Identification and Drug Resistance of Fungal Pathogens in Cystic Fibrosis Patients

Emmaleigh G. Maritz

Bachelor of Science in Microbiology/Cell and Molecular Biology

HONORS THESIS

Advisor: Dr. Karen Wozniak

May 2020

ABSTRACT

Cystic fibrosis (CF) is an autosomal genetic disease that causes infection in the lungs due to a lack of mucus clearance in the airways. Published studies on fungal pathogens in CF patients noted that the use of multiple antibiotics to treat drug-resistant bacterial infections in these patients has created an opening for colonization of the lung with fungal pathogens, and these organisms may also be drug-resistant. **I hypothesized that fungal pathogens can be isolated from CF patient samples, and these fungal organisms will be resistant to antifungal therapies.** Sputum samples were taken from CF patients from the Cystic Fibrosis Clinic in Oklahoma City, OK. Samples were plated using selective media to enrich for fungal growth. Following this, single colonies were isolated and cultured for inoculation of biochemical tests (API AUX20 C test strips) or for isolation of DNA. DNA samples were subjected to PCR for the ITS3 gene followed by gel electrophoresis to identify fungal ITS3 bands. Isolates with positive ITS3 bands were sequenced to identify the fungal species. In addition, MIC assays were conducted with antifungal drugs. Analysis of colonies tested by API test strips showed that several *Candida* species were present, and we further confirmed the species by sequencing the ITS3 gene. MIC assays were conducted with antifungal drugs fluconazole, caspofungin, and novel compound EIPE-1. MIC assays showed that some of these isolates were resistant to typical antifungal drugs and all were sensitive to EIPE-1. Further understanding the fungal pathogens and drug resistance in CF patients as well as identifying alternatives to current antifungal drugs could potentially prolong CF patient lifespan and decrease the severity of CF lung infections.

INTRODUCTION

Cystic Fibrosis is a common, autosomal recessive genetic disease that results in infection in the lungs due to a lack of mucus clearance in the airways [1]. A key feature of CF is the imbalance of salt and water within the cell that results from a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR) [2]. When this gene is disrupted, chloride becomes trapped inside the cells causing cell dehydration and mucus build up. Complications arise when the CFTR gene is mutated meaning the overall function of the body's cells, tissues, organs, and sweat glands are negatively affected [3].

In healthy individuals, the CFTR *gene* is responsible for making the CF transmembrane conductance regulator *protein [3]*. Production of the CFTR protein begins in the cell's nucleus, where the CFTR gene gives instructions responsible for making the protein [2]. Transcription then occurs, and RNA matches these instructions in the DNA. Ribosomes take these instructions from RNA and translate them to make the CFTR protein. After translation, trafficking allows the CFTR protein to move from the cell to the cell's surface where it acts as a channel in the lungs. From there, chloride ions are transported across the cell membrane from inside the cell to the outside. When the chloride ions reach the outside of the cell, they attract water which then allows for cilia to move mucus through the airways. However, if the CFTR gene is mutated then complications with the CFTR protein will arise, and chloride ions will become trapped inside the cell causing thick, viscous mucus to build up and flatten the cilia [2]. Since water is no longer being attracted to the chloride ions and the cilia are flattened, the mucus cannot be swept out of the airways ultimately leading to pathogen colonization and respiratory related complications [4]. Mucus is necessary for lining multiple organs including those of the respiratory tracts, pancreas, intestines,

and sweat glands; however, because individuals with CF have thicker mucus and are more prone to blockage, then organ issues or failure is inevitable.

Approximately 2000 mutations have been discovered that are associated with CF [5]. Deletions or alterations in the protein's amino acid sequence are the most common mutations of the CFTR protein and effect the chloride channel's production, structure, and stability. Other mutations tend to have different effects on the protein; meaning, on a molecular level the variation of CFTR mutations has the ability to impact patient symptoms along with the organs that are affected which can alter patient treatments [5]. In terms of who is at risk for CF, the disease is most common within Caucasian and northern European populations occurring for every 1 in 3,200 births [6]. For African Americans, CF occurs at around 1 in 15,000 births, and is relatively uncommon among Asian, Native American, and Hispanic populations.

CFTR FUNCTION

The CFTR protein channel operation is powered by ATP regulation and phosphorylation of the R domain via cAMP-dependent protein kinase [7]. CFTR has been classified as an ATP-binding cassette protein making it a member of the ABC protein subfamily. The CFTR protein's ATP-gated ion channel is unique in that it transports chloride ions via *passive diffusion* whereas other proteins of this family would normally use free energy of ATP hydrolysis to export anions [8]. Essentially, CFTR is not like other classical ligand-gated ion channels; instead, channel opening and closing is dependent on the uptake of ATP during its gating cycle. The channels open when ATP binds to the ATP binding site on the nucleotide binding domains and they close when ATP is consumed. Since CFTR is found primarily in mucosal and secretory epithelia, the overall

transportation of negatively charged chloride ions through this channel is necessary for water movement in tissues and normal mucus production [8].

DIAGNOSIS

The genetic nature of cystic fibrosis implies that patients may be diagnosed through medical history as well as genetic and molecular testing [6]. Since CF is genetic, gathering medical history along with the patient's family history is helpful for physicians before conducting genetic testing in the laboratory. The goal of genetic testing for CF is to detect any mutations within the CFTR genes, the most common mutation being ΔF508 [9]. However, there are over 1,500 CFTR mutations and genetic testing screens for only a limited portion of these mutations. Prenatal testing involves the same concept through procedures called amniocentesis and chorionic villus sampling (CVS) where amniotic fluid or tissue from the placenta is taken from the mother and the cells are tested for CFTR mutations [6]. If the unborn child tests positive for a CFTR mutation, then further genetic testing will occur after birth. Meanwhile, the most common and standard testing procedure for CF is a sweat test because of abnormally high levels of sodium and chloride in the sweat caused by the mutations in the CFTR. Sweat tests involve increasing sweat production through chemical and electrical stimulation to the arm or leg where sweat is collected and sent off for analysis of chloride concentrations [9]. Chloride levels of greater than or equal to 60 millimoles per Liter are indicative of CF. Levels less than 30 millimoles per Liter suggest that having CF is either unlikely, or the patient is atypical of CF symptoms meaning they may not express all classical CF phenotypes [9].

SYMPTOMS AND PHENOTYPES

Symptoms of cystic fibrosis vary among individuals depending on the severity of the disease, CFTR mutation, and organ sensitivity [5]. The most common organs affected are those within the pulmonary and respiratory tract, primarily the lungs, but organs such as the pancreas, liver, intestines, and reproductive organs are also affected. Mucus buildup in the lung's airways allows bacteria and fungi to colonize and cause ongoing infections such as allergic bronchopulmonary aspergillosis, an allergic reaction that occurs in the lungs from the fungus *Aspergillus* [10]. Bronchiectasis is also very common in CF and results from airway obstruction and chronic inflammation of the lungs. The gastrointestinal system is the next most affected organ system in CF patients, primarily the pancreas. Inside the pancreas, thick mucus is created by mutated exocrine glands and inhibits digestive enzymes from traveling to the small intestine via pancreatic ducts. This inhibition causes digestion and nutrient absorption complications causing diabetes, malnutrition, pancreatitis, abdominal pain, etc. [5, 6] . Other symptoms involve intestinal blockage at birth, abdominal pain, jaundice, low body mass index, muscle and joint pain, delayed growth, infertility, salty skin, salty sweat, sinus infections, heart failure, and liver disease. In addition to genetic mutation, CF symptoms are also subject to several environmental cursors including air pollution, climate, humidity, temperature, dust, second-hand smoke, socioeconomic status, and geographical location [11]. Studies have shown that the rise in air pollution has increased the risk of pulmonary exacerbations in CF patients, and exposure to secondhand smoke can negatively impact mucus clearance and respiratory therapy [12]. Current and future research on the environmental impact on CF patients is beneficial and necessary in understanding CF pathophysiology as well as how quality of life can be improved.

PATHOGENESIS

Airway obstruction and chronic inflammation of the lungs from CF allows bacterial pathogens to occupy lung pathways [10]. Since CF patients are already immune deficient and lack cilia to clear mucus, this makes bacterial infections difficult to treat especially if not treated early on. While the primary pathogens of CF are *Staphylococcus aureus* and *Pseudomonas aeruginosa,* different stages of CF involve different pathogens [10]. Namely, *Hemophilus influenza* and *S. aureus* tend to cause infection in the first decade of life. During the second and third decades, *P. aeruginosa* becomes the main pathogen of the lung where it has the ability to form biofilms, ultimately prolonging infection in the lungs and resisting antibiotic treatment. Previous studies have shown that the lungs of CF patients can also harbor fungal pathogens where treatment of fungal infections can lead to drug-resistance [13]. It is also becoming increasingly recognized that fungal biofilms can persist in the lung and contribute to pathology and are highly resistant to antifungal therapy [10]. Further understanding the composition and drug resistance of bacterial and fungal pathogens in CF patients could potentially prolong CF patient lifespan and decrease the severity of their lung infections.

TREATMENT

When treating CF, addressing the condition of the lungs is usually the first step before infection can increase or worsen since lung disease usually occurs before other symptoms [6]. Clearing the airways of mucus build up, fighting bacterial or fungal infections through antimicrobial drugs, or even surgery are all techniques used to treat CF patients. In order to clear lung airways, airway clearance techniques (ACTs) is a common and chronic treatment among CF patients that loosens mucus through coughing in order to reduce blockage, decrease infections, and

improve overall respiratory function. Medications are also prescribed to CF patients to be used in combination with ACTs including antibiotics, anti-inflammatory drugs, bronchodilators, mucus thinners, and CFTR modulators [6]. Surgery may be necessary in worse cases leading to lung or liver transplants.

In the last 70 years, treatment and diagnosis of cystic fibrosis has evolved drastically due to highly progressive CF research [14]. During the 1940s, CF antibiotic therapy began with penicillin to help with chronic coughing and transitioned into using intravenous drugs such as gentamicin and tobramycin in the 1980s. The early 2000s focused on using azithromycin, a pill taken orally about 3 times a week to help combat chronic bacterial infections and improve lung function [6, 14]. Advancements in drug development have also led to high resolution imaging that has allowed researchers to recognize where and how drugs bind to the CFTR protein giving insight on potential therapies. However, imaging is still limited, and the protein is still unable to be shown in an open position that would further display how chloride ions moves through the ion channel [2]. Proper diet and nutrition is also crucial for nutrient absorption and weight gain for those with pancreatic and gastrointestinal complications. CF diets are normally high in calories, fat, protein, and salt to help immunity and muscle growth; nutritional supplements like pancreatic enzymes and vitamins are also essential for digestion and absorption [6].

FUNGAL-BACTERIAL INTERACTIONS

Fungi are eukaryotic organisms that exist in unicellular or multicellular form in a variety of environments [15]. Unlike bacteria, fungi differ in structure in that fungal cell walls are comprised of cross-linked glucans and contain ergosterol in the membrane, whereas peptidoglycan is the main component in bacterial cell walls [16]. These structural differences are clinically

relevant since antibacterial and antifungal agents are designed to target these different cell wall components. As previously mentioned, respiratory secretions from cystic fibrosis patients are home to certain filamentous fungi and yeasts [17]. Of these, *Candida albicans* is one of the most predominant fungal pathogens isolated from CF sputum [17].

Over 200 *Candida* species have been identified, however, only a few are considered opportunistic pathogens including *Candida albicans* [18]. *Candida albicans* is a normal resident of the human microbiota found on the skin and within the gastrointestinal tract and genital tract [19, 20]. In hosts, *C. albicans* can exist as a yeast or in a filamentous form known as hyphae [21]. Though *Candida albicans* is a normal commensal in humans, colonization is greatly increased in immunocompromised individuals which can then involve other bacterial or fungal opportunists [22]. Since the airways of Cystic Fibrosis are lined with an abnormal layer of viscous mucus, this enables the formation of biofilms, polymicrobial infections, and inter-kingdom interactions [10]. One of the most common yet controversial inter-kingdom relationships of the CF lung involves the interaction between *Pseudomonas aeruginosa* and *Candia albicans*. The controversy of this interactions stems from the fact that it can be either synergistic or antagonistic. It has been shown that *P. aeruginosa* can induce biofilm formation on filamentous hyphae of *C. albicans* causing death [19]. The exception to this is that *P. aeruginosa* does not bind to C. *albicans* in its yeast form likely due to differences in yeast and hyphae cell walls thus causing selective attachment. This fungicidal activity allows *P. aeruginosa* to occupy more surface area within the CF lung *and* obtain nutrients from the fungal filaments, ultimately representing the antagonistic nature of this inter-kingdom interaction [20]. Other *in vivo* murine studies of with *P. aeruginosa* and *C. albicans* have suggested that prior colonization of *Candida* may reduce overall *P. aeruginosa* infection and CF lung injury [23]. Alternatively, *C. albicans* produces the quorum sensing molecule farnesol which

allows the pathogen to inhibit germ tube formation and remain in its yeast form, a defense mechanism against *P. aeruginosa* [24]. Farnesol also prevents *P. aeruginosa* from producing pyocyanin, a virulence factor toxic to eukaryotic cells, which inhibits the morphology transition of *C. albicans* from yeast to hyphae and preventing fungal biofilm formation in the lungs [24].

While *Candida albicans* has undoubtedly been recognized as a predominant fungal pathogen in the CF lung, it has also been questioned whether *C. albicans* colonization is a true reflection of CF fungal pathogenesis or is simply a contamination [10, 17]. Essentially, this clinical uncertainty suggests that sputum samples may be contaminated via the environment, laboratory processing, or through colonization of existing commensal yeasts. In retrospect, sputum samples that test positive for *C. albicans* or any fungal agent may lead to unnecessary antifungal therapy thus resulting in drug resistance [25].

ANTIFUNGAL AGENTS & RESISTANCE

Fungal infections in Cystic Fibrosis lungs are often difficult to treat due to the close evolutionary relationship between eukaryotic cells and human cells [26]. More obviously, because antifungal drugs are toxic to fungal organisms, they are also toxic to human hosts. There has also been a rapid increase in fungal infections and development of antifungal drug resistance limiting effective treatment strategies [26].

Based on their mechanism of action, antifungal agents are grouped into three classes: azoles, polyenes, and echinocandins. Azoles are involved in the biosynthesis of ergosterol, a component necessary for maintaining the integrity of the fungal cell wall [27]. Polyenes bind to ergosterol and form pores in the membrane releasing molecules necessary for ion balance and membrane integrity [26]. Finally, echinocandins are the most recent of the antifungal drug classes and target the

enzyme beta-(1,3)-D-glucan synthase which is needed to synthesize cell wall component beta-(1,3)-D-glucan [15]. For this particular study, antifungal agents fluconazole (azole), caspofungin (echinocandin), and novel antimicrobial EIPE-1 were used to treat and evaluate the drug susceptibility of fungal organisms in CF sputum. The Clinical and Laboratory Standards Institute (CLSI) classifies the resistance breakpoints of *Candida albicans* and *Candida dubliensis* as ≥8 μg/mL for fluconazole and ≥1 μg/mL for caspofungin [28, 29]. For *Candida lusitaniae,* MICs range from 0.125-8 μg/mL for fluconazole and 0.03-1 μg/mL for caspofungin [29]. EIPE-1 is a eumelanin derived antimicrobial compound developed by Dr. Toby Nelson in the Department of Chemistry at Oklahoma State University [30]. Eumelanin belongs to a class of biomacromolecular pigments called melanins that are protective against UV light and contribute to hair, skin, and eye color in humans and animals. Microorganisms have the ability to produce eumelanin as a defense mechanism in hosts, a concept that is clinically relevant since the success of some antimicrobial drugs is dependent on host defenses [30].

MATERIALS & METHODS

Sample isolation: Approximately 24 sputum samples from de-identified CF patients from the Oklahoma City Cystic Fibrosis Clinic were collected and the clinical samples were frozen at -80 in skim milk. After thawing the frozen clinical samples, they were struck onto a selective media consisting of skim milk agar with the antibiotic chloramphenicol and were incubated for two days at 30° C. Isolated colonies were then subcultured onto additional skim milk agar. Skim milk agar promotes fungal growth and inhibits bacterial growth due to its low pH, and the addition of chloramphenicol is a preventative measure for contamination due to bacteria within the samples.

Microscopy: Each sample was viewed microscopically to initiate fungal isolation. Samples that displayed yeast or filamentous morphology were selected for further study. To prevent contamination, samples with similar morphology to *Aspergillus* species were eliminated from the experiment.

DNA purification: Single fungal colonies were isolated and purified using a fungal DNA isolation kit from Zymo Research according to manufacturer's instructions. DNA concentration and purity were determined using the Synergy HTX multimode reader (BioTek) using a Take 3 microvolume plate.

ITS3 PCR: After completing purification, ITS3 PCR was performed. The PCR mixture contained 12.5μL Thermo Scientific DreamTaq Mastermix, 1μL forward primer, 1μL reverse primer, 0.5 μL DNA template, and 10 μL nuclease-free H_2O for a total of 25μL of reaction mixture. The denaturation step was programmed at 95°C for 1 minute, the annealing step was programmed at 52°C for 30 seconds, and the extension step was programmed at 72°C for 1 minute and 30 seconds. The total reaction consisted of 30 cycles, and the final extension was programmed at 72°C for 5 minutes.

DNA electrophoresis: After PCR was completed, DNA gel electrophoresis was performed to identify fungal bands with a size of 500 bp. An 8% agarose gel was prepared, and samples were run at 100 volts for one hour. The gel was then imaged on the Bio-Rad Gel Documentation System in order to view any potential fungal bands.

Fungal identification using API test strips and DNA sequencing: API AUX20 C biochemical test strips (BioMerieux) containing 20 biochemical tests were inoculated with each fungal isolate and incubated for 48-72 hours according to manufacturer's instructions. Identification was based

off a 7-digit numerical profile by adding the numbers corresponding to the positive reactions. Each 7-digit profile was entered into the api*web* identification database.

MIC assays: Fungal growth inhibition was measured by performing Minimum Inhibitory Concentration (MIC) assays for each fungal patient sample CLSI guidelines [CLSI, 2008]. with the antifungal drugs Fluconazole, Caspofungin, and novel drug EIPE-1. On a 96 well plate, 200 µl of each drug was added into row 12 and diluted by 100 µl down each row beginning at row 12 and ending at row 2 where row 1 served as the control and contained no drug. After dilution, 100 µl of fungal samples was added to the entire plate. Each plate was incubated for 48 hours at 35°C and then placed onto a plate reader to measure optical density. The last "clear" row without fungal growth was considered the MIC concentration.

RESULTS

Fungal colonies were identified from cystic fibrosis patient samples. For these studies, 24 patient samples were thawed and streaked onto skim milk agar plates in order to determine the presence of fungal organisms in the patient samples. After 48 hours, colonies were analyzed by microscopy for size (>5 um in diameter) and morphology (yeast or hyphae). Colonies that met these criteria were presumed to be fungi and were subjected to additional assays. We found 8 fungal isolates from the 24 patient samples, and 0 patients had multiple fungal colonies present (Table 1).

Bacteria	32014B	31314A	8714C	21314A	22714D	102314A	102314H	112014B
	102314O	11614D	11614E	112014A	8714C	81414C	9414D	112014C
Fungi	31614A	2515A	8414D	8714E				
Both	22014C	22714E	21314B	22714A				

Table 1. Patient samples were separated according to microbial species found. A total of 16 samples contained bacteria only, 4 samples contained only fungi, and 4 samples contained both bacterial and fungal species

PCR for ITS3 revealed the presence of fungal organisms from CF clinical samples. In order to confirm that fungal species were present in our clinical samples, DNA was isolated from each isolate, followed by PCR for ITS3. Those with bands corresponding to the size of the ITS3 gene (500 kb) were then sequenced. Out of 24 total samples subjected to ITS3 PCR, gel electrophoresis revealed that 8 of these samples contained bands corresponding to the size of the ITS3 gene (Figure 1). Sample 11614D displayed a false positive for band imaging due to environmental contamination and was not included in the study.

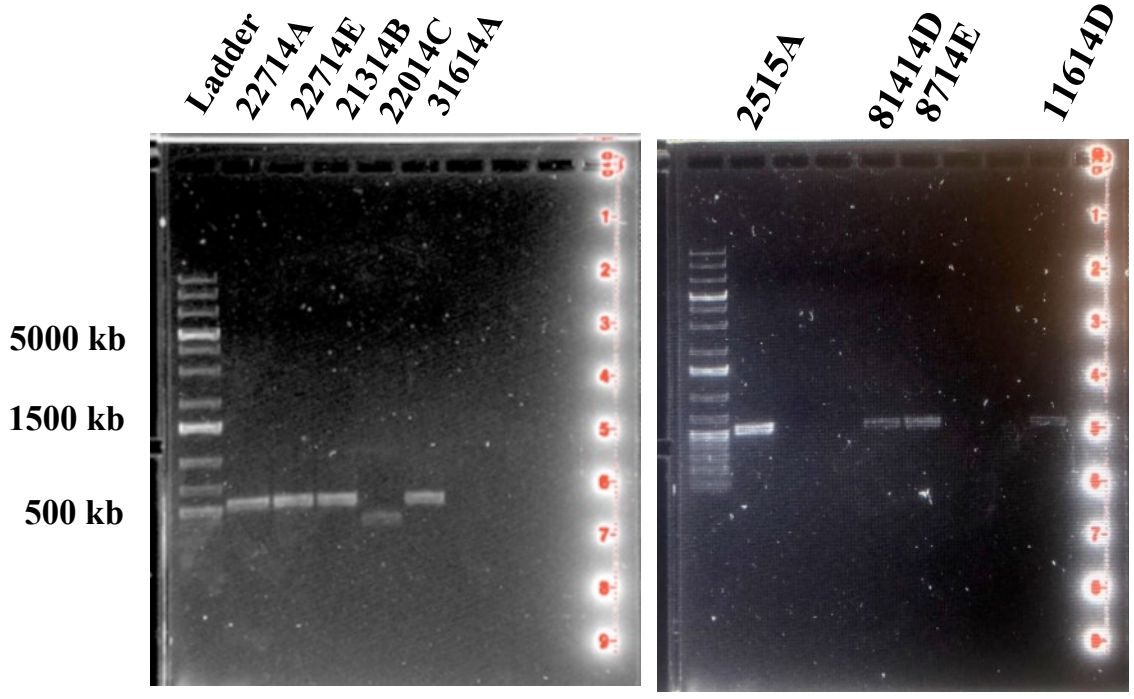


Figure 1. DNA gel showing bands after ITS3 PCR amplification. Following PCR amplification of the ITS3 gene, samples were run on an agarose gel at 100 volts for one hour. The gel was then imaged on the Bio-Rad Gel Documentation System. Lanes 1-5 show potential ITS3 bands.

ITS3 sequencing and API biochemical tests identified the fungal species from CF clinical samples. Following positive ITS3 bands (Figure 1), these fungal isolates were identified to the genus and species level by API test strips and by DNA sequencing of the ITS3 gene. Using the API database, patient samples 22714A and 22014C were identified as *Candida famata*, and 22714E, 21314B, and 31614A as *Candida albicans* (Table 2). However, sequencing results identified patient samples 22714E and 21314B as *Candida albicans*, 22714A and 31614A as *Candida dubliensis*, and 22014C as *Candida lusitaniae* (Table 3).

Patient ID	***API Test***	***DNA Sequencing***
22714A	*Candida famata*	*Candida dubliensis*
22714E	*Candida albicans*	*Candida albicans*
22014C	*Candida famata*	*Candida lusitaniae*
21314B	*Candida albicans*	*Candida albicans*
31614A	*Candida albicans*	*Candida dubliensis*

Table 2. Identification of fungal species using API database and ITS3 DNA Sequencing

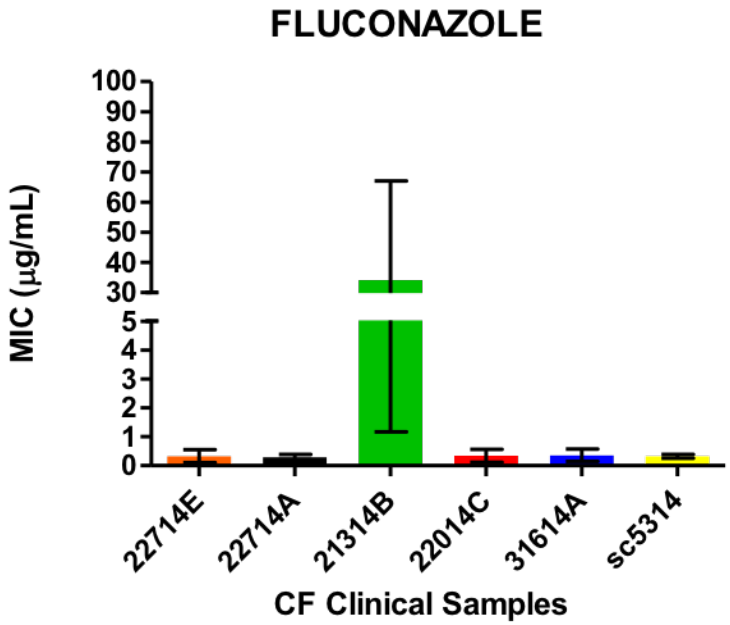
Fungal isolates from the CF clinical samples were resistant to antifungal drugs but sensitive to a novel antimicrobial compound. Minimum inhibitory concentration (MIC) assays were conducted using fluconazole, caspofungin, and a novel antimicrobial compound, EIPE-1. Results showed resistance to fluconazole in patient 22014C (*Candida lusitaniae*) (Table 3 and Figure 2). Patient sample 21314B showed resistance to caspofungin (Table 3 and Figure 3). No resistance was shown against novel, eumelanin derived drug EIPE-1 (Table 3 and Figure 4), suggesting

potential use for *Candida* infections. The wild-type *C. albicans* species SC5314 was used as a control.

Minimum Inhibitory Concentrations: MIC (μg/ml) ± SEM

	22714E	**22714A**	**21314B**	**22014C**	**31614A**	**SC5314**
Fluconazole	0.3268±0.227	0.2943±0.09636	34.11±32.94	0.3418±0.2238	0.3581±0.2135	0.3255±0.0651
Caspofungin	0.2461±0.1445	0.2461±0.1445	12.70±12.30		6.445±6.055	0.3906±0.0
EIPE-1	3.125±0.0	3.125±0.0	4.688±0.9021	2.051±1.403	3.125±0.0	7.292±2.756

Table 3. Minimum inhibitory concentrations for clinical samples and antifungal agents fluconazole, caspofungin, and novel drug EIPE-1.



.

Figure 2. Fluconazole MIC for clinical samples. Samples were tested to determine the MIC of each isolate to fluconazole. Data shown are means +/- SEM from 2-3 experiments with each clinical isolate.

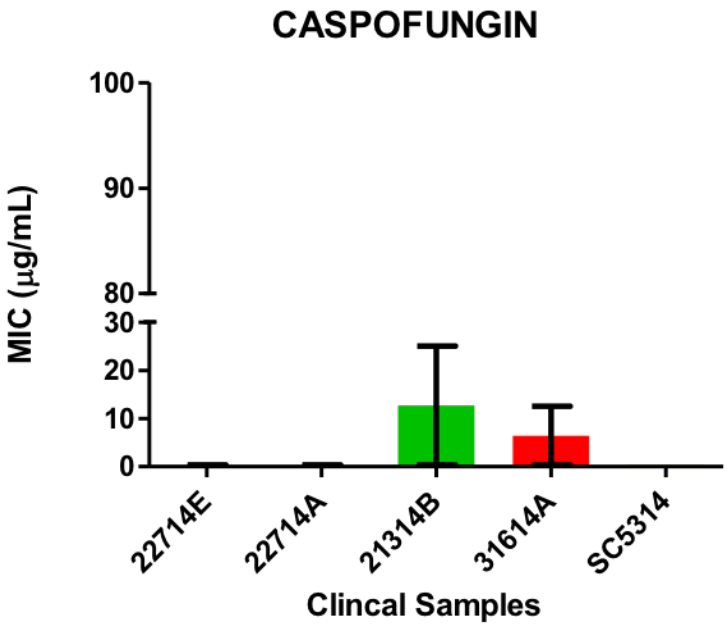


Figure 3. Caspofungin MIC for clinical samples. Samples were tested to determine the MIC of each isolate to caspofungin. Data shown are means +/- SEM from 2-3 experiments with each clinical isolate.

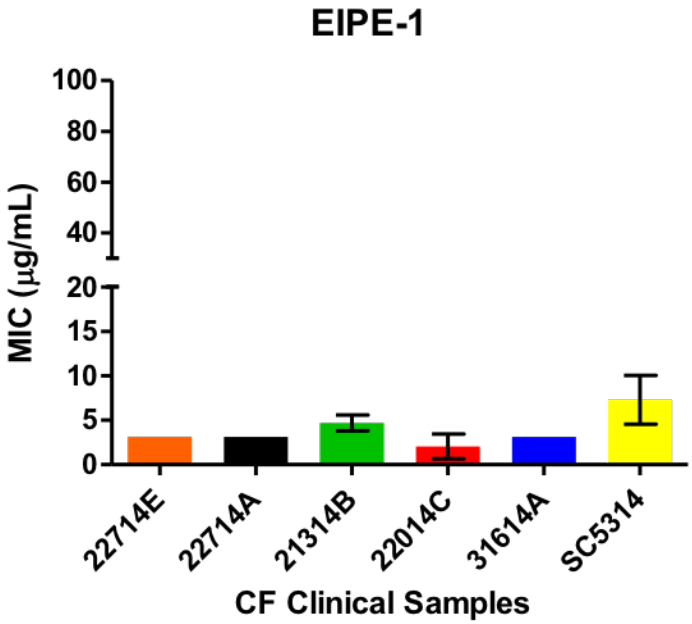


Figure 4. EIPE-1 MIC for clinical samples. Samples were tested to determine the MIC of each isolate to EIPE-1. Data shown are means +/- SEM from 2-3 experiments with each clinical isolate.

CONCLUSION

There is growing concern regarding fungal pathogen colonization within Cystic Fibrosis lungs. Not only do fungal pathogens like *Candida albicans* prolong microbial infections they also give rise to rapid drug resistance [10]. In terms of treatments and drug development, high resolution imaging has offered insight on the pharmacodynamics between drugs and the CFTR protein, however, imaging limitations prevent the view of CFTR protein channel operations [2]. Inter-kingdom interactions between fungal and bacterial species have shown great controversy in CF airways due to antagonistic behaviors. Though *P. aeruginosa* biofilms enable the destruction and killing of *Candida* hyphae, the bacterial pathogen is able to obtain nutrients and survive in the lung [19]. Alternatively, *C. albican's* production of farnesol inhibits growth of *P. aeruginosa* allowing for its own survival. Ongoing polymicrobial infections prolong administration of antimicrobial agents in Cystic Fibrosis patients imposing risk of organisms becoming multi-drug resistant. In turn, increased levels of drug resistance make it harder for researchers to develop novel antifungal drugs.

The initial hypothesis of this study aimed for identification of fungal pathogens in CF patient sputum samples, and that these fungal organisms would show resistance to antifungal therapies**.** However, the results indicate that only some resistance was shown against antifungal drugs fluconazole and caspofungin, while multiple *Candida* species showed consistent sensitivity towards the novel, eumelanin derived antimicrobial EIPE-1 indicating its potential use for antifungal therapies. Although CF still has no cure, the overall improvement of CF patient quality of life is the first step. Effective combination therapy that addresses both CFTR function and pathogenesis of the lungs along with development of enhanced diagnostic tools is imperative and necessary. Understanding the drug resistance behind bacterial and fungal interactions within

the lungs would allow for producing better antimicrobial drugs that inhibit both pathogens from colonizing and increasing CF infections. Further CF research, clinical trials, and *in vivo* studies is crucial for expansion of other combination therapies, especially those that can be used to treat patients of all ages.

REFERENCES

1. Coutinho, H.D., V.S. Falcao-Silva, and G.F. Goncalves, *Pulmonary bacterial pathogens in cystic fibrosis patients and antibiotic therapy: a tool for the health workers.* Int Arch Med, 2008. **1**(1): p. 24.
2. Cystic Fibrosis Foundation, *Basics of the CFTR Protein.* 2019.
3. *U.S. National Library of Medicine,* N.I.o.H. *CFTR gene*. 2008; Available from: https://ghr.nlm.nih.gov/gene/CFTR.
4. Delhaes, L., et al., *The airway microbiota in cystic fibrosis: a complex fungal and bacterial community--implications for therapeutic management.* PLoS One, 2012. **7**(4): p. e36313.
5. Naehrig, S., C.M. Chao, and L. Naehrlich, *Cystic Fibrosis.* Dtsch Arztebl Int, 2017. **114**(33-34): p. 564-574.
6. Cunningham, J.C. and L.M. Taussig, *An Introduction to Cystic Fibrosis for Patients and Their Families*, ed. L. Hazle, J. Marciel, and M. Quirk. 2013: Cystic Fibrosis Foundation
7. Hwang, T.C. and K.L. Kirk, *The CFTR ion channel: gating, regulation, and anion permeation.* Cold Spring Harb Perspect Med, 2013. **3**(1): p. a009498.
8. Moran, O., *The gating of the CFTR channel.* Cell Mol Life Sci, 2017. **74**(1): p. 85-92.
9. Voter, K.Z. and C.L. Ren, *Diagnosis of cystic fibrosis.* Clin Rev Allergy Immunol, 2008. **35**(3): p. 100-6.
10. Williams, C., R. Ranjendran, and G. Ramage, *Pathogenesis of Fungal Infections in Cystic Fibrosis.* Curr Fungal Infect Rep, 2016. **10**(4): p. 163-169.
11. Collaco, J.M., et al., *Quantification of the relative contribution of environmental and genetic factors to variation in cystic fibrosis lung function.* The Journal of pediatrics, 2010. **157**(5): p. 802-7.e73.
12. Ortega-García, J.A., et al., *Smoking prevention and cessation programme in cystic fibrosis: integrating an environmental health approach.* Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society, 2012. **11**(1): p. 34-39.
13. Hector, A., et al., *Microbial colonization and lung function in adolescents with cystic fibrosis.* Journal of Cystic Fibrosis, 2016. **15**(3): p. 340-349.
14. Brad Dell, *C.F.N.T. The Evolution of CF Treatments*. 2017; Available from: https://cysticfibrosisnewstoday.com/2017/11/21/cf-treatments/.
15. McKeny, P.T. and P.M. Zito, *Antifungal Antibiotics*, in *StatPearls*. 2020: Treasure Island (FL).
16. Ghannoum, M.A. and L.B. Rice, *Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance.* Clin Microbiol Rev, 1999. **12**(4): p. 501-17.
17. Borman, A.M., et al., *Lack of standardization in the procedures for mycological examination of sputum samples from CF patients: a possible cause for variations in the prevalence of filamentous fungi.* Med Mycol, 2010. **48 Suppl 1**: p. S88-97.
18. Spampinato, C. and D. Leonardi, *Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents.* Biomed Res Int, 2013. **2013**: p. 204237.
19. Hogan, D.A. and R. Kolter, *Pseudomonas-Candida interactions: an ecological role for virulence factors.* Science, 2002. **296**(5576): p. 2229-32.
20. Thein, Z.M., et al., *Community lifestyle of Candida in mixed biofilms: a mini review.* Mycoses, 2009. **52**(6): p. 467-75.

21. Lo, H.J., et al., *Nonfilamentous C. albicans mutants are avirulent.* Cell, 1997. **90**(5): p. 939-49.
22. Hermann, C., et al., *Bacterial flora accompanying Candida yeasts in clinical specimens.* Mycoses, 1999. **42**(11-12): p. 619-27.
23. Ader, F., et al., *Short term Candida albicans colonization reduces Pseudomonas aeruginosa-related lung injury and bacterial burden in a murine model.* Crit Care, 2011. **15**(3): p. R150.
24. Fourie, R., et al., *Candida albicans and Pseudomonas aeruginosa Interaction, with Focus on the Role of Eicosanoids.* Front Physiol, 2016. **7**: p. 64.
25. Barenfanger, J., et al., *Improved outcomes associated with limiting identification of Candida spp. in respiratory secretions.* J Clin Microbiol, 2003. **41**(12): p. 5645-9.
26. Prasad, R., A.H. Shah, and M.K. Rawal, *Antifungals: Mechanism of Action and Drug Resistance.* Adv Exp Med Biol, 2016. **892**: p. 327-349.
27. Dixon, D.M. and T.J. Walsh, *Antifungal Agents*, in *Medical Microbiology*, th and S. Baron, Editors. 1996: Galveston (TX).
28. Fothergill, A.W., et al., *Impact of new antifungal breakpoints on antifungal resistance in Candida species.* J Clin Microbiol, 2014. **52**(3): p. 994-7.
29. Lockhart, S.R., et al., *Species identification and antifungal susceptibility testing of Candida bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011.* J Clin Microbiol, 2012. **50**(11): p. 3435-42.
30. Santosh, S., et al., *Eumelanin-inspired antimicrobial with biocidal activity against Methicillin Resistant Staphylococcus aureus.* 2020, Oklahoma State University.