of SAG were recovered; however, it appeared as though other members of the viridans streptococci, which are also considered normal oropharyngeal flora, were able to produce an acid environment on McKay agar, resulting in a color change of the media.

Due to the unexpected results obtained during respiratory matrix testing, pure isolates of several organisms that could possibly be encountered in CF respiratory samples, such as SAG and other members of the viridans streptococci, *S. aureus* (ATCC 29213), and NF GNRs were selected and tested. A 0.5 McFarland standard suspension of each organism was prepared and ten microliter aliquots were inoculated to assigned quadrants on McKay agar. Three sets of plates were inoculated in an identical fashion, and incubated for 48 hours under the following conditions: 35°C, 35°C with CO_2, and 35°C in an anaerobic environment. The selected organisms and results obtained under each of the above specified incubation conditions are listed in Table 6.

In addition to SAG, many species of viridans streptococci, which are considered normal respiratory flora, produced yellow colonies on McKay agar, especially when incubated under anaerobic conditions, which is optimal for recovering SAG. Based on these findings, it was evident that McKay agar could only be used to screen CF respiratory samples for the presence of SAG. Any yellow colony growing on McKay agar, regardless of size and texture, should be subcultured to BA and identified by MALDI-TOF.

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Table 6: *McKay Agar Specificity Testing*

Organism	Aerobic 35°C	Aerobic CO2 35°C	Anaerobic 35°C
S. aureus (ATCC 29213)	No growth	No growth	No growth
E. coli (ATCC 25922)	No growth	No growth	No growth
S. anginosus (ATCC 33397)	Growth Yellow colonies	Growth Yellow colonies	Growth Yellow colonies
S. constellatus (ATCC 27823)	No growth	Growth Yellow colonies	Growth Yellow colonies
S. intermedius (ATCC 27335)	No growth	Growth Yellow colonies	Growth Yellow colonies
S. mitis (patient derived)	Growth No yellow colonies	Growth No yellow colonies	Growth Yellow colonies
S. parasanguinis (patient derived)	Growth Yellow colonies	Growth Yellow colonies	Growth Yellow colonies
S. salivarius (patient derived)	Growth Yellow colonies	Growth Yellow colonies	Growth Yellow colonies
S. oralis (patient derived)	Growth No yellow colonies	Growth No yellow colonies	Growth Yellow colonies
P. aeruginosa (ATCC 27853)	No growth	No growth	No growth
P. aeruginosa (mucoid) (patient derived)	No growth	No growth	No growth
B. cepacia (patient derived)	No growth	No growth	No growth
A. xylosoxidans (patient derived)	No growth	No growth	No growth

McKay agar inhibited the growth of *S. aureus* (ATCC 29213) and many NF GNRs routinelyfound in CF cultures. In the routine culture protocol, SAG would be obscured by these organisms, preventing the isolation and identification of these emerging pathogens. Therefore, the addition of McKay agar as a culture-dependent screening method for SAG was warranted.

Anaerobes. Three agar plates, Brucella, PEA, and LKV/BBE, were added to the research protocol for cultivation and isolation of strict anaerobes from CF sputum samples. The standard operating procedure currently in place for deep wounds, tissues, and sinus aspirates was followed

when incubating, cultivating, and identifying strict anaerobes from research samples in this study. (Appendix B). After 48 hours of incubation at 35°C under anaerobic conditions, growth on Brucella, PEA, and LKV/BBE was compared to growth on the BA incubated at 35°C in ambient air. This aided in the determination of true obligate aerobes versus facultative anaerobes. An aerotolerance test was performed on suspected obligate aerobes that were in quantities greater than or equal to the principal CF pathogens. If results of the aerotolerance test indicated that the organism was a true obligate anaerobe by only growing in strict anaerobic conditions, the isolate was definitively identified using MALDI-TOF. All of the obligate anaerobes reported in this study were adequately cultivated and isolated on Brucella agar. Based on this observation, the PEA and LKV/BBE agars were not necessary to cultivate this group of emerging pathogens. Eliminating these additional agars would decrease the cost and labor intensiveness in future studies.

Culture identification of colistin-resistant non-fermenting Gram-negative rods. Two types of agar containing colistin sulfate were developed, optimized, and implemented in the study protocol to select for emerging colistin-resistant NF GNRs. MAC containing colistin was optimized and validated for use with recovery studies. The second agar base, BHI, which contained colistin and oxalic acid, repeatedly failed quality control testing by not inhibiting the growth of *S. aureus* (ATCC 29213). In addition, the agar did not suppress the growth of Gram-positive OF when challenged with clinical matrix (respiratory samples). As a result, an alternative agar base, Tryptic Soy Agar (TSA), was selected and validated for use in the study. Oxalinic acid was replaced with vancomycin to sufficiently suppress the growth of normal OF when selecting for colistin-resistant NF GNRs in CF respiratory samples.

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Several batches of MAC and BHI agar with varying concentrations of colistin were prepared and tested to determine the optimal concentration for use in the study. Initially, the following concentrations of colistin were prepared, sterilized, and incorporated into the MAC and BHI agar bases: 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, and 40 mg/L.

Quality control was performed on all concentrations of each media type which consisted of *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), and a patient derived strains of *B. cepacia, P. aeruginosa,* and *S. marcescens*. These organisms were also used to inoculate both BHI and MAC plates which contained no colistin. Sterility plates were also incubated. Expected QC results for each media type were observed after 48 hours of incubation at 35°C. The sterility plates exhibited no growth, all isolates grew on BHI and MAC with no colistin, indicating the organisms were viable. *B. cepacia* and *S. marcescens* grew on all BHI and MAC at all colistin concentrations, and *E. coli, P. aeruginosa, and S. aureus* (ATCC 29213) did not grow on any of the colistin-containing media.

Seventy-four clinically-derived GNRs, with known colistin susceptibility results (Table 7), were used to determine the optimal colistin concentration for use in the study. Fresh subcultures were used to prepare 0.5 McFarland standard suspensions of each isolate, and 10 microliters were dispensed onto the respective MAC and BHI plates containing various concentrations of colistin. All plates were incubated at 35°C and read at 24 and 48 hours. Results revealed that all colistin-resistant isolates (n=21) grew on either MAC or BHI with 20 mg/L of colistin, five colistin-sensitive strains grew on MAC or BHI with 10 mg/L, and the BHI plate which contained 20 mg/L and 40 mg/L of colistin inhibited the growth of one colistin-resistant strain. In summary, MAC and BHI with 10 mg/L was not inhibitory enough; however,

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Table 7. *Isolates Used for Initial Determination of Optimal Colistin Concentration for MAC and BHI*

Organism	Colistin Sensitive	Colistin Resistant	Total
Achromobacter xylosoxidans	6	7	13
Acinetobacter baumanni	6	0	6
B. cenocepacia	0	1	1
B. cepacia	0	1	1
B. gladioli	0	1	1
Chryseobacterium indologenes	0	2	2
Inquilinus limosus	0	1	1
Myroides odoratus	0	1	1
Pseudomonas aeruginosa	19	0	19
Pseudomonas aeruginosa (mucoid)	9	0	9
Ralstonia pickettii	0	1	1
Serratia marcescens	0	4	4
Sphingobacterium spiritivorum	0	2	2
Stenotrophomonas maltophilia	13	0	13
Total	53	21	74

20 and 40 mg/L was too inhibitory; therefore, an additional concentration of colistin (15 mg/L), was tested.

Thirty-nine of the 74 clinical isolates (Table 8) used in the previous concentration study, representing a mixture of colistin-sensitive and colistin-resistant GNRs, were selected for repeat testing on BHI and MAC containing the following colistin concentrations: 10 mg/L, 15 mg/L, and 20 mg/L of colistin. QC was performed as described above and expected results were observed. The 39 clinically derived isolates were prepared as previously described, incubated at 35°C, and read at 24 and 48 hours. One colistin-sensitive strain of *S. maltophilia* grew on BHI with 10 mg/L of colistin and one colistin-resistant strain of *M. odoratus* was inhibited by 20 mg/L of colistin. These data revealed that 15 mg/L was the optimal concentration of colistin to incorporate into both MAC and BHI agar bases to select for colistin-resistant NF GNR in CF respiratory samples.

Table 8: *Isolates Used for Follow-Up Determination of Optimal Colistin Concentration for MAC and BHI*

Organism	Colistin Sensitive	Colistin Resistant	Total
Achromobacter xylosoxidans	2	3	5
Acinetobacter baumanni	5	0	5
B. cenocepacia	0	1	1
B. cepacia	0	1	1
B. gladioli	0	1	1
Chryseobacterium indologenes	0	2	2
Inquilinus limosus	0	1	1
Myroides odoratus	0	1	1
Pseudomonas aeruginosa	8	0	8
Pseudomonas aeruginosa (mucoid)	4	0	4
Ralstonia pickettii	0	1	1
Serratia marcescens	0	2	2
Sphingobacterium spiritivorum	0	1	1
Stenotrophomonas maltophilia	6	0	6
Total	25	14	39

BHI agar with colistin and oxalinic acid. Once the optimal concentration of colistin was determined to select for colistin-resistant NF GNRs, oxalinic acid was then incorporated into the BHI to inhibit the growth of Gram-positive organisms. Sibley et al. (2011) added 5 mg/L of oxalic acid to achieve desired suppression of Gram-positive organisms. Using this as a starting point, BHI agar with the following concentrations of oxalinic acid were prepared: 2.5 mg/L, 5 mg/L, and 10 mg/L. Two Gram-positive organisms, *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212), and one colistin-resistant NF GNR, *B. cepacia* were used for quality control. Both ATCC strains grew on BHI agar with all concentrations of oxalinic acid and *B. cepacia* was suppressed. Additional BHI plates were prepared with 15 mg/L of colistin and 2.5 mg/L, 5 mg/L, and 10 mg/L of oxalinic acid. QC was performed, and the same results were observed.

Media verification. De-identified respiratory samples were plated to BHI agar with 15 mg/L of colistin and oxalinic acid and BHI containing oxalinic acid alone to determine if other

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members of the OF would be inhibited. After a total of 48 hours of incubation at 35°C, it was evident that oxalinic acid did not sufficiently inhibit the growth of other Gram-positive organisms such as members of the viridans streptococci and *Corynebacterium* spp., both considered normal respiratory flora.

BHI with vancomycin. Based on the poor performance of oxalic acid in BHI agar, a glycopeptide, vancomycin, was selected to suppress the growth of Gram-positive organisms. A stock solution of vancomycin was prepared and diluted to make BHI agar plates with the following concentrations: 1 mg/L, 1.5 mg/L, 2 mg/L, 2.5 mg/L, 3 mg/L and 4 mg/L. Quality control was performed on each BHI plate at the above concentrations using the following organisms: *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), and *E. faecalis* (ATCC 51299). After 24 hours of incubation at 35°C, results were observed and recorded. All isolates grew on each BHI plate with all vancomycin concentrations demonstrating that this media was not suppressing the growth of Gram-positive organisms, as desired. The calculations used to prepare the stock solution of vancomycin and agar plates containing the varying concentrations were checked for accuracy.

After confirming that the calculations were correct, a fresh stock solution of vancomycin was prepared and used to prepare another batch of BHI with vancomycin at the above stated concentrations. The temperature of the BHI agar was allowed to cool to 50°C before adding the vancomycin to ensure that the antibiotic was not heat inactivated. Fresh isolates of the organisms listed above were used for QC testing. After 24 hours of incubation at 35°C, the same results were observed as with the previous batch of media.

At this point, vancomycin was purchased from a different manufacturer for testing. Upon arrival in the laboratory, a stock solution of vancomycin was made and BHI plates with varying

concentrations of vancomycin were prepared as described above, with the addition of three more vancomycin concentrations, 6 mg/L, 8 mg/L, and 20 mg/L. The same QC organisms and incubation conditions were used to assess the agar. Once again, the same results were obtained. At this point it was suspected that the vancomycin may be binding to some component of the agar and is not available to suppress the growth of vancomycin-susceptible organisms. In order to test this theory, vancomycin Etests were performed on two isolates, *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212). Two bases were used to test these isolates. BHI agar without vancomycin and Mueller-Hinton (MH) agar were prepared, which is routinely used for susceptibility testing in the clinical laboratory. All Etests were incubated for 24 hours at 35°C. The minimum inhibitory concentrations (MIC) for *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212) on BHI were 6 ug/mL and 12 ug/mL, respectively. The vancomycin MICs on MH agar for *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212) were 1 ug/mL and 3 ug/mL, respectively, which were within expected ranges. These results supported the suspicion that vancomycin was binding to some component in the BHI agar. As a result, an alternative agar base, Tryptic Soy agar (TSA) was selected for use in the study.

   TSA agar with vancomycin. Tryptic soy agar was selected as an alternative to BHI agar. A stock solution of vancomycin was prepared, sterilized, and diluted to achieve TSA agar plates with the following concentrations: 1 mg/L, 1.5 mg/L, 2 mg/L, 2.5 mg/L, 3 mg/L, 4 mg/L, 6 mg/L, and 8 mg/L. Three organisms, *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), and *E. faecalis* (ATCC 51299) were selected for QC. The QC organisms were prepared and inoculated to the TSA agar with varying vancomycin concentrations. After 24 hours of incubation at 35°C, plates were observed for growth. Expected results were observed; this agar

suppressed the growth of *S. aureus* (ATCC 29213) and *E. faecalis* (ATCC 29212), and supported the growth of *E. faecalis* (ATCC 51299).

Five de-identified respiratory samples were used for matrix testing. TSA agar plates with the following concentration of vancomycin were prepared and used: 2 mg/L, 4 mg/L, 6 mg/L, and 8 mg/L. Each respiratory sample was plated to TSA without vancomycin, and TSA agar plates containing the concentrations listed above. After a total of 48 hours of incubation at 35°C, it was evident that vancomycin inhibited Gram-positive organisms and normal OF. However, scant growth was still seen on the TSA agar containing 8 mg/L of vancomycin. A Gram stain of colonies growing on TSA with 8 mg/L of vancomycin revealed Gram-positive rods and Gram-negative diplococci, both normal members of OF. Since the purpose of this media was to inhibit these types of organisms, testing TSA with a higher concentration of vancomycin was warranted. As a result, TSA with 16 mg/L of vancomycin was prepared. QC was performed on TSA with 16 mg/L of vancomycin and expected results were observed. This media inhibited the growth of *S. aureus* (ATCC 29313) and *E. faecalis* (ATCC 29212) and supported the growth of *E. faecalis* (ATCC 51299). An additional five de-identified respiratory samples were used and matrix testing was repeated. Normal flora was abundant in all five samples on the TSA plate without vancomycin and completely inhibited on the TSA plate with 16 mg/L of vancomycin after 48 hours of incubation. Based on these results, it was determined that 16 mg/L of vancomycin was the optimal concentration needed to inhibit the growth of Gram-positive organisms and OF.

TSA agar with colistin. Since BHI was replaced with TSA, the optimal concentration of colistin needed to select for colistin-resistant NF GNRs needed to be determined. The 74 isolates listed in Table 7 were used to test TSA with colistin at the following concentrations: 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, and 40 mg/L. Testing was performed in the same manner as

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previously described with MAC agar. Similar results were obtained. Some colistin-sensitive isolates, such as *P. aeruginosa*, grew on TSA with 10 mg/L of colistin; many of the colistin-resistant isolates were inhibited at 40 mg/L; and *M. odoratus*, a colistin-resistant NF GNR, was inhibited at a colistin concentration of 20 mg/L. To narrow down the optimal colistin concentration, a subset of the 74 isolates (Table 8) was used to test TSA with the following colistin concentrations: 10 mg/L, 15 mg/L, and 20 mg/L. As seen with BHI agar, a few colistin-sensitive strains grew at 10 mg/L and *M. odoratus* was inhibited at 20 mg/L of colistin in TSA. Therefore, 15 mg/L was determined to be the optimal concentration of colistin in TSA to select for colistin-resistant NF GNR in CF respiratory samples.

  TSA agar with colistin and vancomycin. The optimal concentration of colistin (15 mg/L) and vancomycin (16 mg/L) in TSA were determined separately. Therefore, TSA plates containing optimal concentrations of both antibiotics were prepared and tested to ensure expected results were achieved. Quality control organisms for this prepared media consisted of the following: *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *E. faecalis* (ATCC 51299), *E. coli* (ATCC 25922), and a patient derived strain of *B. cepacia*. QC organisms were prepared and tested as previously described. After 24 hours of incubation the agar suppressed the growth of all QC organisms, except *E. faecalis* (ATCC 51299) and the patient derived strain of *B. cepacia,* indicating that the agar was functioning as desired.

  The TSA plates with 15 mg/L of colistin and 16 mg/L of vancomycin were used to test the organisms in Table 7. This was performed to ensure that none of the colistin-resistant NF GNR were inhibited by the addition of vancomycin. The TSA with colistin and vancomycin suppressed the growth of colistin-sensitive isolates and supported the growth of colistin-resistant isolates. In addition, five de-identified respiratory samples were plated to TSA without colistin

and vancomycin and TSA with 15 mg/L of colistin and 16 mg/L of vancomycin. After a total of

48 hours of incubation at 35°C, growth was observed on TSA without antibiotics, and growth

was suppressed on TSA with colistin and vancomycin. Results indicated that Gram-positive

organisms and normal respiratory flora were inhibited as desired.

Performance characteristics of MAC with colistin and TSA with vancomycin and

colistin. Two patient derived colistin-resistant strains of *B. cepacia* and *S. marcescens* were used

to assess the performance characteristics of MAC with colistin and TSA with vancomycin and

colistin. McFarland standard suspensions (0.5) of each organism was prepared and 10-fold

serially diluted. One-hundred microliters of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated to four

MAC with colistin plates and four TSA with vancomycin and colistin plates. One set of plates

(two of each respective type for each respective organism) was incubated in ambient air and the

other at 35°C with CO_2. Plates were removed and visually inspected at both 24 and 48 hours, at

which time colonies were counted and recorded (Tables 9 and 10).

Table 9. *Performance Characteristics of MAC with Colistin*

Incubation Conditions	Colony Counts (cfu/mL) Aerobic CO_2 35°C			Colony Counts (cfu/mL) Aerobic 35°C		
Dilution Factors	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
B. cepacia	90	19	1	86	19	1
S. marcescens	95	14	1	102	20	1

Table 10. *Performance Characteristics of TSA with Colistin and Vancomycin*

Incubation Conditions	Colony Counts (cfu/mL) Aerobic CO_2 35°C			Colony Counts (cfu/mL) Aerobic 35°C		
Dilution Factors	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
B. cepacia	90	19	1	94	26	1
S. marcescens	98	15	1	99	47	2

Due to size of the organisms, the plates inoculated with *B. cepacia* had to be incubated for an addition 24 hours in order to accurately count colonies on the agar. There was no difference in incubation conditions with regard to colonies counted or characteristic morphology of the tested organisms. Therefore, 35°C was selected as the atmospheric incubation condition used in this study. Similarly, MAC plates used in the routine CF protocol are also incubated at 35°C without CO_2.

Media verification. Both TSA with colistin and vancomycin and MAC with colistin were developed to screen for colistin-resistant NF GNRs in CF respiratory samples. In order to assess their specificity, de-identified patient respiratory samples were pooled and used as a diluent when performing serial 10-fold dilutions. Two patient derived colistin-resistant isolates, *A. xylosoxidans* and *S. marcescens* were prepared and serially diluted. One-hundred microliters of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were plated to MAC with colistin and TSA with colistin and vancomycin as described above. At both 24 and 48 hours of incubation at 35°C, plates were removed and colonies were counted. Expected recovery of organisms was achieved (Table 11), and Gram-positive organisms and normal OF flora were inhibited. The colistin-containing media was validated for use in the study protocol.

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Table 11. *Media Verification of MAC with Colistin and TSA with Colistin and Vancomycin*

Media Type	MAC w/ Colistin (cfu/mL) 35°C			TSA w/ Colistin and Vancomycin (cfu/mL) 35°C		
Dilution Factors	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
A. xylosoxidans	83	15	0	79	11	1
S. marcescens	91	17	0	96	11	1

Preliminary trial and study protocol. Once all media had been developed, optimized, and validated, a test run of the study was completed. Over a two-week period, a total of 10 de-identified CF respiratory samples were plated to routine and study media. This was performed to develop a workflow and procedure for culture workup during the study period (Table 12).

Table 12. *Research Study Protocol for Culture Workup*

Media	Study Protocol
McKay Agar	• Incubate for 48 hours in anaerobic conditions (35°C) • Subculture any yellow colonies to BA • Send colonies resembling SAG to MALDI for ID
MacConkey w/ Colistin	• Incubate for 48 hours in aerobic conditions (35°C) • Subculture any GNR to BA • Send to MALDI for ID
TSA w/ Colistin and Vancomycin	• Incubate for 48 hours in aerobic conditions (35°C) • Subculture any colony resembling GNR to BA • Confirm colony morphology on BA as GNR with GS • Send to MALDI for ID
Brucella, PEA, BBE/LKV	• Incubate for 48 hours in anaerobic conditions (35°C) • Observe plates for growth of anaerobes by comparing to growth on BA • Perform aerotolerance test on any suspected anaerobe • Send strict anaerobes to MALDI for ID

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Specific Aim 2

The prevalence of emerging pathogens in the CF population at VCUMC was determined based on culture results obtained from utilizing the optimized and validated media.

Population and sample. During the study period, 101 CF respiratory samples from a total of 75 (38 females, 37 males) patients were tested. The residual CF respiratory samples included in the study originated from 35 adult and 40 pediatric patients. The average age in the adult population, defined as those ≥18 years of age, was 28.37 years. The pediatric population was defined as patients <18 years of age, with a mean of 8.25 years. Age range of patients in the study was one to 56 years of age. PE exacerbation samples were defined as those that were collected while a patient was admitted to the hospital, determined by location.

At least two respiratory specimens were collected on 23 of the 75 patients. As a result, 101 CF respiratory samples were tested throughout the duration of the study period. Refer to Figure 2 for characteristics of the 101 patient samples analyzed.

SAG. Thirteen of the 75 patients were culture-positive for SAG. Three patients had more than one specimen submitted during the study period; therefore, a total of 16 samples were analyzed. Refer to Table 13 for a summary of SAG isolates detected in culture. The prevalence of SAG in the CF population during the study time frame was 17.33%. This was calculated based on the total number of non-repeated patients culture positive for SAG (n=13) divided by the total number of CF patients included in the study (n=75) multiplied by 100. Approximately 54% of SAG recovered were from CF patients experiencing PE. Coinfection with *P. aeruginosa* was also recorded.

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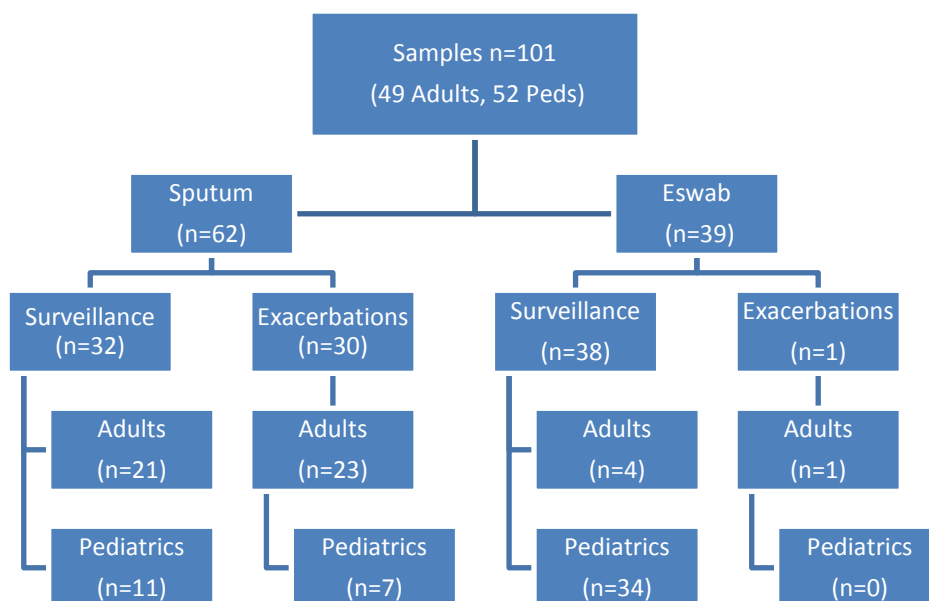
Figure 2. CF Respiratory Sample Characteristics

Table 13. *SAG Isolates Cultivated on Study Media*

Sample ID	Age	Sample Tested	Episode	Coinfection with *P. aeruginosa*?
S. anginosus				
20	A	Sp	Ex	Yes
35	A	Sp	Ex	Yes
38	A	Sp	S	Yes
49	A	Sp	Ex	Yes
S. constellatus				
8b	P	Sp	Ex	No
30	P	Es	S	No
41	P	Es	S	Yes
51	P	Sp	S	No
52	P	Es	S	No
52b	P	Es	S	Yes
56	A	Sp	Ex	No
S. intermedius				
36	P	Sp	Ex	No
38	A	Sp	S	Yes
42	P	Sp	S	Yes
52	P	Es	S	No
74b	A	Sp	Ex	Yes

A=adult, P=Pediatric, Sp=Sputum, Es=Eswab, S=surveillance, Ex=Exacerbation

Four *S. anginosus* isolates were identified from four different adult patients. Three of these adult patients were experiencing a PE. Only one *S. anginosus* was isolated in a surveillance culture. All four patients were co-colonized with *P. aeruginosa*. Some research groups have stated that SAG can possibly upregulate the virulence factors of classic CF pathogens.

S. constellatus was recovered in culture from six different patients. One patient, with two data points during the study period, was repeatedly culture positive for *S. constellatus*. Five of the patients were pediatrics, and of those, four were surveillance cultures. Only two of the pediatric patients who were culture positive for *S. constellatus* were co-infected with *P. aeruginosa*. *S. constellatus* was isolated from only two samples collected during PE: one from a pediatric patient, and one from an adult patient. Five of the six patients culture positive for *S. constellatus* were part of the pediatric population. Unlike *S. anginosus*, this member of the SAG was predominantly found in those less than 18 years of age during a period of routine surveillance.

S. intermedius was isolated from five different CF patients during the study time frame. Three of those were pediatric patients; two during a time of surveillance and one experiencing a PE. *S. intermedius* was isolated from two adult patients, both via surveillance cultures. Two of the adult patients and one of the pediatric patients were co-colonized with *P. aeruginosa*.

In two patients, two members of the SAG were isolated in culture. One was an adult patient, whose surveillance culture was positive for *S. anginosus* and *S. intermedius*. The other, a pediatric patient, was co-colonized with *S. constellatus* and *S. intermedius*.

Anaerobes. Brucella, PEA, and LKV/BBE agars were used to determine the prevalence of strict anaerobes in the CF population at VCUMC. Thirty-one of the 75 patients included in the

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study were culture positive for anaerobes in greater than or equal to quantities than the classic CF pathogens and/or normal respiratory flora. This was determined by comparing growth on the anaerobic plates to growth on the BA at 35°C in ambient air. Therefore, the prevalence of anaerobes in the CF population at VCUMC during the study time frame was 41.33%.

Thirty-three of the 101 samples tested were positive for anaerobes in greater than or equal to quantities than the principle CF pathogens and/or normal respiratory flora. Twenty-eight of the 75 patients (30 total CF respiratory specimens tested), were culture positive for *Veillonella* species (Table 14). Of these 28 patients, 18 were pediatrics and 10 were adults. In both age groups, the majority of anaerobes were recovered during periods of surveillance and only eight were coinfected with *P. aeruginosa*. The remaining anaerobic species cultivated were *Lachnoanaerobium umeaense, Lanchnoanaeroboculum ovale, Prevotella pallens, Prevotella melaninogenica*, and *Fusobacterium naviforme,* which were all isolated from pediatric surveillance cultures. One sample from one patient grew both *L. umeaense* and *P. pallens*. The CF patient respiratory sample that grew *F. naviforme* was coinfected with *P. aerugonisa*.

Colistin-Resistant NF GNR. Ten of the 75 patients (15 total specimens) were culture positive for colistin-resistant NF GNRs recovered on both study media and in routine culture (Table 15). Only four colistin-resistant NF GNRs were isolated. Moreover, repeated samples on the same patients did not reveal new organisms. However, three unique colistin-resistant NF GNRs were in four specimens using study media alone: *Sphinogomonas paucimobilis* and *Chryseobacterium gleum* recovered from one patient (two separate samples), and *A. xylosoxidans* recovered from two separate patients (Table 16). Therefore, the prevalence of colistin-resistant NF GNRs only on study media is 4%. When both emerging and classic CF

Table 14. *Veillonella species Cultivated on Study Media*

Sample ID	Age	Sample Tested	Episode	Coinfection with *P. aeruginosa*?
V. aytpica				
1	A	Sp	S	Yes
1b	A	Sp	Ex	Yes
9	P	Sp	S	No
10	A	Sp	Ex	Yes
14	P	Es	S	No
14b	P	Es	S	No
18	A	Sp	Ex	No
24	P	Es	S	No
25	A	Sp	S	Yes
38	A	Sp	S	Yes
39	P	Es	S	No
44	A	Es	S	No
45	P	Sp	S	No
46	A	Sp	S	Yes
59	P	Sp	S	No
60	P	Es	S	No
64	P	Es	S	No
V. parvula				
12	P	Es	S	No
28	P	Es	S	No
49	A	Sp	Ex	Yes
61	P	Es	S	No
V. dispar				
7b	P	Es	S	No
31b	P	Es	S	No
36	P	Sp	Ex	No
66	A	Sp	S	Yes
68b	P	Sp	Ex	No
70	P	Es	S	No
72	P	Es	S	No
74	A	Sp	S	Yes
V. rogosae				
52	P	Es	S	No

A=adult, P=Pediatric, Sp=Sputum, Es=Eswab, S=surveillance, Ex=Exacerbation

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Table 15. *Colistin-Resistant NF GNR Cultivated on Routine and Study Media*

Sample ID	Age	Sample Type	Episode	Organism ID
4	A	Sp	Ex	*B. cenocepacia*
4b	A	Sp	Ex	*B. cenocepacia*
4c	A	Sp	Ex	*B. cenocepacia*
5b	A	Sp	Ex	*A. xylosoxidans* *P. aeruginosa*
16	A	Sp	Ex	*A. xylosoxidans*
16b	A	Sp	S	*A. xylosoxidans*
26	P	Es	S	*S. maltophilia*
33	A	Sp	Ex	*A. xylosoxidans*
40	A	Sp	S	*A. xylosoxidans*
41	P	Es	S	*S. maltophilia*
56	A	Sp	Ex	*B. cenocepacia*
56b	A	Sp	Ex	*B. cenocepacia*
74	A	Sp	S	*A. xylosoxidans*
74b	A	Sp	Ex	*A. xylosoxidans*
75	A	Sp	Ex	*B. cenocepacia*

A=adult, P=Pediatric, Sp=Sputum, Es=Eswab, S=surveillance, Ex=Exacerbation

Table 16. *Colistin-Resistant NF GNR Cultivated Only on Study Media*

Sample ID	Age	Sample Type	Episode	Organism ID
14	P	Es	S	*S. paucimobilis*
14b	P	Es	S	*C. gleum*
18	A	Sp	Ex	*A. xylosoxidans*
38	A	Sp	S	*A. xylosoxidans*

A=adult, P=Pediatric, Sp=Sputum, Es=Eswab, S=surveillance, Ex=Exacerbation

pathogens are combined, the prevalence of colistin-resistant NF GNRs in the population at

VCUMC over the study period is 17.33%. Colistin-resistant *A. xyolsoxidans* was isolated from

seven of 13 patients, *B. cenocepacia* in three of 13 patients, and *S. maltophilia* in two of 13

patients. *Providencia rettgeri and Serratia marcescens*, members of the *Enterobacteriaceae*,

were recovered from two separate patients. Both isolates are colistin-resistant; however, since

they are not considered NF GNRs, they were not included in the calculation for prevalence. It

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was noted that *P. rettgeri* was found in greater quantities than classic CF pathogens and normal respiratory flora.

Specific Aim 3

Specific Aim 3 was accomplished by prospectively evaluating the current CF culture procedure routinely performed in the clinical microbiology laboratory at VCUMC with the CF research protocol. Traditional CF cultures were read and reported by clinical laboratory scientists in the clinical microbiology laboratory at VCUMC. These were compared with results obtained from the study protocol for consistency in reporting of pathogens.

SAG. The presence of SAG was not reported in any of the routine CF respiratory culture results. Members of the SAG are not specifically sought, and, the current procedure does not include a selective media for recovery of this group of organisms.

Anaerobes. The routine CF respiratory culture protocol does not include media or incubation conditions to cultivate and isolate anaerobic bacteria from this population. Therefore, all anaerobes reported were only from the research protocol.

Colistin-resistant NF GNR. Four specimens from three different patients yielded colistin-resistant NF GNRs when cultivated and isolated on the study media. These consisted of the following genus and species: *A. xylosoxidans*, *S. paucimobilis*, and *C. gleum* (Table 16). Two isolates of *A. xylosoxidans* were recovered from adults; whereas, *S. paucimobilis* and *C. gleum* were recovered from one pediatric CF patient.

Additional findings. Seven respiratory samples from seven different CF patients grew fungus only on the study media, specifically TSA with 15 mg/L of colistin and 16 mg/L of vancomycin (Table 17). The majority of these samples were collected from the pediatric CF population. In addition, most of these samples were collected during periods of surveillance.

Table 17. *Culture Discrepancies Between Routine and Research CF Protocols*

Sample ID	Age	Sample Type	Episode	Discrepancy *Isolated in Research Protocol Only
3	P	Sp	S	Fungus
10	A	Sp	Ex	Fungus
18	A	Sp	Ex	*A. xylosoxidans*
38	A	Sp	S	*A. xylosoxidans*
42	P	Sp	S	Fungus
51	P	Sp	S	Fungus
59	P	Sp	S	Fungus
68	P	Sp	Ex	Fungus
71	A	Es	Ex	Fungus
14	P	Es	S	*S. paucimobilis*
14b	P	Es	S	*C. gleum*

A=adult, P=Pediatric, Sp=Sputum, Es=Eswab, S=surveillance, Ex=Exacerbation

Summary

This chapter discussed the results of Specific Aims 1 through 3. Specific Aim 1 highlighted the development, optimization, validation, and implementation of the culture-dependent media used to target emerging pathogens in the CF population at VCUMC. Recovery, media verification, and optimal incubation conditions were determined for McKay agar, MAC with 15 mg/L of colistin, and TSA with 15 mg/L of colistin and 16 mg/L of vancomycin. McKay agar performed best under anaerobic conditions, and both MAC with colistin and TSA with colistin and vancomycin performed well in both 35°C ambient air and 35°C CO_2. The prevalences of SAG, anaerobes, and colistin-resistant NF GNRs for the CF population at VCUMC over the study period were calculated as 17.33%, 41.33%, and 4%, respectively. Finally, discrepancies between the routine and research protocol culture, consisting of additional colistin-resistant NF GNRS recovered in the research protocol only as well as the unexpected cultivation of fungus on TSA containing colistin and vancomycin were described.

Chapter 5: Discussion

Introduction

A summary of the research is presented in this chapter, and findings of the study are discussed and interpreted. The significance of these results, in the context of emerging CF pathogens is examined and compared to the literature. Implications of the results to current practice and recommendations for CF culture protocols is presented. Limitations and future research directions are discussed.

Molecular profiling studies have revealed the vast number and complexity of microbial communities in the CF lung. Based on these studies, several emerging pathogens, hypothesized to play a direct or indirect role in pulmonary exacerbations, have been widely recognized and discussed (Sibley et al., 2011; Zhao et al., 2012). Currently, it is not feasible to perform molecular profile analysis on CF surveillance cultures. In fact, molecular profiling studies are only recommended when epidemiologically indicated (CF Foundation, www.cff.org). Therefore, in order to determine the role of these potential emerging pathogens in CF lung disease, and potentially add to the reporting scheme in the clinical rather than research setting, culture-dependent microbiology screening protocols were developed. Several emerging pathogens, thought to play a direct or indirect role in PEs, were specifically targeted in culture. In order to recover these targeted CF pathogens from respiratory samples, selective media was developed and/or purchased for use in the study protocol.

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SAG

McKay agar was able to cultivate and isolate SAG in CF respiratory cultures. Although anaerobic incubation conditions were assessed in previous studies (Sibley et al., 2008; Sibley et. al, 2011), results were not discussed. Therefore, in this study, performance of McKay agar was assessed at three different atmospheric conditions: 35°C, 35°C CO_2, and 35°C in anaerobic environment. Initial limit of detection studies, utilizing three ATCC strains of SAG, revealed that recovery was increased when McKay agar was incubated at 35°C under anaerobic conditions. In addition, appearance of the colonies were larger in size on McKay agar under anaerobic conditions. It was noted that McKay agar did not support the growth of *S. intermedius* (ATCC 27335) or *S. constellatus* (ATCC 27823) when incubated in ambient air at 35°C. It is important to note that SAG was not observed on BA included in the study protocol (35°C with CO_2) due to possible overgrowth by normal respiratory flora and CF pathogens, such as *S. aureus* and mucoid *P. aeruginosa*.

Members of the SAG have been labeled as a CF respiratory pathogens capable of playing a role in acute PE by several groups (Sibley et al, 2008; Parkins et al, 2008; Grinwis et al., 2010; Sibley et al., 2010; Sibley et al., 2011;. Filkins et al., 2012; Asam and Spellerberg, 2014; Navratilova et al., 2016). The reported prevalence of SAG in the CF population at VCUMC during the study period was 17.33%. Sibley et al. (2008) presented multiple case reports in which SAG was the numerically dominant pathogen during periods of PE. When SAG was specifically targeted for treatment, clinical response was observed. In this study, 53.8% of SAG recovered in culture were from CF patients experiencing PE. Recurrent episodes of PE in CF patients are associated with long term decline in lung function and shortened survival. Selecting for SAG in culture with McKay agar would allow cultivation and identification of this emerging

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pathogen and provide clinicians with an additional treatment target when managing CF patients experiencing PE.

In the current study, percent species breakdown of SAG in the CF population at VCUMC is as follows: *S. anginosus* (25%), *S. intermedius* (31%), and *S. constellatus* (44%). These results are similar to those reported by Sibley et al. (2010). An overall SAG prevalence of 40.6% in 108 CF patients over a six-month period was reported by incorporating McKay agar in the culture protocol. These isolates were further classified by species. *S. anginosus* constituted 40.7% of the recovered SAG isolates, *S. intermedius* comprised 34.3%, and *S. constellatus* accounted for 25%. These results indicate that SAG is present and persistent in CF patients.

Similar to the findings in this study, Navratilova et al. (2016) reported a SAG prevalence of 17.6% in the CF population studied. Recognizing the potential of SAG to cause PEs, this emerging pathogen was sought in patients with other lung disorders, such as chronic obstructive pulmonary disease (COPD). SAG prevalence in this population was 10.3%. Acknowledging the role of SAG in PE as well as modifying culture protocols to detect this group in CF pulmonary samples is necessary to provide optimal patient care.

Anaerobes

Three different types of anaerobic media, Brucella, PEA, and BBE/LKV were incorporated in the study protocol. This media combination is expensive, and after conducting the study, it was concluded that only one anaerobic media type, Brucella, was actually needed. Anaerobes were only identified if they were in greater than or equal quantities than the classic CF pathogens and/or normal flora. This was easily assessed on Brucella agar alone, which could reduce future costs and labor intensiveness of reading culture plates.

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Based on the results of molecular profiling studies in research settings, there has been a renewed interest in anaerobes and the role they play in CF lung disease (Sibley et al., 2012, Zhao et al., 2012; Sherrard et al., 2016; Tunney et al., 2008; Pustelny et al., 2015).The prevalence of anaerobes in the CF population at VCUMC was 41.33%. Interestingly, *Veillonella* spp. was isolated from 28 patients. Only six of these 28 patients were experiencing a PE. Only seven of the patients from which *Veillonella* species was recovered were coinfected with *P. aeruginosa*.

A recent study reported on the interaction of *Veillonella parvula* with *P. aeruginosa* in a murine tumor model (Pustelny et al., 2015). Significantly higher cell numbers of *P. aeruginosa* were recovered from tumor tissue when the mice were coinfected with both organisms. In vitro studies suggested that *V. parvula* may promote the development of robust *P. aeruginosa* aggregates and mature biofilm structures. Again, this supports the claim that organisms previously considered to be normal respiratory flora in CF patients, can potentially upregulate the virulence factors of the principle pathogen, *P. aeruginosa*. Further studies are required to determine if anaerobes contribute to the microbial diversity of the CF lung, corresponding with periods of clinical stability; or, if in fact, they, too, could be potentially used as a microbiologic marker of PE.

CF pulmonary infections are polymicrobial; and it is possible that anaerobic bacteria, not detected by routine aerobic culture, reside within infected airway mucous. Tunney et al. (2008) conducted a study to determine whether anaerobic bacteria was present in the sputum of patients with CF. Anaerobes in quantities greater than 10^4 were identified and reported. The prevalence of obligate anaerobes in the 50 CF patients studied was 66%. A total of 66 samples were collected from these 50 patients. Sixty-three percent of those revealed anaerobes in quantities greater than or equal to $10^{4.}$ Anaerobes detected were similar to those involved in anaerobic

pulmonary infections such as nosocomial pneumonia, lung abscesses, and empyema, where the presence of aerobic and anaerobic bacteria were considered to be a significant finding. Twenty-seven samples were positive for both *P. aeruginosa* and anaerobes. Eleven of the samples culture negative for *P. aeruginosa* were positive for anaerobes. These data suggest that anaerobes may cause early infection, and over time, produce a hospitable environment for subsequent infection with *P. aeruginosa*.

In the current study, anaerobes were identified if present in greater than or equal to quantities than the principal CF pathogens. The population prevalence of anaerobes was 41.33%. Thirty-three of the 101 (32.67%) samples collected from 75 CF patients were positive for anaerobes during the study period. Ten of the 33 samples were culture positive for both *P. aeruginosa* and anaerobes (30.33%) and 23 (69.69%) were culture negative for *P.aeruginosa* and positive for anaerobes. High quantities of anaerobes may be unique to CF patients and play a possible role in lung disease.

Field et al. (2010) detected *Prevotella* spp. in combination with SAG from CF patients at the onset of PE. *Prevotella* spp. were isolated in two patients in this study, resulting in a prevalence of 6.45%. Both were pediatric patients during a period of routine surveillance and cultures were negative for *P. aeruginosa* and SAG. A pediatric respiratory sample collected during PE in this study was culture positive for *F. naviforme*, *P. aeruginosa*, and *S. constellatus*. These findings further support the claim that interspecies interactions among emerging pathogens such as anaerobes and SAG with classic pathogens, such as *P. aeruginosa*, may result in periods of clinical instability or PE.

Colistin-Resistant NF GNRs

Isolation and identification of all colistin-resistant NF GNRs, regardless of quantity, should be identified by methods such as MALDI-TOF or DNA sequencing. Colistin is used as a last line of defense to treat colistin-resistant *P. aeruginosa*, and any NF GNR exhibiting resistance in culture should be reported to clinicians (Kiska and Riddell, 2012). The prevalence of colistin-resistant NF GNRs found only on study media was 4%, and the prevalence of those found in both the routine and research culture protocols was 17.33%. There was one colistin-resistant NF GNR isolated that was unable to grow on MAC, *C. gleum*. However, this organism was cultivated and isolated on TSA with colistin and vancomycin. In two instances, the study media isolated two colistin-resistant strains of *A. xylosoxidans* from two separate patients that was not reported in routine culture. The selective nature of the study media prevented the mixed respiratory flora and classic CF pathogens from overgrowing and obscuring the growth of *A. xylosoxidans*. Both samples were obtained from adults experiencing a PE, where *A. xylosoxidans* would be targeted for antimicrobial therapy. Two colistin-resitant NF GNRs, *S. paucimobilis* and *C. gleum*, were recovered from one pediatric patient separately at two different time points during periods of surveillance. These organisms are opportunistic and ubiquitous in the environment. Their presence in culture may indicate improper maintenance or cleaning of respiratory equipment.

I. limosus, an emerging CF pathogen, is a colistin-resistant NF GNR unable to grow on MAC. The appearance of this organism on BA and CHOC agars resemble the appearance of mucoid *P. aeruginosa*. This organism was specifically sought in culture; however, it was not isolated from any patient during the study time frame.

Fungus

One unexpected finding was the cultivation of yeast and fungi on the colistin-containing media, particularly TSA. Incorporation of colistin and vancomycin suppressed the growth of Gram-positive organisms and colistin-sensitive bacteria allowing the slower growing yeast and fungus to grow without competition. This media recovered all fungi reported in the routine CF culture protocol. In addition, fungus was present in seven patient samples on study media only, three of which were collected during periods of PE. Therapeutic regimens differ for bacteria and fungus. The presence of fungus in CF respiratory cultures should be reported and considered by physicians when treating patients, as it offers an additional therapeutic target. *Aspergillus fumigatus* can cause allergic bronchopulmonary aspergillosus in up to 15% of CF patients. This complication can cause repeated acute exacerbations leading to an accelerated decline in lung function.

A. fumigatus and *P. aeruginosa* both form biofilms in vivo and in vitro which provide organisms with protection from host defenses as well as tolerance to some drugs. *P. aeruginosa* secretes molecules such as homoserine lactones, pyocyanin, and fluorescent green pigments which have all been studied for their antifungal activities (Ferreira et al., 2015). Approximately 80% of adult CF patients are infected with *P. aeruginosa*. When this principal pathogen is present with *A. fumigatus* in culture, it may inhibit the growth of the fungus preventing cultivation and identification of this CF pathogen. Granillo et al. (2015) reported the same type of phenomenon with *S. aureus*, a classic CF pathogen, and *A. fumigatus*. *S. aureus* inhibited the development of the biofilm formed by *A. fumigatus* in vitro. This pathogen could also be responsible for inhibiting the growth of fungus in routine CF culture. Failure to identify and report fungus in CF respiratory cultures can impact patient outcomes as antifungals instead of

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antimicrobials are implicated for treatment. Further prospective studies are needed to determine the ability of TSA with colistin and vancomycin to reliably recover fungus from CF patient respiratory samples.

Limitations

Ideally, in order to get a representative sample of the lower airways, sputum should be obtained from each patient. In the event that a patient cannot produce sputum, which is common in the pediatric CF population, an Eswab is collected for analysis. Excluding samples collected with Eswabs would decrease the number of participants and inadequately represent the population of CF patients at VCUMC. Emerging pathogens were frequently recovered from CF respiratory samples collected by Eswabs in this study.

When anaerobes are suspected in culture, transport, receipt, and plating should occur as soon as possible to ensure optimal recovery. In this study, either sputum or Eswabs were collected and transported to the lab. According to the manufacturer, Eswabs maintain the viability of aerobic and anaerobic bacteria for up to 72 hours. When sputa is collected, it is not feasible for the specimen to be collected and plated within an hour of collection; therefore, this may impact the recovery of anaerobes. All CF respiratory samples included in this study were plated to the study media within 48 hours of receipt in the clinical microbiology laboratory.

Another noted limitation is sample size. This study determined the prevalence of emerging pathogens in 75 CF patients, from which a total of 101 respiratory samples were collected. Multiple data points were obtained on 23 of the 75 patients. Increasing the sample size and obtaining multiple culture data points, ideally four to five per patient, would determine if the presence of these emerging pathogens in culture represent transient versus chronic colonization. In addition, multiple data points would further describe the association of these

pathogens with periods of PE and allow more unique analysis of subgroups. However, the sample size of 75 in this study is comparable to other studies in the literature. In this study, periods of PE were defined by hospitalizations. A clear-cut definition of PE does not exist; therefore, periods of PE may be defined in different ways by other researchers.

The prevalence of emerging pathogens in the CF population at VCUMC was calculated. Prevalence of emerging pathogens may differ among CF clinics by patient population and even geographic distribution, which impacts the generalizability of these findings. Results are specific to the population studied; however, the method to determine prevalence of emerging pathogens can be easily reproduced in other settings.

Future Studies

The results of this study confirmed that emerging pathogens cited in the literature are present in the respiratory samples of CF patients at VCUMC. A longitudinal study, conducted in the same fashion, would be helpful to further describe the role of SAG in CF respiratory cultures. In addition, in order to determine the role emerging pathogens play in PE and if culture can serve as a microbiologic indicator of lung function decline, clinical data should be correlated with culture results. Results of pulmonary lung function tests (FEV), body mass index (BMI), and antimicrobial therapeutic regimens have been used in previous studies. Collaboration with CF pulmonologists would assist in bridging this gap in order to gain a better understanding of the role of emerging pathogens in this population. A prospective study, evaluating the use of TSA with colistin and vancomycin to recover fungus in CF respiratory cultures is warranted. Study protocol results could be compared for consistency with fungus culture results. Another type of selective media, NAS agar, has been used in recent studies to recover SAG from clinical samples cultures. Raclavsky et al. (2016) compared McKay agar to NAS agar using 123 patient samples.

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Overall, NAS was able to recover greater numbers of SAG. However, it was noted that a fraction of strains grew better on McKay agar. A future study could assist in comparing these two selective media for the recovery of SAG in CF respiratory samples.

Conclusion

Molecular profiling studies have identified potential emerging pathogens, such as SAG, anaerobes, and colistin-resistant NF GNRs, and suggest their involvement in the progression of CF lung disease. In the clinical setting, these methods are not routinely used or recommended to monitor CF patients. Therefore, culture-dependent screening techniques were developed, optimized, and implemented in this study to determine the prevalence of targeted emerging pathogens in the CF population at VCUMC.

Results revealed that emerging pathogens are present in CF respiratory samples, many of which were collected from patients during periods of clinical instability. Expanding the list of CF pathogens sought in culture could provide additional therapeutic targets for physicians. Initiating treatment earlier, specifically directed against emerging pathogens thought to play a role in PE, could improve patient outcomes by decreasing hospitalizations and associated costs as well providing more targeted versus broad spectrum antibiotic therapy.

The dynamic microbiome within the CF airway, consisting of classic CF pathogens as well as emerging pathogens, must be considered to effectively manage and treat patients. Therefore, the routine culture protocol needs to be revised to select for emerging CF pathogens thought to play a role in PE and lung function decline. In order for definitive conclusions to be made, the presence of emerging pathogens in CF cultures coupled with clinical data should be evaluated to further define their role in PE and lung function decline.

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Appendix A

Routine CF Culture Protocol

I.   PRINCIPLE

The culture of respiratory secretions from cystic fibrosis (CF) patients presents unique challenges for the microbiologist that requires patience and determination. The most significant organism associated with CF is a highly mucoid, nonpigmented strain of *Pseudomonas aeruginosa*. The nature alone of this mucoid *P. aeruginosa* necessitates the use of a vigorous and selective protocol to ensure isolation of the other major CF pathogens. Refer to section VII., Procedure Notes for more detailed information on cystic fibrosis.

II.   CULTURE MEDIA

Media	Incubation		
	$35^{o}C$-CO_2	$35^{o}C$-nonCO_2	LOI
BAP	✓		4 days
MAC		✓	
CHOC	✓		
CNA	✓		
BCSA		✓	
MS		✓	

LOI=Length of Incubation.

III.   POTENTIAL PATHOGENS/NORMAL FLORA
   A.   Potential Pathogens
      1.   Mucoid *Pseudomonas aeruginosa*
      2.   Non-mucoid *Pseudomona aeruginosa*
      3.   *Staphylococcus aureus* (including small colony variant)
      4.   *Haemophilus influenzae*
      5.   *Burkholderia cepacia*
      6.   Fungi – mould
      7.   Beta-hemolytic *Streptococcus*
      8.   *Stenotrophomonas maltophilia*
      9.   *Streptococcus pneumoniae***
      10.   Other non-fermenting gram-negative bacilli**
      11.   Other *Haemophilus* species**
      12.   *Enterobacteriaceae*****
      13.   *Moraxella catarrhalis*****
      14.   *Neisseria meningitidis*****
      15.   Yeast*****

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*Quantity = moderate-many; **Quantity = predominating

B. Normal Flora
1. Alpha-hemolytic (viridans) *Streptococcus*
2. Non-hemolytic *Streptococcus*
3. *Enterococcus* species
4. *Staphylococcus* species, coagulase-negative
5. *Micrococcus* species
6. *Corynebacterium* species and other gram-positive rods
7. *Neisseria* species (saprophytic)
8. *Streptococcus pneumoniae**
9. Other non-fermenting gram-negative bacilli*
10. Other *Haemophilus* species*
11. *Enterobacteriaceae***
12. *Moraxella catarrhalis***
13. *Neisseria meningitidis***
14. Yeast**

*Quantity = few; **Quantity = not predominating over normal flora

IV. SAFETY: If any organism resembling *Neisseria meningitidis* or *Haemophilus* spp. (grey, translucent colony morphology on CHOC agar) is isolated, or if gram-negative diplococci are predominating in the direct Gram stain of the culture:

A. Take all plates to a BSL-2 hood.

B. Perform all work, including MALDI setup, under the hood until *N. meningitidis* is ruled out.

C. Shrink-seal all plates.

V. CULTURE EXAMINATION AND INTERPRETATION
A. Culture Exam
1. <u>At 24 hours</u>:
   a. Examine all plates for normal respiratory flora and the appearance of any significant CF pathogens (see table, page 3-4).
   b. If significant pathogens are present, see interpretation section below for extent of workup.
   c. Reincubate all plates.
   d. Issue a preliminary report as described in section VII.B.1.
2. <u>At 48 hours and 72 hours</u>:
   a. Re-examine all plates as above (section V.A.1.a-b).
   b. Reincubate all plates.
   c. Issue preliminary reports as described in section VI.B.2-3.
3. <u>At 96 hours (4 days)</u>:
   a. Re-examine all plates as above (section V.A.1.a-b).
   b. Issue preliminary reports as described in section VII.B.4.

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c.   If no apparent CF pathogens have appeared, issue a final report as described in section VII.C.

B.   Interpretation and Extent of Workup

1.   Special considerations for CF cultures:

a.   Wait until there is sufficient growth before attempting any subcultures; this can take 3-4 days.
b.   Subculture all isolates prior to identification and susceptibility testing and incubate until growth is sufficient to ensure a pure culture.
c.   Hold all original media <u>and</u> all plates that have been subcultured and worked from in the plate reader's "workup stack" until the culture is complete.
d.   Review the patient's previous cultures in **Micro Inquiry**. CF patients usually grow the same organisms for extended periods of time. The susceptibility patterns often remain constant, but can change over time.

2.   Examine all media for appearance of mucoid *Pseudomonas aeruginosa*:
a.   Slow grower (48 to 72 hours) on BAP, CHOC, MAC.
b.   Non-pigmented, non-lactose fermenter.
c.   Starts out wet; gradually turns into sticky gum-like slime.

3.   Examine **MS** for *S. aureus*: Most strains of *S. aureus* grow on MS and produce yellow zones around the colonies as a result of mannitol fermentation.
a.   Occasionally, some strains produce white colonies on **MS**.
b.   If both parent and small colony variant *S. aureus* are present, only perform ID and sensis on parent colony.

4.   Examine **BCSA** for *Burkholderia cepacia* complex:
a.   Usually slow grower (3 to 4 days).
b.   Colonial Morphology: variable = small to large, dry to moist.
c.   Colonial Appearance:

i.   Colonies that absorb the crystal violet dye = purple to purple-gray.

ii.   Acid production from carbohydrate oxidation = yellow zone in the medium surrounding the colony.

iii.   Peptone utilization = pink zone in the medium surrounding the colony.

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d. Key biochemical reactions for *B. cepacia*: Oxidase = positive, but may appear negative initially.

e. This media is highly selective against gram-negative rods, but some breakthrough growth can occur. Examples are:

    i. *Burkholderia gladioli*, which produces a natural yellow, pigment, but is oxidase-negative.

    ii. *Stenotrophomonas maltophilia*.

    iii. *Achromobacter xylosoxidans*.

5. If *B.* cepacia, *Burkholderia gladioli,* or *Ralstonia pickettii* cannot be ruled out, check **Micro Inquiry** to determine if the patient has grown the organism previously.

    a. If no previous cultures have grown these organisms, send the isolate to the *Burkholderia cepacia* Research Laboratory and Repository for confirmation.

        i. Refer to page 6 for instructions.

        ii. Reporting: see section VI.B.5, page 4.

    b. If the organism has been identified in previous cultures, report as described in section VI., page 4.

6. Use the following table as a guide to differentiate normal respiratory flora from potential CF pathogens and to determine extent of workup:

Organism	Quantity	Workup
Alpha-hemolytic (viridans) *Streptococcus*	Any	None = Normal respiratory flora
Non-hemolytic *Streptococcus*	Any	None = Normal respiratory flora
Enterococcus species	Any	None = Normal respiratory flora
Staphylococcus, coagulase-negative	Any	None = Normal respiratory flora
Micrococcus species	Any	None = Normal respiratory flora
Corynebacterium species and other gram-positive rods	Any	None = Normal respiratory flora

Neisseria species (saprophytic)	Any	None = Normal respiratory flora
Moraxella (*Branhamella*) *catarrhalis*	Predominant	ID and β-lactamase
Neisseria meningitidis	Predominant	ID
Enterobacteriaceae	Predominant	ID and AST

Organism	Quantity	Workup
Yeast	Predominant	"Feet" [1] = *C. albicans* No "feet" = send to Mycology
Streptococcus pneumoniae	Moderate	ID & AST
Haemophilus species	Moderate	ID, β-lactamase; AST if *H. influenzae*
Burkholderia gladioli	Any	ID and AST
Ralstonia pickettii	Any	ID and AST
Fungi – mould	Any	Send to Mycology (Mycology will update report)
Stenotrophomonas maltophilia	Any	ID and AST
Other non-lactose-fermenting, gram-negative bacilli	Moderate	ID and AST
Beta-hemolytic *Streptococcus* (large colony)	Any	ID
Staphylococcus aureus	Any	ID and AST
Mucoid *Pseudomonas aeruginosa*	Any	ID and AST

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non-Mucoid *Pseudomonas aeruginosa*	Any	ID and AST
Burkholderia cepacia	Any	ID and AST

[1]"Feet" = Pseudoprojections from colony on BAP or CHOC within 48 hours.

VI.   IDENTIFICATION AND SUSCEPTIBILITIES
A.   Refer to Manual 105, as needed for identification procedures.

B.   Refer to Manual 106, as needed for susceptibility testing.

VII.   REPORTING
A.   Report Panic Values as indicated in procedure 100.7.2.

B.   Preliminary Reports
    1.   <u>At 24 hours</u>, report:
        a.   **CI** = "Continuing Incubation".
        b.   Do not report any growth of normal flora or potential pathogens at this point.
    2.   <u>At 48 hours</u>, issue another report:
        a.   If no apparent growth of CF pathogens at this time, quantitate the normal respiratory flora and report:
            i.   **negp** = "_____ Mixed Respiratory Flora, Continuing Incubation."
            ii.   If CF pathogens are present, quantitate and report normal respiratory flora as above and report the pathogen(s).
    3.   <u>At 72 hours</u>: report any CF pathogens that appear and update the report as needed.

    4.   <u>At 96 hours</u>: report any CF pathogens that appear and update the report as needed.

    5.   For first-time isolates of *B. cepacia*, *B. gladioli*, or *Ralstonia pickettii* report:

        a.   **Send Buce** = "Presumptive _____ Sent to CFF *Burkholderia cepacia* Research Laboratory and Repository for confirmation."

        b.   DO NOT FINALIZE THE CULTURE until confirmation is returned.

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      c.   Do not report susceptibility results until identification is received from *Burkholderia cepacia* Research Laboratory & Repository.

    C.   Final Reports

        1.   If no apparent CF pathogens have appeared after 96 hours (4 days), finalize the culture and report: **negf** = "_____ Mixed Respiratory Flora."

        2.   For positive cultures, finalize the culture when all identification and susceptibilities are completed.

VIII. PROCEDURE NOTES

    A.   Cystic Fibrosis (CF) is the most common lethal genetic disorder seen in Caucasians. Progressive pulmonary disease, commonly associated with infections in conducting airways, is the principle cause of morbidity and mortality in CF. Another condition associated with CF is pancreatic insufficiency with malnutrition. Both syndromes are the result of the production of extremely viscous secretions. These secretions cause blockage of the pancreatic ducts resulting in an inability to secrete digestive enzymes and, more significantly, the development of chronic lung infections due to several types of organisms. The most significant organism associated with CF is a highly mucoid, nonpigmented strain of *Pseudomonas aeruginosa*. During the past decade, another nonfermenter, *Burkholderia cepacia*, has also been recognized as a significant pathogen in these patients. Two factors are extremely important when considering *Burkholderia cepacia*. First, approximately 20% of patients who become infected will have a rapid decline in their pulmonary function, and death will occur in 1 to 6 months. Second, there is accumulating evidence that some clones of *Burkholderia cepacia* can be transmitted person-to-person. These patients are often isolated from all other CF patients, making them social outcasts in the tightly-knit CF community.

    B.   *Burkholderia cepacia* complex is a group of organisms that are genetically and sometimes biochemically distinct from each other. Although nine genomovars are now recognized in the *B. cepacia* complex, over 85% of the isolates recovered from CF patients belong to either genomovar III (50%) or genomovar II (38%). Several genomovars have species names while others are still awaiting designation.

IX. REFERENCES

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SEND-OUT INSTRUCTIONS FOR *Burkholderia* spp.

I. BACKGROUND

The Burkholderia cepacia Research Laboratory and Repository was established in 1998 and is funded by the CF Foundation. The laboratory is located currently at the University of Michigan in Ann Arbor and is directed by Dr. John LiPuma. The laboratory makes the following services available free of charge to all CF care centers:

A. <u>Confirmation of species identification</u> – Putative *Burkholderia* spp. recovered from CF sputum undergo polyphasic analyses to confirm species identity. The laboratory can rule out *Burkholderia* spp. among nonenteric, nonfermenting, gram-negative bacteria for which species identification is uncertain.

B. <u>Genomovar designation</u> – Isolates confirmed as *B. cepacia* complex are analyzed to determine their species (genomovar) within the complex.

C. <u>Isolate genotyping</u> (DNA fingerprinting) – Upon request the laboratory will provide typing by RAPD and/or PFGE to assess strain identity among isolates submitted. This allows investigation of possible person-to-person transmission or nosocomial acquisition.

D. *Isolate respository* – All isolates are catalogued and stored. This provides an important resource for future epidemiologic studies.

II. PROTOCOL FOR SENDING ISOLATES

A. Send any isolate identified as *Burkholderia* spp. or *Ralstonia pickettii* for the first time from any CF patient.

B. Send only pure isolates from fresh overnight cultures on a TSA slant.

C. Complete a **PHYSICIAN AND PATIENT INFORMATION REFERRAL FORM** for each isolate sent.

    1. Forms can be found in a labeled notebook (CF Send-Outs) located on Respiratory Bench.

    2. See example form in front of notebook.

    3. Make a copy of each form and file in notebook behind the front tab – PENDING.

    4. Send the original form with the isolate.

D. Send copies of our ID and Susceptibility reports for each isolate.

E. Isolates must be packed/shipped by a person in the laboratory certified to ship hazardous goods. Multiple isolates can be shipped in the same box if necessary.

F. Send by UPS 2nd Day Air and bill to recipient's account **No. WO4Y00**. Send to:

Dr. John LiPuma	For more information:
1150 W. Medical Center Drive	**Tel:** 734-936-9767
8323 MSRB III, SPC 5646	**FAX:** 734-615-4770
Ann Arbor, MI 48109-5646	

H. When results are returned: enter the results in a Final report for the isolate as follows:

    1. **Buce Confirm** = "_____ Confirmed by CFF *Burkholderia cepacia* Research Laboratory and Repository."

    2. Attach report to referral form. File in COMPLETE section of CF Send-Outs notebook.

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Appendix B

Anaerobe Protocol

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I. PRINCIPLE

Wound colonization and/or infection is often polymicrobic, with both aerobes and anaerobes involved. A hallmark of local infection within an abscess or deep wound is an accumulation of inflammatory cells. Subsequent pus formation can be documented by the presence of PMNs in the gram-stained smear. Therefore, the quality of a wound or tissue specimen can be assessed by Gram stain, which should be used to guide the extent of microbiology testing. The presence of epithelial cells indicates contamination of the specimen with skin or mucous membrane microbiota and may compromise the significance of the culture results.

Obviously, this procedure cannot possibly outline every possible scenario for wound or tissue culture examination and interpretation. A Composite Q Score algorithm (pg 4) is used to help the technologist integrate all available data. A certain amount of microbiological intuition is required to interpret each culture. In an effort to offer the physician clinically relevant, accurate and cost-effective information, the technologist must integrate direct observations of bacterial growth (aerobic and anaerobic) with direct Gram stain, patient diagnosis, and specimen source. This procedure also details reporting guidelines for the most common potential pathogens (pg 4-5), as well as cultures with mixed organisms.

II. MATERIALS

Media	Incubation			
	35°C-CO_2	35°C-nonCO_2	ANA	LOI
CNA	✓			3 days
MAC		✓		
TM*	✓			
BA	✓			5 days
BR			✓	
CHOC	✓			
KV			✓	
PEA			✓	

*Add for genital and joint sources.

III. POTENTIAL PATHOGENS AND NORMAL FLORA
    A.   Potential Pathogens
          1.   *Actinomyces* spp. (must be incubated for 14 days in an anaerobic pouch in the anaerobic chamber)
          2.   Anaerobes
          3.   *Bartonella* spp.
          4.   Beta-hemolytic *streptococci***
          5.   Coagulase-negative Staphylococci (needs to be evaluated in each circumstance)
          6.   *Eikenella spp.*
          7.   *Enterococcus* spp.
          8.   Enteric gram-negative rods

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9. *Erysipelothrix* spp.
10. Fungus
11. *Haemophilus* spp.
12. *Listeria* spp.
13. *Mycobacterium* spp.
14. *Neisseria gonorrhoeae*
15. *Neisseria meningitidis*
16. *Nocardia* spp.
17. Non-fermenting gram-negative rods including *Pseudomonas aeruginosa*** and *Acinetobacter* spp.
18. *Pasteurella* spp.
19. *Staphylococcus aureus**
20. *Staphylococcus lugdunensis*
21. *Streptococcus milleri* group
22. *Streptococcus pneumoniae*
23. Yeast

NOTE: Always report organisms with an ***** regardless of Q score. Always report organisms with a ****** if predominating, regardless of Q score.

B. Potential Bioterrorism Pathogens
1. *Francisella* spp.
2. *Yersenia pestis*
3. *Bacillus anthracis*
4. *Brucella* spp.

C. Normal Flora
1. *Aerococcus* spp.
2. *Corynebacterium* spp.
3. *Lactobacillus* spp.
4. *Micrococcus* spp.
5. Non-pathogenic *Neisseria* spp.
6. *Peptostreptococcus* spp.
7. *Propionibacterium* spp.
4. Coagulase-negative *Staphylococcus*
5. Viridans streptococci

NOTE: Under some circumstances, depending on the Q score and the body site, these organisms may need to be worked up.

IV. SPECIAL PRECAUTIONS AND SAFETY
A. Refer to procedure 114.8 for a list of select agents that require special handling.

B. Day 2 culture examination
1. If there is faint or newly appearing growth, move plates to biological safety cabinet, then perform a Gram stain (GS).
2. If GS reveals tiny amorphous or small gram-negative coccobacilli, proceed with all further workup under biosafety hood in AFB laboratory

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until *Brucella* spp. and/or *Francisella* spp. are ruled out. Refer to SOPs in Manual 114 (Bioterrorism).

3. If GS reveals beaded or faintly staining rods, seal plate and give to the Mycology/AFB laboratory until *Mycobacterium* spp. or *Nocardia* spp. are ruled out.

C. If any organism resembling *Neisseria meningitidis* or *Haemophilus* spp. (grey, translucent colony morphology on CHOC agar) is isolated, or if gram-negative diplococci are predominating in the direct GS of the culture:
1. Take all plates to a BSL-2 hood.
2. Perform all work, including MALDI set-up, under the hood until *N. meningitidis* is ruled out.
3. Shrink-seal all plates.

V. CULTURE EXAMINATION AND INTEPRETATION
A. Culture Exam
1. <u>At 24 hours</u>:
    a. Examine aerobic plates for growth. Do not examine anaerobe plates at this time.
    b. If no growth, reincubate plates.
    c. If growth has occurred, see section V., Interpretation.
2. <u>At 48 hours to 5 days</u>:
    a. Re-examine aerobic plates, and simultaneously open and examine any anaerobic plates for that culture.
    b. Remove anaerobic plates from the anaerobic chamber immediately prior to examining.
       NOTE: Do not allow anaerobic plates to sit out on bench longer than 30 minutes.
    c. For no growth cultures:
       i. Reincubate the ANA, BA and CHOC plates for a total of 5 days.
       ii. Discard MAC, CNA and TM plates at 72 hrs.
       iii. No additional LIS reports need to be issued until the culture is final at 5 days.
       iv. Continue to examine remaining plates on Days 3, 4 and 5.

B. Interpretation and extent of work-up
1. <u>For cultures positive at 24 hours</u>:
    a. Note the direct GS result, the source, and the patient diagnosis (click on the patient demographic icon on the menu bar).
       NOTE: For positive lymph node cultures, perform all work in a biological safety cabinet.
    b. Determine the number of organisms to workup (identification and susceptibility) using the Q score algorithm (pg 4).
       NOTE: Remember that the Q score algorithm is a guideline and may not work for every culture. It is equally important to

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remember that our goal is to offer accurate and timely information for quality patient care.

    c.   Work up at 24 hr any culture growing *S. aureus*, β-hemolytic streptococci, or a predominating *P. aeruginosa* regardless of the Q score.

    d.   Subculture cultures that are mixed to fresh media and reincubate.

    e.   The total number of organisms worked up includes both aerobic and anaerobic organisms; therefore, it is necessary to observe all plates together at ≥ 48 hrs to accurately compare predominating organisms.

        NOTE: Be careful not to work up too many aerobic organisms at 24 hours.

2.   <u>For cultures positive at ≥ 48 hours</u>:

    a.   Recalculate the Q score (pg 4).

        NOTE: If at 24 hrs, you have begun working up aerobic organisms, and at 48 hrs you have a number of anaerobes, it is acceptable at this time, after bringing up on Lab Rounds, to report a mixed statement.

    b.   If the number of potential pathogens exceeds the Q score, determine what is to be worked up by the organisms observed in the direct GS.

    c.   If the organisms grown in culture do not correlate with those seen in GS, review the direct GS. Refer to procedure 104.2.5.

    d.   For suspected anaerobes:

        i.   Perform a GS and save the slide for later reference.

        ii.   Subculture immediately to BA, BR and CHOC. Incubate as follows:

            (a)   BR – anaerobic incubation.

            (b)   BA – 35-37°C non-CO_2.

            (c)   CHOC – 35-37°C CO_2.

    e.   After 24-48 hrs of incubation, observe aerotolerance testing results using the following table. If organism is anaerobic, send BR plate to MALDI for identificaton. Use GS to assist in interpretation.

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Aerotolerance Results:

	Growth on BA in non-CO_2	Growth on CHOC in CO_2	Growth on BRU in ANA
Aerobe	+	+	–
Capnophilic Aerobe	–	+	–
Facultative Anaerobe	+	+	+
Obligate Anaerobe	–	–	+

3.   To determine the number of organisms to workup in positive cultures, refer to the Q score algorithm below:

     a.   Compare the number of WBCs to the number of squamous epithelial cells seen in the direct GS. The point at which the numbers intersect on the table is the composite quality or Q score.

     b.   Any specimen with no squamous epithelial cells is scored as 3, regardless of the number of WBCs. This scoring takes into account the neutropenic patient who may be unable to mount an immune response.

		Squamous Epithelial Cells			
WBCs	**Report**	0	1-9	10-24	≥ 25
		None	Few	Mod	Many
0	None	**3**	0	0	0
1-9	Few	**3**	0	0	0
10-24	Mod	**3**	1	0	0
≥ 25	Many	**3**	**2**	**1**	**0**
		Composite Q Score			

     c.   If the composite Q score equals:

        0, work up 0 organisms and use mixed statements.

        1, work up 1 organism according to GS.

        2, work up to 2 organisms according to GS.

        3, work up to 3 organisms according to GS.

NOTE: This guideline may not work for every culture. Consult rounds as needed.

4.   To determine the extent of workup necessary on each organism, refer to Table of Organism workup below.

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Organism	Quantity	Workup and Report
Actinomyces spp.	Predominating/Pure	ID
Anaerobes	Predominating/Pure	ID and AST
Arcanobacterium spp.	Predominating/Pure	ID
Bacillus anthracis[1,2]	Any	Rule out/refer
Bartonella spp.	Any	ID
β-hemolytic streptococci (large colony)[3]	Any	ID
β-hemolytic streptococci (small colony)	Predominating/Pure	ID and AST
Brucella spp.[1,2]	Any	Rule out/refer
Corynebacterium spp.	Pure	Consult rounds
Enterobacteriaceae	Predominating/Pure	ID and AST
Enterococcus spp.	Pure ----------------- All others	ID and AST ----------------- Consult rounds
Eikenella spp.	Predominating/Pure	ID
Erysipelothrix spp.	Predominating/Pure	ID
Francisella spp.[1,2]	Any	Rule out/refer
Fungus	Any	Consult rounds
Haemophilus spp.	Predominating/Pure	ID and B-lact
Listeria spp.	Any	ID and AST
Mycobacterium spp.[1]	Any	ID
Neisseria gonorrhoeae	Any	ID
Neisseria meningitidis[1]	Predominating/Pure	ID
NF GNRs	Predominating/Pure	ID and AST

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Nocardia spp.	Any	ID
Pasteurella spp.	Predominating/Pure	ID
Pseudomonas aeruginosa [3]	Predominating/Pure	ID and AST
Staphylococcus aureus [3, 4]	Any	ID and AST
Staphylococcus aureus small colony variant	Any	If only variant, perform ID and AST. If parent and variant present, report only parent ID and AST.
Staphylococcus coagulase-negative [4]	Predominating/Pure	Consult rounds
Staphylocuccus lugdunesis	Predominating/Pure	ID and AST (use *S. aureus* interpretation for oxacillin and cefoxitin).
Streptococcus milleri group	Predominating/Pure	ID and AST
Streptococcus pneumoniae	Predominating/Pure	ID and AST
Yersenia pestis [1, 2]	Pure	Rule out/refer
Viridans streptococci	Pure	ID and AST
Yeasts	Predominating/Pure	ID

[1] Always work in biosafety hood.

[2] If GS is a poorly staining GNR with pinpoint colonies at 2-4 days, perform all work in BSC in AFB laboratory.

Refer to manual 114 for workup of potential bioterrorism organisms.

[3] Always work up regardless of Q score.

[4] If pure, refer to procedure 105.3.1, Flow Chart II.

VI. IDENTIFICATION AND SUSCEPTIBILITES
   A. Refer to manual 105, as needed, for identification procedures.
B. Refer to manual 106, as needed, for susceptibility testing guidelines and procedures.

VII. REPORTING
   B. Report Panic Values as indicated in procedure 100.7.2.
   C. Preliminary reporting
      1. If negative, report **NEGP** = "No growth to date, continuing incubation."
      2. If positive, report organism identification (descriptive or definitive) with quantitation.
      3. If hazy or young growth, report **SCGRCURE** = "Scant growth, culture reincubated."

D.   Final reporting
1.   If wound culture is negative, report **NEGF** = "No aerobes or anaerobes isolated."
2.   If tissue culture is negative, report **NEGF** = "No growth after 5 days."
3.   If positive, report:
a.   Organism identification with quantitation.
b.   Site-appropriate mixed statements with quantitation, if applicable:
i.   **MAB** = "Mixed aerobic bacteria".
ii.   **MNB** = "Mixed anaerobic bacteria".
iii. **MANB** = "Mixed aerobic/anaerobic bacteria".
iv.   **MRB** = "Mixed respiratory bacteria".
v.   **MIB** = "Mixed intestinal bacteria".
vi.   **MXB** = "Mixed bacteria, including...".
vii. **MXFF** = "Mixed fecal flora".
viii. **MSF** = "Mixed skin flora.
ix.   **MUF** = "Mixed urogenital flora".
x.   **MVF** = "Mixed vaginal flora".
NOTE: Freetext in organisms on workcard to be included in mixed statements.
c.   If no anaerobes are seen on culture but aerobes are reported, add **NOANA** = "No anaerobes isolated," to the final report.
d.   If no aerobes are seen on culture but anaerobes are reported, add **NOAER** = "No aerobes isolated," to the final report.

VIII. PROCEDURE NOTES
A.   Dialogue between the clinician or nurse and the microbiology laboratory should be encouraged for proper interpretation of results.

B.   The results of wound cultures will only be as valuable as the quality of the specimen submitted, its transport, and expedient processing.

C.   Reporting selected organisms in mixed cultures can lead to erroneous interpretation of the number and variety of infecting pathogens.

E.   Performance of AST is not indicated in cases of mixed microbiota indicative of infection of the abdominal cavity with bowel contents. Treatment should include broad-spectrum coverage for normal intestinal microbiota.

E.   Use of the Gram stain can improve the accuracy of evaluating the importance of each potential pathogen. Organisms present in the Gram stain of an appropriately collected specimen correlate with $\geq 10^5$ organisms per g of tissue.

F.   Clinical studies have demonstrated that the microbial load in an acute wound can predict delayed healing or infection. The more numerous the organisms, the more likely they are to be indicative of infection.

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G.   Many wound infections are polymicrobic, and the isolation of an organism in culture may or may not correlate with infection of the wound.

H.   The microbiologist plays a critical role in the treatment of wound infections because practitioners often consider the report from the laboratory as definitive proof of infection. Providing inappropriate identifications and susceptibility results can prompt unnecessary treatment.

I.   The presence of PMNs is an indication of an inflammatory or infectious process, while the presence of epithelial cells indicates surface contamination of the specimen. Specimens containing numerous epithelial cells yield culture results of questionable accuracy in the diagnosis of the infectious process.

J.   If a patient is immunocompromised or has poor vascular supply, inflammatory cells may not be present in the specimen as a guide to the extent of workup of the culture.

K.   Low levels of organisms or fastidious organisms that grow poorly on the direct plates may be missed in culture.

IX.   REFERENCES

A. Isenberg, Henry D. 2010. Clinical Microbiology Procedures Handbook. Vol. 1, pp. 3.13.1.1-18. ASM Press, Washington, D.C.

B. Sharp, Susan. 1999. Algorithms for Wound Specimens. Clin. Microbiol. Newsletter 21:14, 118-120.

Appendix C

MALDI-TOF Protocol

I.   PRINCIPLE
The Bruker MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) Biotyper allows fast and reliable identification of microorganisms. Bacterial colonies are directly spotted onto a MALDI steel target. An overlay of Formic Acid (for extraction) followed by HCCA (matrix) is applied on top of the bacteria for protection from the laser beam. The steel plate is inserted in the instrument where microorganisms are identified by their molecular composition of proteins and peptides and are arranged in a spectrum with increasing mass. This resulting characteristic peak pattern allows for reliable identification of microorganisms to the species level.

II.   SPECIMENS
A.   *Bacteria - Pure organism from a 24-hr to 7-day-old culture plate submitted on CHOC, BAP or Brucella (Remel or PRAS).*
   NOTE: No potential Bioterrorism (BT) organisms should be tested on the MALDI-TOF. If *Neisseira meningitidis* is suspected, spot plate and overlay with Matrix under a Biosafety Hood. Perform testing on the MALDI-TOF <u>only</u> after Matrix is thoroughly dry.

B.   Yeast – Pure organism from a 24-hr to 4-day-old culture plate submitted on Sabouraud, Chocolate or Blood agar.

III.   REAGENTS AND MATERIALS
A.   Bruker MALDI Biotyper Steel Target Plate.
B.   Bruker Bacterial Test Standard (BTS).
C.   Bruker HCCA (matrix).
D.   Toothpicks.
E.   Eppendorf Brand pipettes/tips (1-10 μL; 10-100 μL; 100-1000 μL).
   NOTE: Use <u>only</u> Eppendorf brand pipettes/tips with the Bruker Maldi-TOF.
      Other plastics may loosen and cause issues inside the mass spectrometer.

F.   MALDI Biotyper Target Worksheet (pg 7).
G.   Biological Safety Cabinet (BSC).
H.   100% Ethanol, HPLC/MS Grade.
I.   100% Formic Acid, HPLC/MS Grade.
J.   100% Acetonitrile, HPLC/MS Grade.
   NOTE: Acetonitrile is highly flammable.

K.   100% Trifluoroacetic acid, HPLC/MS Grade.
   NOTE: Trifluoroacetic acid is very corrosive. Double nitrile gloves and eye
      protection must be worn when handling.

L.   Organic Solvent Standard Solution.
M.   Ultra-Pure water, HPLC/MS Grade.

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M.   Bruker MALDI Biotyper Steel Target Plate.
N.   Bruker Bacterial Test Standard (BTS).
O.   Bruker HCCA (matrix).
P.   Toothpicks.
Q.   Eppendorf Brand pipettes/tips (1-10 µL; 10-100 µL; 100-1000 µL).
        NOTE: Use only Eppendorf brand pipettes/tips with the Bruker Maldi-TOF.
             Other plastics may loosen and cause issues inside the mass spectrometer.

R.   MALDI Biotyper Target Worksheet (pg 7).
S.   Biological Safety Cabinet (BSC).
T.   100% Ethanol, HPLC/MS Grade.
U.   100% Formic Acid, HPLC/MS Grade.
V.   100% Acetonitrile, HPLC/MS Grade.
        NOTE: Acetonitrile is highly flammable.

W.   100% Trifluoroacetic acid, HPLC/MS Grade.
        NOTE: Trifluoroacetic acid is very corrosive. Double nitrile gloves and eye
             protection must be worn when handling.

X.   Organic Solvent Standard Solution.
M.   Ultra-Pure water, HPLC/MS Grade.

Y.   Eppendorf 1.5 µL microfuge tubes.
Z.   Two plastic squeeze bottles (for 70% ethanol and water).
AA.   Vortex.
BB.   Microfuge tube rack.
CC.   Sharpie pen.
DD.   Microcentrifuge.
EE.   Safety glasses or face shield.
FF.   Kim Wipes

IV.   CALIBRATON AND QUALITY CONTROL
A.   Calibration is performed each day and with each change in the target plate. BTS
        is used as the calibrator and calibration needs to pass for runs to be acceptable.
        Refer to section VI.D. for calibration instructions.

B.   QC organisms
        NOTE: Refer to procedure 110.3.4 for maintenance of MALDI QC organisms.
             QC organisms will be kept at room temperature on the MALDI bench.

      1.   *E. coli*, ATCC 25922.
      2.   *S. aureus*, ATCC 29213.
      3.   *C. albicans*, ATCC 10213.
      4.   Blank control (Formic Acid and Matrix).

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> **NOTE:** If a tube extraction is performed, select a QC organism whose Gram reaction resembles the organism being extracted. Run a tube extraction on that QC organism.

C.   QC testing – Perform each day and with each change in target plate.

D.   QC results
  1.   Expected results: QC organism should have the correct identification with a score > 1.7 for bacteria and 1.5 for yeasts.
  2.   Record results on QC Form (pg 8).
  3.   Unacceptable results:
      a.   Notify supervisor/senior technologist.
      b.   Complete QC deviation.
      c.

V.   SAMPLE PREPARATION

A.   Label MALDI Target Worksheet as follows

  1.   Project – Date.initials.serial number of target plate. Run #1, e.g., 7.16.13.KN.0056391.1.
  2.   Select unused spots on plate; on worksheet label 1 spot with BTS.
  3.   Label the next 4 spots with QC organisms (*E. coli*, *S. aureus*, *C. albicans*, blank, tube extraction (if needed).
  4.   Label the next spot with 1 accession number, excluding dashes, including zeros:

      a.   Repeat until all accession numbers are on the worksheet.

      b.   Each isolate will have 1 single spot.

  5.   Do not remove BTS from freezer until ready to pipette.

B.   All samples and QC will use the Direct Colony with Formic Acid overlay first. Then perform tube extraction, if needed.
  1.   <u>Direct Colony with Formic Acid:</u>
      a.   Smear biological material (single colony) onto a single spot on the steel target plate with a toothpick. Allow to air dry.
      b.   Overlay with 1 µL of 70% formic acid and let air dry at room temperature.
      NOTE: For blank control, apply formic acid on spot.

      c.   Overlay with 1 µL of matrix solution. Air dry completely.
      d.   Analyze on Biotyper (see section VI.)

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2.   Formic Acid Tube Extraction:

NOTE: Perform tube extraction on the QC organisms that resembles organism being extracted. Record on QC Sheet (pg 8).

a.   Add 300 µL of MS Grade water to each Eppendorf microfuge tube.

b.   Transfer a large, single colony of microorganism to the tube (more than one colony may be needed if microorganism is small; choose isolated colonies). Vortex thoroughly.

c.   Add 900 µL of 100% ethanol. Vortex thoroughly.

d.   Centrifuge at maximum speed for 2 minutes.

e.   Pour off excess ethanol. Decant remaining ethanol using a pipette; centrifuge for 2 minutes.

f.   Remove all excess ethanol with fine-tipped pipette (completely remove all ethanol. Let sit for 10 minutes to complete evaporation of any remaining ethanol.

g.   Add 50 µL of 70% formic acid (if only a small amount of microorganism is available, decrease the formic acid volume to 10 µL). Vortex thoroughly.

h.   Add 50 µL of 100% acetonitrile (if only a small amount of microorganism is available, decrease the formic acid volume to 10 µL). Vortex thoroughly.

NOTE: The volume of 70% formic acid and acetonitrile must be equal volumes.

i.   Centrifuge at maximum speed for two minutes.

j.   Pipette 1 µL of supernatant onto steel target plate; air dry.

k.   Overlay with 1 µL of matrix; air dry (ensure target is completely dry before it is inserted into MBT).

l.   Analyze on the Biotyper.

NOTE: For any organism that is extracted, the accession # should be followed by "tfa" (tube formic acid), e.g., 1319801234tfa.

D.   Applying BTS and Matrix

1.   Remove a tube of reconstituted BTS from freezer and give it a quick spin in the microcentrifuge (2-5 seconds).

2.   Pipette 1 µL of BTS onto one spot on the target.

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> **NOTE:** Apply BTS to target plate **last** because it is important not to let BTS be exposed directly to air (protein oxidization) for a long period of time.

    3.   Allow BTS spots to completely air dry before adding HCCA matrix.
    4.   Remove HCCA matrix from RT storage drawer.
    5.   Vortex the reconstituted HCCA matrix for 10 seconds.
    6.   Apply 1 µL of HCCA (matrix) to each spot on target plate and allow to air dry.
    7.   When BTS is dry, pipette 1 µL of HCCA matrix onto BTS spot.

E.   Open the MALDI-BIOTYPER RTC APPLICATION

> **NOTE:** If Windows is logged off, log on using the following:
> User name: **Administrator**
> Password: **bruker12**

    1.   Click on New Classification (icon looks like a piece of paper).
    2.   Name the project: **Date.Initials.target#.Bench.Run#**.
    3.   Click on NEW → NORMAL PROJECT → OK.
    4.   Click on NEXT and maximize the screen.
    5.   Highlight the spots to be tested. Left click and drag to cover the spots → right click → Add Analytes. Repeat to add partial rows.
    6.   Under ID, type in or scan the accession numbers (e.g., 1303304437-1). Press ENTER.
    7.   Once the run is built, minimize RTC. Accession numbers can be entered while matrix is drying.

VI.   MALDI-TOF BIOTYPER AND COMPUTER SOFTWARE

A.   <u>Instrument</u> - The Biotyper instrument should always be ON: the system should have a yellow light beside warm-up and a green light beside access.

> **NOTE:** When the Biotyper is not in use, a target plate must be in the chamber to keep the vacuum stable.

Can load/eject using green

B.   <u>Software</u>

    1.   Open the flex control 3.3 application.

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2. Click on the "Load/Eject" button to move the target from the vacuum to the access door.

   a. When the target is moving, there will be a yellow light beside "in process" on the instrument.

   b. When the target plate is at the access door, a green light will be beside "Access".

3. Dust entry door surface with a Swiffer or Kimwipe.

4. Open the door and carefully remove the old target plate and place in the carrying case:

   a. Carefully put the target plate to be tested in the slot.

   b. Dust O-ring with a Kimwipe.

   c. Make sure the target is laying flat. If it is not flat, the target could jam the chamber. If jammed, call the manufacturer.

5. Click on the "Load/Eject" button. A yellow light beside "in process" appears while the target is moving.

6. When the target is ready, a green light appears beside "Access."

C. Check the vacuum

   1. Click on Status → Details → Vacuum:
      a. Make sure the vacuum status says READY and that there are green lights beside "Lock Rough", "Source Rough" and "Source High."
      b. If there is a red light, wait a few minutes and when the vacuum is stabilized, the lights will turn green.
         NOTE: If the vacuum does not stabilize, check to see if there is dust near the access door. If there is dust, wipe the surface O-ring with a Kimwipe.

   2. Close the status window (not the software).

D. Calibration – perform whenever a target plate is placed in the Biotyper

   1. Click on the spots where the BTS was applied.
   2. Click on Calibrate.

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3. When the calibration is complete, a box pops up on the screen stating that calibration is successful.
4. Save the calibration once a day. Press CTRL + Print Scrn.
   a. Open the Paint application: Click START → PROGRAMS → ACCESSORIES → PAINT.
   b. Click on EDIT → PASTE.
   c. To SAVE, click File → SAVE AS → DESKTOP → Calibration folder → use today's date as the name → SAVE.
   d. Close the paint application.
5. After the calibration is saved, click OK.
6. If calibration fails:

   a. Thaw a new BTS aliquot and repeat.

   b. If repeat calibration is out of range:

      (1) Reconstitute new BTS.

      (2) Prepare new organic solvent and the fresh BTS.

   c. If repeat calibration with new BTS fails, inform a senior technologist/supervisor.

7. Minimize flex control.

E. Start run
   1. Maximize RTC software.
   2. Click FINISH.
   3. Run will process.

F. When the run is complete, print the results
   1. Click on the "View Results" icon (looks like a calculator).
   2. Right click on the screen.
      3. Select PRINT from the pop-up box.
      4. Type in the number of pages to print.
         NOTE: Only print the pages that have the identification and score.
      5. Click on PRINT.
      6. If yeast identifications were performed and the scores for the yeast spots were between 1.5 and 1.7, then manually record the yeast ID on the Maldi printout:
         a. Scroll through the Maldi report looking for any yeast identifications that appear in red and whose first score is between 1.5 and 1.7.
         b. Record the identification on the Maldi sheet in the corresponding spot location.
   G. When finished, close the flex control and MALDI RTC applications.

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X. REFERENCES

A. Bruker Diagnostics, Daltonics Dispatch "International Life Science Edition", Vol. 2, Issue 1–2009. Bruker Daltonics, Freemont, CA.

B. Bruker Quick Reference Guide. Bruker Daltonics, Freemont, CA.

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Vita

Emily Marie Hill was born January 6, 1980, in Fredericksburg, Virginia, the daughter of Larry Edward Crabtree and Donna Kimmitz Crabtree. After graduating from Stafford Senior High School in 1998, she entered Virginia Commonwealth University (VCU), receiving the degree Bachelor of Science in Clinical Laboratory Sciences (CLS) in 2003. After working in the clinical microbiology laboratory at VCU Medical Center (VCUMC) for five years, she entered the Advanced Track of the graduate VCU CLS program and earned a Master of Science degree in 2007. Emily currently serves as Assistant Chairman and Assistant Professor of clinical microbiology in the Department of Clinical Laboratory Sciences at VCU.