AN ARTIFICIAL SPUTUM *IN VITRO* MODEL FOR EVALUATION OF ANTIBIOTICS FOR TREATMENT OF CYSTIC FIBROSIS

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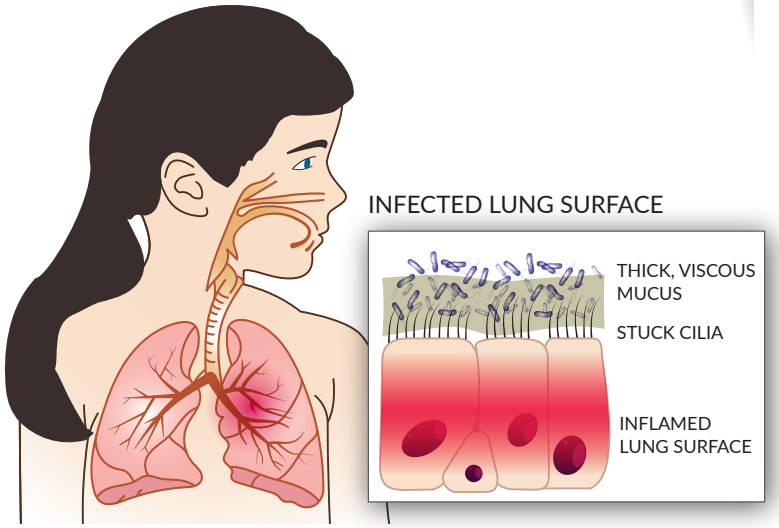
INTRODUCTION

Chronic lung infections are the leading cause of mortality in cystic fibrosis (CF) patients. These infections incite tissue damage and inflammation of the airways, ultimately ending in respiratory failure.[1] Although *Staphylococcus aureus* and *Haemophilus influenzae* frequently infect patients,[2] *Pseudomonas aeruginosa* is the major pathogen responsible for respiratory failure, infecting as many as 80% of patients by the age of 20.[3] Once established, *P. aeruginosa* infections are extremely difficult to eradicate.

The conditions of the CF lung significantly contribute to the persistence of these infections. As a species, *P. aeruginosa* has several hypermutable strains which can rapidly develop antibiotic resistance.[4] In the CF lung, these strains respond to their stressful environment by mutating into a mucoid form.[4,5] Mucoid *P. aeruginosa* produces an alginate material that protects individual bacteria from the destructive mechanisms of antibiotics and the immune system.[5] In addition, the presence of viscous sputum in the lung facilitates the formation of microcolonies— small communities of bacteria protected by an extracellular matrix.[6,7] This complex environment contributes to the multidrug resistant nature of *P. aeruginosa* in the CF lung, rendering antibiotic therapies ineffective at preventing lung failure and patient death.[1]

Development of new antimicrobial agents to eradicate these infections is an essential component to the advancement of CF healthcare. Unfortunately, current models for testing antimicrobials are either impractical or irrelevant to cystic fibrosis. *In vivo* models using mice fail to replicate the CF phenotype and cannot sustain chronic lung infections.[8] *Ex vivo* models using CF patient sputum yield highly variable results.[9] Traditional *in vitro* methods fail to produce suspended microcolonies like those found in CF sputum.



iFyber has implemented an *in vitro* model based on Artificial Sputum Medium (ASM)[6,7] that mimics *P. aeruginosa* growth in the CF lung. ASM reflects the nutritional content of CF sputum, with high levels of mucin, DNA, and amino acids, and *P. aeruginosa* has been shown to form mucoid microcolonies within the ASM closely resembling those found in the CF lung.[6,7] Furthermore, this model is amenable to high-throughput screening, allowing assessment of multiple antibiotics and a range of concentrations simultaneously to provide a comparison of antibiotic efficacy against different species and strains of bacteria.

APPLICATION: ASSESSING ANTIBIOTIC EFFICACY

To assess the efficacy of common antibiotics against CF biofilms, *P. aeruginosa* BAA-47 was grown for 6 days in ASM. Inoculated ASM was aliquoted into a 24-well plate and incubated with daily addition of ASM to counter evaporation and provide additional nutrients to the biofilm.

After 6 days, the biofilms were treated with tobramycin or gentamicin at a range of concentrations for 24 hours, followed by recovery of surviving bacteria. Tobramycin is commonly used to treat CF lung infections and has been measured at a concentration of 80 µg/mL in sputum in clinical samples.[10] Gentamicin was selected due to its broad-spectrum antimicrobial activity. After treatment, the remaining biofilms were disrupted through sonication, serially diluted, and plated for enumeration. For both antibiotics, *P. aeruginosa* survival was reduced with increasing antibiotic concentration. However, both gentamicin and tobramycin failed to eradicate *P. aeruginosa* biofilms at 25x and 80x the MIC values, respectively.

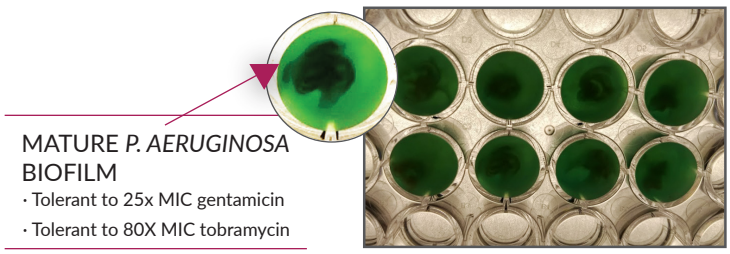
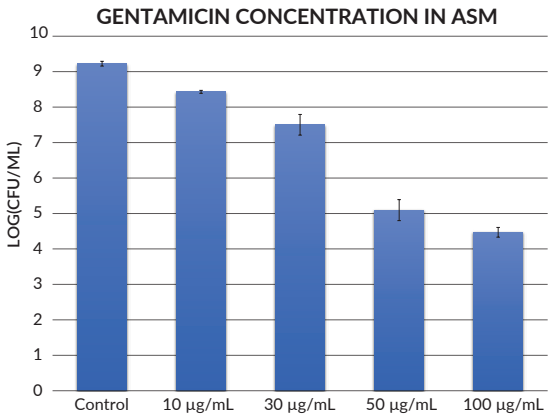


Figure 1. P. aeruginosa BAA-47 in ASM (24-well assay format) forms visible clumps which do not adhere to a surface. Note the green color of the ASM due to *P. aeruginosa* production of pyocyanin.



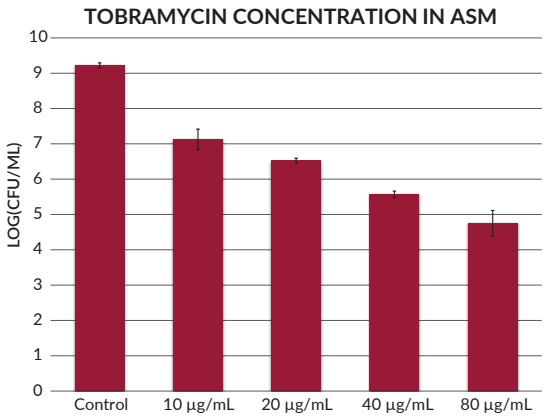


Fig. 2. Efficacy of antibiotics against 6-day old *P. aeruginosa* BAA-47 biofilms in ASM. A: Gentamicin B: Tobramycin

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APPLICATION: STRUCTURAL CHARACTERIZATION OF *P. AERUGINOSA* BIOFILMS GROWN IN ASM

Microscopic analysis was used to examine the structure of *P. aeruginosa* biofilms grown in ASM. Untreated 6-day old biofilms were fixed in paraformaldehyde and processed for histological assessment, paraffinized, and sectioned. Individual sections were stained with Gram stain to highlight the bacteria present in the samples, and Alcian Blue stain which stains the polysaccharides present in the ASM. The images in Figure 3 highlight the presence of biofilm and planktonic bacteria in the ASM, both of which are found in chronic infections, as well as the presence of a thick extracellular matrix.[9]

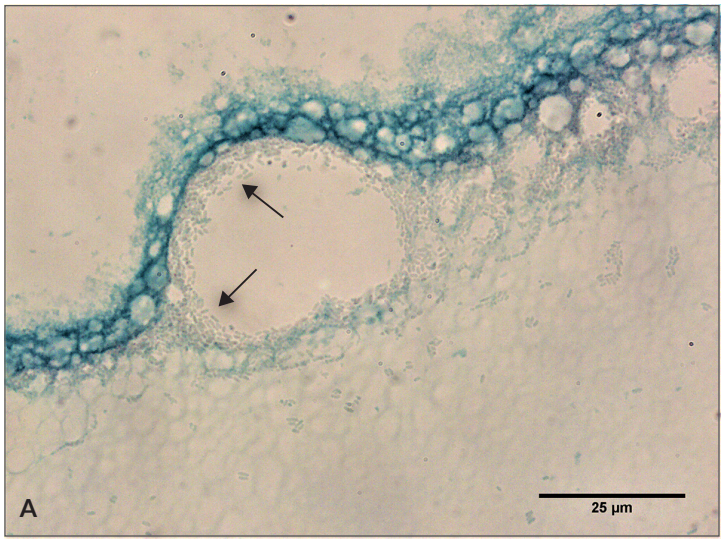
TYPICAL STUDY PARAMETERS

- Bacterial strains
- Time-kill studies
- Combination treatment
- Biofilm age: 3-7 day old biofilms
- Aerobic or microaerophilic conditions

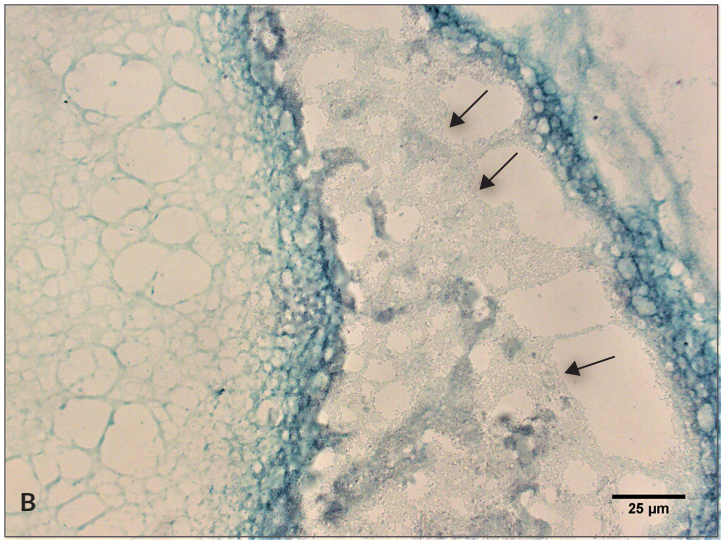
CONCLUSIONS

iFyber has successfully implemented an *in vitro* model for evaluation of various treatments geared towards eradication of infections related to cystic fibrosis. The artificial sputum model produces antibiotic tolerant *P. aeruginosa* biofilms resembling those found in the CF lung. This model allows for rapid screening of a number of antibiotic concentrations and/or combinations, making it a valuable tool for the development of new antibiotics specific to CF lung infections.

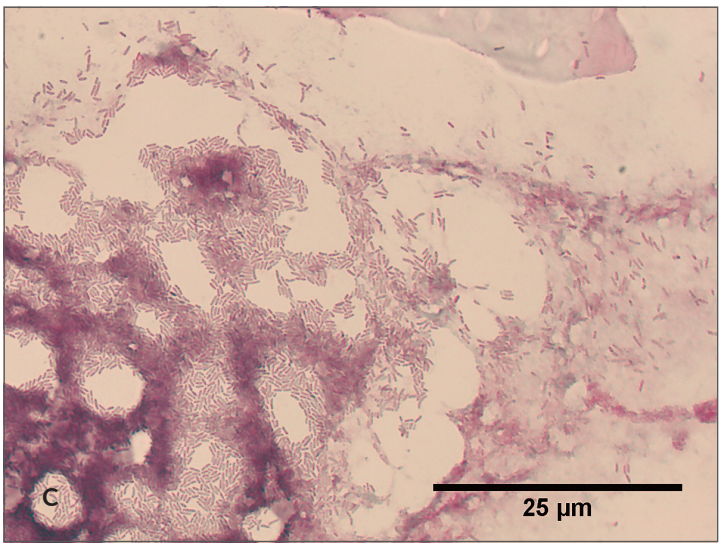
Fig.3 Stained cross sections of 6-day old *P. aeruginosa* BAA-47 biofilm grown in ASM. The alcian blue stain (A-B) highlights the presence of polysaccharides, with the intensity of the stain corresponding to the density of polysaccharides in the extracellular matrix. The Gram stain (C-D) is a general stain for the visualization of bacteria. Gram negative rod-shaped *P. aeruginosa* is stained red.



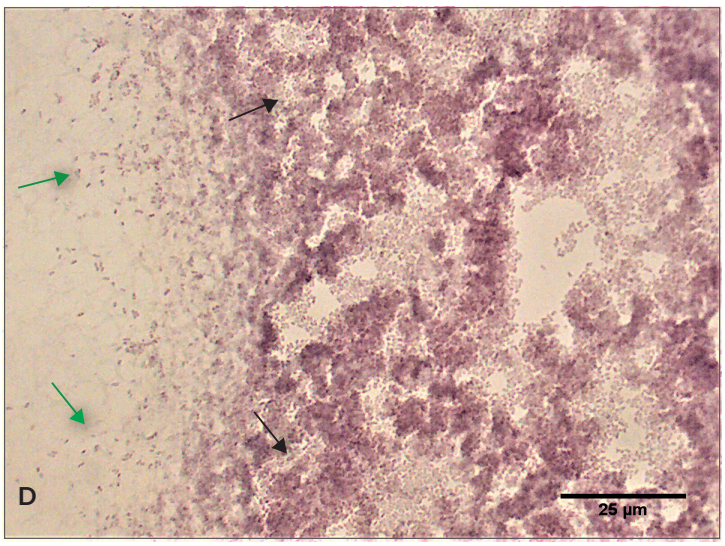
Alcian blue stain. Arrows identify rod-shaped *P. aeruginosa*, which are protected by a thick extracellular matrix (darker blue areas). Bacteria is lightly stained due to polysaccharides present in the cell wall.



Alcian blue stain. Arrows point to aggregates of *P. aeruginosa* surrounded by the thick extracellular matrix (darker blue areas).



Gram stain. Representative section of the biofilms showing a high density of *P. aeruginosa* within the thick extracellular matrix formed in the ASM model. Darker red areas correspond to dense bacterial aggregates within the biofilm.



Gram stain. Green arrows identify planktonic *P. aeruginosa* present within the sample while black arrows point to biofilm-associated bacteria protected by a thick extracellular matrix.

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