MODIFICATION OF THE NEXT GENERATION IMPACTOR AND INCORPORATION OF BACTERIAL CULTURES AS AN IN VITRO PNEUMONIA MODEL

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A THESIS

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Abstract

Pneumonia continually ranks in the top ten causes of death in the United States, despite continual development of new antibiotics and streamlined therapeutic treatment of patients. One approach to the treatment of pneumonia that has recently once again gained prominence is inhaled delivery of antibiotics to the lungs, with the development of new drug formulations. Despite the development of new products and potential for improved therapy, inhaled antibiotics, especially for the treatment of pneumonias, are under-utilized. This is mainly due to the poor correlation between in vitro and in vivo/ex vivo studies. Current methods to do not evaluate drug deposition and aerodynamic properties in conjunction with pharmacokinetic and pharmacodynamics studies. Therefore, there is a need for an improved tool to evaluate aerosol testing.

Building off the work done by a previous graduate student, the development of an in vitro model to evaluate antibiotic drug formulation efficacy was created and validated, utilizing large volume liquid impaction surfaces (LVLIS) incorporated into the Next Generation Impactor (NGI). This model allowed for the correlation of deposition and aerodynamic parameters of an antibiotic solution and antibacterial effects. Amberlite® beads were incorporated into the model to absorb the antibiotic so that time kill studies could be performed. Ceftazidime was chosen as the aerosolized antibiotic formulation due to its broad spectrum of activity as a third generation cephalosporin and its use clinically to treat pneumonias, specifically hospital-acquired pneumonias. ‘Laboratory’ strains of both E. coli and Klebsiella pneumoniae were incorporated into the LVLIS modified NGI. Time-kill studies showed decreased bacteria counts over an eight hour time period, and in some strains, complete eradication of the organism was seen.
Clinically relevant, susceptible and non-susceptible strains with various resistance mechanisms, of both *E. coli* and *Klebsiella pneumoniae* were incorporated into the LVLIS modified NGI. Killing was observed in the susceptible strains and at least a two-log killing was not observed in the non-susceptible strains. A laboratory strain of *Pseudomonas aeruginosa* was unsuccessfully incorporated into the model. Studies were performed to determine the reason why killing was not observed that suggest that this could have been due to the production of a biofilm by a *P. aeruginosa* when exposed to the nebulized antibiotic in the LVLIS modified NGI.

The modification to the NGI allowed the correlation of both deposition and aerodynamic parameters with antibacterial effects, which was seen in several bacteria strains. However, further studies need to be completed to widen the scope of the usefulness of this model by incorporating additional clinically relevant bacteria strains, gram negative and gram positive, and characterizing and testing additional antibiotic formulations appropriate for patients with pneumonia.
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Dedicated to my family and loved ones
Table of Contents

Abstract ........................................................................................................ iii
Acknowledgements ......................................................................................... v
Dedication ......................................................................................................... vii
Table of contents ............................................................................................ viii
List of tables ................................................................................................... xiii
List of figures .................................................................................................. xiv
List of abbreviations ....................................................................................... xvi

Chapter 1: Introduction .................................................................................. 1
1 Introduction .................................................................................................. 2
  1.1 Pneumonia ............................................................................................ 3
    1.1.1 Relevant bacterial organisms ......................................................... 5
      1.1.1.1 Escherichia coli ................................................................. 5
      1.1.1.2 Klebsiella pneumoniae ....................................................... 5
      1.1.1.3 Pseudomonas aeruginosa ................................................. 6
    1.1.2 Treatment and management of pneumonia ................................. 7
      1.1.2.1 Typical treatment strategy ............................................... 8
      1.1.2.2 Empiric therapy .............................................................. 8
      1.1.2.3 Changing therapy based on culture results ....................... 10
  1.2 Multidrug resistant pathogens and resistance mechanisms ............. 11
  1.3 Targeted drug delivery ....................................................................... 13
  1.4 Devices for pulmonary drug delivery ................................................. 15
Chapter 2: Incorporation of bacterial cultures into the Next Generation Impactor (NGI)

2.1 Modification to the NGI collection cup

2.2 Evaluation of bacterial cell viability in the LVLIS within the NGI

2.2.1 Material and methods

2.2.2 Results and discussion

2.2.3 Conclusion

2.3 Characterization and analysis of ceftazidime solution in normal and LVLIS collection cups within the NGI

2.4 Nebulization of ceftazidime solution in LVLIS modified NGI containing *E. coli* K12

2.4.1 Materials and methods

2.4.2 Results and discussion

2.4.3 Conclusion

2.5 Incorporation of an antibiotic binding resin into the LVLIS modified NGI

2.5.1 Materials and methods

2.5.2 Results and discussion

2.5.3 Conclusion

2.6 Conclusion
Chapter 3: Incorporating clinical strains of bacteria to build a pneumonia model

3 Incorporating clinical strains of bacteria to build a pneumonia model

3.1 Characterization and choice of an antibiotic solution

3.2 Determining the MIC for bacterial cultures

3.3 Incorporation of various strains of E. coli

3.3.1 Materials and methods

3.3.2. Results and discussion

3.3.2.1. Results and discussion for laboratory strain, E.coli K12

3.3.2.2. Results and discussion for clinically susceptible strains, E. coli ATCC 25922 and B/r*, and CUMC 243 CMY-2

3.3.2.3. Results and discussion for clinically non-susceptible strains, E. coli 261 DHA(+) 

3.3.2.4. Results and discussion for clinically relevant ESBL producing, Misc. 238 TEM-10

3.3.3. Conclusion

3.4. Incorporation of various strains of Klebsiella pneumoniae

3.4.1 Materials and methods

3.4.2. Results and discussion

3.4.2.1. Results and discussion for ‘laboratory strain’, Klebsiella pneumoniae KP23
3.4.2.2. Results and discussion for clinically susceptible strains, *Klebsiella pneumoniae* Cu 111 and Cu 129

3.4.2.3. Results and discussion for clinically non-susceptible strains, *Klebsiella pneumoniae* 249 CMY-2-like and 225 ACT-1

3.4.2.4. Results and discussion of a clinically non-susceptible, ESBL producing strain of *K. pneumoniae*, Misc. 233 TEM-5

3.4.3. Conclusion

3.5 Incorporation of *Pseudomonas aeruginosa* cultures into the LVLIS modified NGI

3.5.1. Incorporation of a ‘laboratory strain’ of *P. aeruginosa*, PAO-1, utilizing a time kill study

3.5.2. Aerosolized versus instilled deposition of ceftazidime to *P. aeruginosa* PAO-1

3.5.3. Time kill study with PAO-1 suspended in normal saline
3.5.4. Time kill study of a decreased inoculum of PAO-1 with nebulized ceftazidime

3.5.4.1 Materials and methods

3.5.4.2 Results and discussion

3.5.4.3. Conclusion

3.5.5. Time kill study of an increased concentration of ceftazidime with PAO-1

3.5.5.1 Materials and methods

3.5.5.2 Results and discussion

3.5.5.3. Conclusion

3.5.6. Mimicking a ‘re-dosing’ of ceftazidime utilizing PAO-1

3.5.6.1 Materials and methods

3.5.6.2 Results and discussion

3.5.6.3. Conclusion

3.6 Conclusion

Chapter 4: Summary and Future Studies

4. Summary and future studies

4.1 Summary

4.2 Future studies

References
List of Tables

Table 1.1. Initial empiric therapy for HAP, VAP, and HCAP in patients with risk factors for MDR pathogens___________________________________________________________9

Table 1.2. Median aerodynamic diameters for the various stages in the NGI for a nebulized solution_________________________________________________________19

Table 2.1. Average colony forming units per milliliter (LOG(CFU/ml)) for exposed and non-exposed bacterial cultures_______________________________29

Table 2.2. Average colony forming units per milliliter (LOG(CFU/ml)) for utilizing Amberlite® beads and Control________________________________35

Table 3.1 E. coli strains incorporated and tested in the LVLIS modified NGI ______41

Table 3.2 Klebsiella strains incorporated and tested in the LVLIS modified NGI ____________________________________________________________49

Table B.1. Average aerodynamic parameters in the unmodified NGI (n=3) and LVLIS (n=3) _____________________________________________Abstract B

Table B.2. Change of height of water after exposure to airflow in the NGI. Control: LVLIS water placed on desktop. Sample: LVLIS containing water placed in the NGI ______________________________Abstract B

Table B.3. Change in height of MHB after exposure to airflow in the NGI Control: LVLIS containing MHB placed on desktop. Sample: LVLIS containing MHB placed in the NGI __________________________Abstract B
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Pseudomonas aeruginosa alginate biofilm</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Summary of the management strategies for a patient with suspected HAP, VAP, or HCAP</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Open configuration of the Next Generation Impactor (NGI) with seven stages and the MOC</td>
<td>17</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic of the nozzle piece of the NGI</td>
<td>17</td>
</tr>
<tr>
<td>1.5</td>
<td>Diagram of a collection cup in the NGI. B: Critical impaction distance</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>Diagram of LVLIS within NGI with bacterial culture. (B=critical impaction distance)</td>
<td>25</td>
</tr>
<tr>
<td>3.1</td>
<td>Photograph of an E test after 18 hours of incubation</td>
<td>40</td>
</tr>
<tr>
<td>3.2</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and <em>E. coli</em> K12 in LVLIS modified NGI (n=3), with standard error bars</td>
<td>43</td>
</tr>
<tr>
<td>3.3</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and clinically susceptible <em>E. coli</em> in LVLIS modified NGI (n=3), with standard error bars</td>
<td>44</td>
</tr>
<tr>
<td>3.4</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and non-susceptible <em>E. coli</em> in LVLIS modified NGI (n=3), with standard error bars</td>
<td>46</td>
</tr>
<tr>
<td>3.5</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and <em>E. coli</em> Misc. 238 TEM-10 in LVLIS modified NGI (n=3), with standard error bars</td>
<td>47</td>
</tr>
<tr>
<td>3.6</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and <em>K. pneumoniae</em> KP23 in LVLIS modified NGI (n=3), with standard error bars</td>
<td>51</td>
</tr>
<tr>
<td>3.7</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and clinically susceptible <em>Klebsiella</em> in LVLIS modified NGI (n=3), with standard error bars</td>
<td>52</td>
</tr>
<tr>
<td>3.8</td>
<td>Average LOG (CFU/ml) vs. time of nebulized ceftazidime and non-susceptible <em>Klebsiella</em> in LVLIS modified NGI (n=3), with standard error bars</td>
<td>53</td>
</tr>
</tbody>
</table>
Figure 3.9  Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *K. pneumoniae*, Misc. 233 TEM-5 in LVLIS modified NGI (n=3), with standard error bars ____________________________54

Figure 3.10  Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *P. aeruginosa* PAO-1 in LVLIS modified NGI (n=3), with standard error bars ____________________________57

Figure 3.11.  Comparison of nebulized delivery and controlled delivery of the same amount of ceftazidime onto a *P. aeruginosa* PAO-1 culture in the LVLIS ______________________60

Figure 3.12.  Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *P. aeruginosa* PAO-1 in LVLIS modified NGI, suspended in normal saline (n=3), with standard error bars ______________________62

Figure 3.13.  Photograph of *P. aeruginosa* PAO-1 in modified LVLIS collection cup after nebulization of ceftazidime into MHB/PAO-1 inoculum; production of biofilm _________________________________63

Figure 3.14.  Comparison of 10⁶ and 10⁸ PAO-1 inoculum of the same amount of ceftazidime onto a *P. aeruginosa* PAO-1 culture in the LVLIS ________65

Figure 3.15.  Comparison of 100 mg/ml and 10 mg/ml of ceftazidime solution that was nebulized onto a *P. aeruginosa* PAO-1 culture in the LVLIS ________67

Figure 3.16.  Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *P. aeruginosa* PAO-1 in LVLIS modified NGI, after a second dose of ceftazidime was administered after hour eight _______________________69

Figure B.1.  Comparison of deposition in unmodified NGI and LVLIS after theophylline solution nebulization _______________________ Appendix B
List of Abbreviations

BIC: Biofilm inhibitory concentration
BCS: Biopharmaceutics Classification System
CAP: Community-acquired pneumonia
CFU: Colony forming units
ESBL: Extended spectrum beta-lactamases
FPF: Fine particle fraction
GSD: Geometric standard deviation
HAP: Hospital-acquired pneumonia
HCAP: Healthcare-associated pneumonia
IDSA: Infectious Disease Society of America
LVLIS: Large volume liquid impaction surface
KPC: *Klebsiella pneumoniae* carbapenemases
MDR: Multidrug-resistant
MHA: Mueller-Hinton agar
MHB: Mueller-Hinton broth
MIC: Minimum inhibitory concentration
MMAD: Mean aerodynamic diameter
MOC: Micro-orifice collector
MRSA: Methicillin-resistant *Staphylococcus aureus*
NGI: Next generation Impactor
TFTC: Too few to count
TNTC: Too numerous to count
USP: United States Pharmacopeia

VAP: Ventilator Associated Pneumonia
Chapter 1: Introduction
1 Introduction

As antibiotic resistance continues to develop during the treatment of clinical pneumonias, the use of older and newer drugs are evaluated and re-evaluated for potential formulation into novel drug products. Another avenue for expanded therapeutic options is the preparation of drug products for delivery via the pulmonary route of administration. This route has been explored to deliver drug to targeted areas of the body and to a smaller degree, systemically. The structure of the lung, the aerodynamic properties of the drug formulation, and the deposition of the drug formulation on the structures in the lung all need to be understood to create a formulation that is successful at delivering a known, consistent concentration of drug. While this route does pose some challenges, the benefits of topical administration apply here; the amount of drug is decreased because it is directly applied to the site of action, rather than administered systemically, the side effects are decreased and/or different based on a lower amount of drug and a different site that it is applied to, and increased absorption at the desired site of action, when typically some antibiotics show poor distribution into the lungs.\(^1\) Again, as resistance continues to develop, the need for antibiotics to treat one of the most common infections, pneumonia, continues to grow. Therefore, exploring the pulmonary route for targeted delivery of antibiotics to the site of infection is a logical step.

Pulmonary administration has many advantages, but to a large degree the research to understand the parameters that affect drug administration are siloed into two different areas. One silo revolves around the characterization of the aerosolized formulations, specifically their aerodynamic parameters, deposition patterns, and particle size distribution. The other silo revolves around studying how these formulations behave in
patient models, specifically efficacy, pharmacokinetic parameters, and pharmacodynamics properties. This second silo of studies are carried out as in vivo, ex vivo, or in vitro studies. There is a need to combine these two silos together so that aerosol formulation testing can be done in one step and more closely mimic that of a human lung. This Master’s thesis will continue to build the robustness of an in vitro pneumonia model which can evaluate antibiotic efficacy via deposition patterns and aerodynamic parameters in combination with bacterial cultures.

1.1 Pneumonia

Pneumonia is one of the leading causes of death in the United States, typically ranking in the top ten, but it is the number one infectious cause of death in children and adults.\(^2\) The mortality rate is between 30-40%, despite antibiotic treatment.

In pneumonia, microorganisms proliferate in the lower respiratory tract, typically the lower airways and alveoli, which is where gas exchange occurs. This proliferation causes immune cell migration and fluid retention to the lungs resulting in impaired gas exchange, increased lung weight, and typically difficulty breathing.\(^3\)

The occurrence of pneumonias vary throughout the year and the causative microorganism can also change with the seasons. These various organisms also have differing degrees of pathogenicity and mortality. Due to this, four classifications of pneumonia exist dependent upon where the pneumonia was contracted which then affects the treatment approaches for these various infections. Community-acquired pneumonia (CAP) is the most common type of pneumonia, accounting for up to 75% of acute cases. This type of pneumonia can typically be treated successfully in the outpatient setting with oral
antibiotics. A second type is healthcare-associated pneumonia (HCAP). This type of pneumonia is seen in patients that have been hospitalized for at least two days in the past ninety days, reside in a nursing home, or have received chemotherapy or hemodialysis in the last 30 days. These patients are at a higher risk of contracting pneumonia from some of the more pathogenic organisms, when compared to patients with CAP. A third type of pneumonia is hospital-acquired pneumonia (HAP). Patients with HAP develop pneumonia within a minimum 48-72 hour window of entry to a hospital. Finally, ventilator-associated pneumonia (VAP) is a pneumonia where symptoms develop within 48 hours after the patient is placed on a ventilator.4

Each classification varies in treatment, likely causative organisms, and expected outcomes. Of the three pneumonias in the hospital/healthcare setting, hospital-acquired pneumonia is the second most common nosocomial infection and is associated with high mortality and morbidity and has an average increase in hospital stay of 7-9 days.4 It is associated with an increased cost of more than $40,000 per patient and, while not a reportable illness, is thought to occur at a rate between 5-10 cases per 1,000 hospitalized patients. If looking at VAP, the rate is thought to increase as much as 6 to 20 fold.5 HAP is thought to be associated with up to half of all antibiotics prescribed in the intensive care unit setting.6

The most common organisms associated with HAP include P. aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Acinetobacter species, of which are all gram negative organisms. Gram positive organisms are emerging as causative organisms, especially methicillin-resistant Staphylococcus aureus (MRSA), but overall gram negative
organisms are still the most common causative agent of HAP.\textsuperscript{4} Polymicrobial infections and multidrug-resistant pathogens are becoming more common as well.\textsuperscript{3}

1.1.1 Relevant bacteria organisms

As mentioned above, the most common gram negative organisms that cause HAP are: 
\textit{P. aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Acinetobacter} species. The first three will be discussed in greater depth and will be utilized throughout the rest of the Master’s thesis.

1.1.1.1. \textit{Escherichia coli}

\textit{Escherichia coli}, or \textit{E. coli}, is a gram-negative, rod-shaped bacteria that is flagellated. It belongs to the Enterobacteriaceae class of bacteria. It is normally found in the intestines of animals, but is the most common pathogen in human bacterial infections. It is one of the most studied and investigated bacteria organisms.\textsuperscript{7} It is readily isolated in culture. Most strains are susceptible to at least some antibiotics, again, aiding in the ease of its use.

1.1.1.2 \textit{Klebsiella pneumoniae}

\textit{Klebsiella pneumoniae}, is a gram-negative, encapsulated, rod-shaped bacteria. It is normally found in the normal flora of the mouth, skin, and intestines, but can be very pathogenic if aspirated to the lungs. The presence of an external polysaccharide capsule gives \textit{Klebsiella sp.} a mucoid character, making it appear slimy. This is due to the polysaccharide capsule, which readily absorbs water.\textsuperscript{8} It like \textit{E. coli}, belongs to the
Enterobacteriaceae class of bacteria. Of Enterobacteriaceae, various strains of *Klebsiella* have developed resistance to most antibiotics.\(^7\)

1.1.1.3. *Pseudomonas aeruginosa*

Unlike the previous two bacteria, *Pseudomonas aeruginosa*, or *P. aeruginosa*, is not found in any part of a human’s normal flora. It is part of the Pseudomonadaceae family and like the others is a gram-negative rod. It is aerobic, motile, and can be grown in a wide range of environments compared with the two previous organisms. Its outer membrane contains lipopolysaccharide and porin proteins, resembling many gram negative bacilli. The membrane structures of Pseudomonas discourage permeability to exogenous molecules, most notably antibiotics. All *P. aeruginosa* organisms produce alginate to some degree. The alginate helps form a mucoid exopolysaccharide slime layer to help protect the organism. Some strains of *P. aeruginosa* have mutations in genes that overproduce alginate and provide one way to sub-divide the organisms, into ‘mucoid’ and ‘non-mucoid’ strains.\(^7\) These strains commonly form microcolonies of bacteria and, working together, form a biofilm. Biofilms are “multicellular communities held together by a self-produced extracellular matrix.”\(^9,10\) Figure 1.1 attempts to describe the mechanism of *P. aeruginosa* biofilms.\(^7\)
While the mechanisms are not completely understood for the production of biofilms, it is known that strains are present that over produce alginate and quorum-sensing is involved, where the group of bacteria work together despite being distinct organisms. Production of a biofilm is one of the many ways that *P. aeruginosa* continues to show resistance to antibiotics and can accumulate many resistance mechanisms leading to a very resistant pathogen. Currently, there are known isolates of *P. aeruginosa* that are susceptible to only polymyxin B and some not susceptible to any antibiotic.

### 1.1.2. Treatment and management of pneumonia

The Infectious Diseases Society of America (IDSA) publishes guidelines, based on evidenced based medicine, on the management and treatment of various infectious processes that are updated as therapies change. One of the guidelines published explains the proper management of HAP.
1.1.2.1. Typical treatment strategy

The IDSA guidelines for the treatment of HAP state that treatment of pneumonias is best approached utilizing a combination of clinical and bacteriological strategies. Figure 1.2 shows a summary of the management strategies for a patient with suspected HAP, VAP, or HCAP.\(^4\)

![Figure 1.2. Summary of the management strategies for a patient with suspected HAP, VAP, or HCAP.\(^4\)](image)

The algorithm utilizes both clinical response of the patient and collection of cultures (from various types of samples) to determine the course of treatment and length of treatment with antibiotics. Figure 1.2 states to begin empiric antibiotic therapy according to Figure 2 from the guidelines, which is summarized in Table 1.1.

1.1.2.2. Empiric therapy

Following the above algorithm, if it is determined that a patient has suspected pneumonia it is recommended that the patient be started on microbial empiric therapy. Initial choice
of an antibiotic and the dose of the antibiotic is chosen based upon time of onset of the disease, risk for multidrug-resistant (MDR) organisms, and allergies of the patient. Broad spectrum empiric therapy is the most common, with hopes of de-escalating antibiotic choice once microbiology cultures are obtained from the patient.\textsuperscript{3} Empiric therapy for treatment of HAP from the IDSA guidelines are found in Table 1.1. While the guidelines give suggestions on potential empiric therapy it is important to consider site specific resistance patterns and antibiotic susceptibility when choosing an antibiotic.\textsuperscript{4}

Table 1.1. Initial empiric therapy for HAP, VAP, and HCAP in patients with risk factors for MDR pathogens\textsuperscript{4}

<table>
<thead>
<tr>
<th>Potential Pathogens</th>
<th>Combination Antibiotic Therapy</th>
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</thead>
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Or  
Antipseudomonal carbapenem (imipenem or meropenem)  
PLUS  
Antipseudomonal fluoroquinolone (ciprofloxacin or levofloxacin)  
Or  
Aminoglycoside (amikacin, gentamicin, or tobramycin) |
| If MRSA is suspected or potential risk factors are present | ADD on Linezolid or vancomycin |

The table states that antipseudomonal antibiotics should be used. This refers to the discussion above that \textit{Pseudomonas} has a high rate of developing resistance mechanisms and therefore empiric therapy must cover this organism. \textit{Pseudomonas} is the most challenging bacteria to kill and therefore it must be assumed that a patient is infected with this bacteria until shown otherwise. An appropriate choice for initial empiric therapy is
critically important because improper selection of an antibiotic with no to poor activity against an organism can increase antibiotic resistance, by giving the organism the opportunity to innately develop resistance mechanisms due to the presence of the drug at sub-inhibitory concentrations.\textsuperscript{12}

1.1.2.3. Changing therapy based on culture results

As stated above, most empiric antibiotic choices are broad spectrum antibiotics that are chosen to ensure adequate coverage and killing of bacteria. Empiric therapy is chosen once the patient starts exhibiting signs and symptoms of pneumonia, not when cultures come back positive. Before antibiotic therapy is started bacteria cultures from the patient are taken. Typically these samples in pneumonia patients are from the blood and sputum. The samples are taken in an attempt to determine what organism is causing the infection. Unfortunately the samples do not always grow the causative organism, especially sputum cultures.\textsuperscript{13} However, if the samples do grow the causative organism, susceptibility tests are completed on the organism. Susceptibility tests determine which antibiotics should effectively kill the organism in the patient by determining the minimum inhibitory concentration (MIC) of the drug for a particular organism. The MIC is the smallest concentration of drug needed to inhibit visible growth. Culture results are reported with the various antibiotics tested and are reported as either S (sensitive), I (intermediate), or R (resistant). Each organism has a unique MIC to every antibiotic.\textsuperscript{14,29} For example, if an organism has an MIC of 2 µg/ml it would be susceptible to any antibiotic that was able to achieve serum concentrations of 8 µg/ml or higher; there is always a one-fold dilution between MIC and susceptible concentrations to ensure adequate bacteria killing, because
there is a one-log error with the test. To utilize antibiotics appropriately, to conserve their use, and to help prevent resistance from developing it is appropriate to alter antibiotic therapy once culture results are available and to appropriately choose an antibiotic to which that the causative organism is sensitive, if it is sensitive to any antibiotic.

1.2. Multidrug-resistant pathogens (MDR) and resistance mechanisms
The presence of MDR pathogens are continuing to increase, especially in intensive care and transplant units. Risk factors for contracting multidrug resistant pathogens include the following: antimicrobial therapy in preceding 90 days, current hospitalization of 5 days or more, high frequency of antibiotic resistance in the community or specific hospital unit, present of risk factors for HCAP (hospitalization for 2 days or more in the last 90 days, residence in a nursing home, home infusion therapy, chronic dialysis within the last 30 days, home wound care, family member with MDR pathogens, or on immunosuppressive therapy). With the development of resistant pathogens has also come a better understanding and characterization of the resistance mechanisms associated with these organisms. These resistance mechanisms are continuing to be studied. A variety of resistance mechanisms will be described below.

One of the first resistance mechanisms that was discovered was a beta-lactamase, specifically penicillinase. This mutation in *E. coli* coded for an enzyme that was capable of hydrolyzing the lactam ring of the penicillin molecule, inactivating many penicillin class antibiotics. Beta-lactamases are the most common resistance mechanism present in gram negative bacteria. Beta-lactamases are classified based upon the genes that encode
for the particular enzyme. For example, one of the first isolated beta-lactamase enzymes and one of the most common is called TEM-1. TEM-1 is commonly seen in *E. coli* and *Klebsiella pneumoniae*, however it can be present in many organisms. Other common beta-lactamases include TEM-2 and SHV-1, with each enzyme having varying degrees of resistance associated with it. Upon continued use of beta-lactam antibiotics and the production of newer, novel antibiotics, extended spectrum beta-lactamases (ESBLs) emerged in the 1980s. These ESBLs are capable of hydrolyzing the oxyimino side chain present on later generation cephalosporins. Some ESBLs have derived due to mutations in either the TEM-1, TEM-2, or SHV-1 enzymes because the amino acid sequences are similar. Worldwide the most common ESBL enzyme is CTX-M-15; other ESBL enzymes that are common in the United States are TEM-10 and TEM-12. Additionally, ESBLs tend to be encoded on plasmids which makes them easier to share between bacteria and also tend to have multiple resistance mechanisms present on the plasmid, including other classes of antibiotics. Treatment options for ESBL producing organisms are few, with a carbapenem being first line choice. The various types of ESBL enzymes are thought to be due to the various antibiotic prescribing of different countries. Interestingly, some cephalosporin antibiotics, including ceftazidime, have been shown to be more likely to select for ESBL mutants. In addition, AmpC-type beta-lactamases are an enzyme that when produced by *E. coli* and *Klebsiella pneumoniae* can mimic the phenotypic susceptibility test of an ESBL producer. AmpC genes are typically carried on the chromosome of organisms such as *Serratia* and *Enterobacter* sp. and are inducible, however they can also be found on plasmids. Like ESBLs, AmpC enzymes can hydrolyze the ring present on these antibiotics, but they are not inhibited by inhibitors, such as
clavulanic acid. Carbapenems are active against AmpC producers.\(^{32,35}\) Emergence of beta-lactamases against carbapenems has also occurred. Denoted, as carbapenemases, these enzymes are active against carbapenems, which traditionally have been active against ESBLs and AmpC producers. One specific type, *Klebsiella pneumoniae* carbapenemase (KPC), is the most common type of carbapenemase in Enterobacteriaceae. It was reported in 1996 and this resistance mechanism is becoming more common.\(^{44}\) Bacteria with the KPC gene should be treated with a non-beta-lactam antibiotic such as tigecycline, polymixin B, or any aminoglycoside, however it is common to have KPC producers be resistant to other classes of antibiotics as well.\(^{11}\)

### 1.3 Targeted drug delivery

Current therapy for treatment of pneumonia utilizes intravenous drug delivery that requires systemic distribution through the blood, to the lungs, to the site of infection. Some antibiotics, including cephalosporins, have poor lung penetration and only about half of the serum concentration is obtained in the lung.\(^{18}\) This is much different than other classes of antibiotics, such as fluoroquinolones that distribute well into peripheral tissues and fluids and have been reported to achieve almost exactly the same concentration in the lungs as is found in the serum.\(^{18}\) Additionally, when given intravenously, an antibiotic with poor distribution has the potential for increased systemic side effects in order to reach adequate levels of drug into the lungs. In addition, if adequate levels are not reached the potential for emergence of resistance is increased. Conversely, when looking at specific delivery of a drug to the site of action, there is a decreased requirement of drug, decreased metabolism of the drug because the liver and GI system is bypassed, and
the side effect profile is different. Targeted drug delivery has been explored in various types of therapy due to its ability to overcome Class III and IV substances of the Biopharmaceutics Classification System (BCS). Class III and IV substances both have a low permeability, with Class III having a high solubility and Class IV having a low solubility. Drugs in Class III or IV have a low permeability and are therefore pose a challenge to get to the site of action if administered oral. These drugs tend to have poor absorption, lipophilicity, and dissolution. Therefore, to get these drugs to have maximal action at the specified sight, administering them to the specified site is one way to overcome the formulation challenges they present.46

Utilizing inhaled delivery of a drug has the potential to overcome the disadvantages that are present with systemic delivery of the same drug.3 The pulmonary route of administration has been explored, especially for patients with chronic lung diseases and cystic fibrosis. Currently there are two inhaled antibiotic products that are available on the market, which makes this route a potential option for targeted antimicrobial therapy for other infective lung diseases.19,20 While there are potential disadvantages to utilizing the pulmonary route, including variability in doses, requiring deep breathing in patients with an altered physiologic lung, and utilization of various medical devices, technologies are being explored and developed to potentially use this route for drug administration.39

There have been a few anecdotal reports utilizing nebulized antibiotics in the treatment of *P. aeruginosa* VAP that has been unresponsive to systemic therapies. These patients saw clinical improvement and it is thought to due to a higher amount of drug penetrating the lungs and cultures that could not be attained systemically.31,40,41 However, despite these successes, there is still concern about increased resistance to bacteria with utilization of
nebulized antibiotics. These concerns mainly come from studies using inhaled antibiotics as prophylaxis, rather than treatment.\textsuperscript{24,30,42} There is a need to explore this route further.

1.4 Devices for pulmonary drug delivery
Delivery of a drug to the site of action in the lungs requires the use of a nebulizer, metered dose inhaler, or dry powder inhaler to produce a respirable aerosol of the drug so that it can be inhaled by the patient. The current available inhaled antibiotic therapies that are on the market utilize the technology of nebulization to deliver the solution in an aerosolized form. Nebulizers aerosolize a liquid medication into smaller particles that form a fine mist that can be easily and passively inhaled by the patient. Each nebulizer is different and has different deposition characteristics, aerodynamic properties, and efficiency. Of the various technologies available to deliver a medication to the lungs, nebulizers allow the patient to receive the medication by breathing normally and do not require a deep inspiration or holding of the breath like other devices.\textsuperscript{21} Because of this, nebulizers are more ideal for patients with decreased lung function, as in patients with pneumonia. While nebulizers are larger than other devices and require an external compressor or electrical source, therefore outputting noise and heat, they are utilized extensively in the hospital and in outpatient settings.

1.5 Next Generation Impactor
As described in the last section, deposition characteristics and aerodynamic properties are unique to each nebulizer. Therefore it is necessary to quantify and evaluate these parameters to determine if the nebulizer produces an aerosol that can adequately
penetrate the lung. One of the most common tools utilized to determine the aerodynamic parameters of aerosol devices are known as cascade impactors. There are multiple types of cascade impactors, but they all utilize the same principle of inertial impaction. Inertial impaction is the process of separation of particles based on their inertia, which is affected by the particle’s velocity and size. By controlling for velocity, the aerodynamic particle size distribution is determined. These impactors measure the particle distribution of the entire sample that is delivered to the impactor.

All three of these cascade impactors vary slightly in use, but the principles behind their use are very similar. An aerosol is drawn into the device and pulled through various orifices, via a constant flow rate, typically regulated by a vacuum pump. Each orifice has a decreasing pore size, to separate the particles based on size. The aerosol travels through the orifice flowing down towards a collection surface, or stage, where, if the inertia is great enough, the particle impacts on the collection surface. The particles that did not impact on that specific stage continue to flow through the next orifice and potentially impact on a subsequent stage. Therefore particles with a lower inertia pass through an orifice and particles with a high inertia will impact on the collection surface. Again, because velocity is controlled for, the particles are separated based on aerodynamic size, with the larger particles with a corresponding higher inertia, depositing on the early stages and particles with smaller size, or inertia, impacting later.

The Next Generation Impactor (NGI) is one of the approved cascade impactors defined by the United States Pharmacopeia (USP). It is a planar cascade impactor that has a horizontal arrangement of impaction surfaces with seven different stages and a micro-orifice collector (MOC). The configuration of the NGI can be seen in Figure 1.3.
Figure 1.3. Open configuration of the Next Generation Impactor (NGI) with seven stages and an MOC

The body of the NGI is comprised of a nozzle piece that contains the various orifices of the NGI. This is the top portion in Figure 1.3. The orange parts in Figure 1.3 are O-rings that ensure a tight closure of the nozzle piece to the collection cups when the NGI is closed. The last portion, seen in the bottom portion of figure 1.3, are the seven collection cups and MOC collection cup. These collection cups are the surfaces on which the aerosol is impacted and the particles are collected. Figure 1.4 is a schematic of the nozzle piece of the NGI.

Figure 1.4. Schematic of the nozzle piece of the NGI

Figure 1.4 also shows the number of holes present in each orifice, along with the size of each hole. For example, the nozzle above Stage 2 contains 6 holes while the nozzle
above Stage 7 contains 4032 holes. These two characteristics correlate to the deposition patterns and aerodynamic properties of the NGI.\textsuperscript{22}

One of the most important parameters in the NGI is the critical impaction distance noted as distance ‘B’ in Figure 1.5.

![Diagram of a collection cup in the NGI](image)

**Figure 1.5. Diagram of a collection cup in the NGI**

\textbf{B: Critical impaction distance}

The critical impaction distance is the distance between the collection surface and top of the NGI. This distance must remain constant to maintain consistent deposition of an aerosol throughout the NGI. The NGI was studied and the aerodynamic properties were determine based on this given distance, any alteration in the NGI must maintain a constant distance B. If this distance changes by getting larger or smaller the particles that impact upon each given stage will be altered. For example, if distance B is smaller, the space that the particles travel from the orifice to the collection cup is less. This decrease in distance would impact larger particles on the surface than would have done so with the non-altered distance B. This would skew the known particle size distribution of the NGI, altering the known aerodynamic properties.\textsuperscript{21,22}
In addition to determining deposition patterns of an aerosol, the NGI is used to determine the aerodynamic properties of an aerosol. The USP defines the following aerodynamic parameters that can be determined using the NGI: the mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), and fine particle fraction (FPF). Table 1.2 shows the median aerodynamic diameters for the various stages of the NGI when a nebulization solution is deposited onto the NGI at an airflow rate of 15 L/min.\textsuperscript{21,22}

Table 1.2. Median aerodynamic diameters for the various stages in the NGI for a nebulized solution

<table>
<thead>
<tr>
<th>Stage</th>
<th>$D_{50,0} , (\mu m)$ at 15 L/min flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td>8.61</td>
</tr>
<tr>
<td>3</td>
<td>5.39</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>2.08</td>
</tr>
<tr>
<td>6</td>
<td>1.36</td>
</tr>
<tr>
<td>7</td>
<td>0.98</td>
</tr>
<tr>
<td>MOC</td>
<td>$&lt;0.98$</td>
</tr>
</tbody>
</table>

The aerodynamic diameter cutoffs, in addition to the other aerodynamic parameters, are important for understanding how the particle size separation is similar in the NGI and in the human lung. Particles that have a larger diameter than 6 $\mu$m typically get filtered out by the lung through cilia and impaction on the upper airways. Particles that have a diameter of around 1-2 $\mu$m can get phagocytized in the alveoli. Therefore, particles in the 3-6 $\mu$m range are ideal for drugs that are targeted to reach the deeper airways.
Additionally, FPFs in the range of 40-60% are ideal, denoting that many particles are less than 5 µm. GSDs in the range of 1.2-1.3 are ideal to show consistency in the particles.\textsuperscript{21,22}

1.6 Evaluating targeted drug delivery to the lungs

As described in the last section, the USP has determined \textit{in vitro} aerosol properties and different cascade impactors that can be used to test aerosols.\textsuperscript{21} However, these \textit{in vitro} tests are not able to be correlated well with \textit{in vivo} evaluation. The current methods do not adequately allow for the determination of pharmacokinetic and pharmacodynamics parameters in correlation with aerodynamic parameters.\textsuperscript{23} These methods lack the ability to mimic patient parameters such as breathing patterns, lung clearance, aerosol formulation, and airflow. Due to these limitations various \textit{in vitro, ex vivo} and \textit{in vivo} methods have been developed to correlate aerosol deposition and aerodynamic properties with patient specific parameters.

\textit{In vitro} analysis is completed by separating the system studied with the biological environment it is normally found in. Regarding \textit{in vitro} analysis, methods are typically simplified in order to control critical variables especially when relating the studies to biological systems or humans. The data analysis tends to be straight forward and the cost of the experiments is decreased. A common \textit{in vitro} way to evaluate aerosols is through the use of cell cultures, which allows for the analysis of absorption of a drug.\textsuperscript{23} Most commonly, the way to evaluate pharmacodynamics effects of an antibiotic is through the use of bacterial cultures. Cultures are grown in either broth or petri dishes and antibiotic efficacy is determined by applying a known concentration of antibiotic to the culture. Bacterial growth, is determined by colony counts or correlating optical density of the
solution with the amount of colonies present. These methods are well-established and are used both in the laboratory setting to study emerging resistance and clinically to determine susceptibility in a variety of settings, include the hospital. Additionally, antibiotics are studied via a time-kill curve, which looks at correlating the reduction in colonies over time with the efficacy of an antibiotic. This allows for understanding of how quickly the antibiotic works and when it would need to be re-dosed to maintain adequate levels.

Utilizing these in vitro methods allows for determination of some pharmacokinetic and pharmacodynamics properties, but the ability to correlate these properties with deposition and aerodynamic parameters is lacking; therefore, a complete picture of how these formulations will act and work in the clinical setting is incomplete. There are multiple examples of drugs working excellent in in vitro models that have failed when implemented into in vivo studies.

In vivo analysis is completed by evaluating pharmacokinetic and pharmacodynamics properties in a living animal. These studies are done in animals and an attempt to correlate the data to humans is often undertaken. Despite their higher fidelity to biological systems, these studies pose many disadvantages, including the need to develop a device to deliver drug to the animals, the cost associated with working with animals, and a lack of correlation between animals, especially rodents, and human lung deposition. While these studies do allow for the study of how a drug or drug formulation might behave in a human, there are many disadvantages that need to be considered when exploring this potential type of study.
Ex vivo analysis is completed by performing studies outside of an organism. Typically tissues are isolated and kept alive in an artificial environment. Advantages of their use include the fact that whole tissues are analyzed, not just cells as in in vivo studies, giving them the ability to more accurately correlate their results to in vitro models. Disadvantages include the viability of the lungs to be isolated outside of an organism. The lungs have one of the shortest viability times of all of the organs outside of the body. Due to the short viability time, many of the same disadvantages are present here that are seen in in vitro models, including a specialized delivery device, increased cost and lack of correlation among different study organisms.  

1.7 Objective and hypothesis

The separation of current aerosol testing methods from the evaluation of pharmacokinetic principles or pharmacodynamics effects is a limiting factor in the design and evaluation of potential aerosol formulations for the treatment of bacterial lung infections. Additionally, with the development of various resistance mechanisms in bacteria the need for antimicrobial therapy that are able to combat these, either through new antibiotics or new formulations exists. The objective of this Master’s thesis was to validate an in vitro model of pneumonia utilizing a modification to the Next Generation Impactor that incorporates aerosol testing methods with antimicrobial effects. The NGI was modified and validated in previous studies to incorporate a large volume impaction surface (LVLIS). Continued validation of incorporation of bacterial cultures into the LVLIS modified NGI was needed. Additionally, to build the robustness of this LVLIS modified NGI, clinically relevant strains that could be causative organisms for pneumonia needed
to be incorporated into the model. Therefore, based on the objective of the project, the following hypothesis was proposed:

“Incorporation of bacterial cultures in the Next Generation Impactor (NGI) will allow for evaluation of aerosolized antibacterial formulation efficacy as an in vitro model for bacterial pneumonia.”
Chapter 2: Incorporation of bacterial cultures into the Next Generation Impactor (NGI)
2 Incorporation of bacterial cultures into the Next Generation Impactor (NGI)

The Next Generation Impactor (NGI) was selected as the basis for the integration of antimicrobial efficacy methods with aerodynamic evaluation.

2.1 Modification to the NGI collection cup

To allow for the evaluation of antibiotic efficacy of inhaled drug products, the NGI collection cups were modified to be able to contain bacterial cultures. Adding bacterial cultures to the current NGI collection cups would alter the deposition and aerodynamics of the inhaled drug product by altering the critical impaction distance (Figure 1.5). Therefore, new collection surfaces were created with a deeper cup that allowed for added liquid bacterial culture while maintaining the critical impaction distance on the surface of the liquid, Figure 2.1.

![Figure 2.1. Diagram of LVLIS within NGI with bacterial culture](image)

(B=critical impaction distance)

This modification was designated ‘large volume liquid impaction surfaces, or LVLIS.’ Custom LVLIS were machined from block aluminum from Benson Machine Works, Inc. (Omaha, NE) (Appendix A). The original NGI plate has a depth of 16.63 mm and the new LVLIS has a depth of 24.57 mm; to maintain the critical impaction distance, 42 milliliters of liquid needed to be added to the LVLIS. Preliminary studies were performed...
with theophylline solutions, a model aerosolized drug, and showed that there was no statistical significance in the fine particle fraction (FPF), geometric standard deviation (GSD), and mean median aerodynamic diameter (MMAD). Evaporative loss studies were also performed to determine if airflow affected the height of the liquid in the LVLIS, thereby potentially decreasing the critical impaction distance and affecting deposition and aerodynamic properties. These studies showed that there was a height (evaporative loss) difference of 0.389 mm between the control and sample cups with water and a height difference of 0.178 mm with Mueller-Hinton Broth. The USP specifies that the difference in critical impaction distance should not be more than 0.1 mm. The difference was larger than that of the USP specification, but the specification is specifically for collection cups with solid surfaces. Despite this discrepancy in height, there were no statistically significant differences in observed particle deposition patterns in the LVLIS modified NGI.

2.2 Evaluation of bacterial cell viability in the LVLIS within the NGI

Prior to evaluation of aerosolized antibiotic efficacy, it was important to evaluate for changes in baseline bacterial growth based on the LVLIS modification. Specifically, any change in bacterial growth in cultures incorporated within the NGI needed to be confirmed to be directly attributed to the antibiotic alone. Therefore the need to evaluate these cultures incorporated in the NGI and exposed to airflow compared to cultures that were not incorporated was necessary.

For this experiment, a laboratory strain of *Escherichia coli* was chosen, strain K12. The minimum inhibitory concentration (MIC) for *E. coli* K12 and ceftazidime is 0.06, and is
susceptible to almost all antibiotics. Due to its low MIC, the evaluation of antibiotic
efficacy will be inadequate in the modified NGI, but it is a great strain to evaluate for
proof of concept.

2.2.1 Materials and methods

Mueller-Hinton broth and agar (MHB and MHA, respectively) were obtained from Fisher
Scientific (Pittsburg, PA, USA). Sterile bacterial petri plates were obtained from Fisher
Scientific (Pittsburg, PA, USA). E. coli K12 was provided by Dr. Nancy Hanson’s
laboratory. The Next Generation Impactor was obtained from MSP Corporation
(Shoreview, MN, USA). Calibration of airflow in the NGI was carried out using the TSI
4000 series airflow meter, obtained from TSI (Shoreview, MN, USA). LVLIS that held
the bacterial cultures were custom made from Benson Machine Works, Inc. (Omaha, NE,
USA). Disposable flint glass test tubes, O.D. x L: 16 x 100 mm, were obtained from
Fisher Scientific (Pittsburg, PA, USA). Sterile disposable serological pipets were
obtained from Fisher Scientific (Pittsburg, PA, USA). Propylene test tube disposable
closures (Kim-Kap) with an O.D. of 16 mm were obtained from Fisher Scientific
(Pittsburg, PA, USA).

MHB was prepared and heated in an Erlenmeyer flask by adding 22 grams of MHB
powder to one liter of distilled water, allowing the solution to come to a boil. The desired
amount was pipetted into side arm Erlenmeyer flasks and then sterilized by autoclaving at
121ºC for 20 minutes. MHA was prepared and heated in an Erlenmeyer flask by adding
38 grams of MHA powder to one liter of distilled water, allowing the solution to come to
a boil. Ten milliliters of MHA were added to each glass test tube, capped, and then
autoclaved at 121°C for 20 minutes. Saline tubes were prepared by adding 9 grams of sodium chloride, obtained from Fisher Scientific (Pittsburg, PA, USA), to one liter of distilled water, for a final concentration of 0.9%. Nine milliliters of the saline solution was pipetted into each test tube, capped, and autoclaved. The NGI collection cups and LVLIS were wrapped in aluminum foil and sterilized via the autoclave at 121° for 20 minutes. All other parts of the NGI that were not autoclavable were sterilized using 70% ethanol. The NGI was assembled according to the USP. An external filter was attached to the NGI to catch fine particles that did not get impacted on the MOC. Airflow throughout the NGI was calibrated to 15 L/min using the TSI 4000 series airflow meter. This meter was attached to the induction port via an airtight silicone elastomer fitting.

Frozen stock of *E. coli* K12 was plated onto a blood agar plate (Tryptase soy agar (TSA) with 5% sheep blood, Fisher Scientific, Pittsburg, PA, USA) using the streak plate method, inverted, and incubated for 24 hours before use. The bacterial colonies were added to the MHB in the sidearm flask stepwise, until the optical density of the suspension was around 0.132, ensuring a known bacterial cell inoculum.

Forty-two milliliters of inoculated MHB was added to stage 4 in the NGI, which had the normal collection cup replaced with a LVLIS. The NGI was operated for 10 minutes. At the end of the NGI run, a sample of one milliliter was pipetted out of stage 4 and into a saline tube. Serial dilutions were performed until a dilution of $10^{-9}$ was obtained. One milliliter from each saline dilution tube was pipetted out onto a petri plate, using the pour plate method. This process was done for two petri plate dilutions, so a total of two milliliters from each saline tube was used. A MHA tube was poured into each petri plate, swirled, and allowed to solidify. The plates were inverted and incubated for 24 hours. For
the control, a sample of *E. coli* suspension was used that was not exposed to the conditions of the NGI. Again, the same dilution and pour plate method were used. After incubation for 24 hours, the colonies on the plates were counted by hand using a colony counter.

### 2.2.2 Results and discussion

Each plate was removed from the incubator and the colonies were counted. The plates that contained fewer than 300 colonies and more than 30 colonies were used to determine bacteria count, to ensure accurate counts. Plates that had fewer than 30 colonies were denoted ‘too few to count (TFTC)’ and plates that had more than 300 colonies were denoted ‘too numerous to count (TNTC).’

The dilution $10^{-6}$ met the criteria for the number of colonies present to count for both the plate exposed to the NGI and not exposed to the NGI. The non-exposed plate had an average colony count of 242 and the NGI exposed plate had an average count of 292. The colony forming units per milliliter (CFU/ml) were calculated and the log was taken. The two plates had similar counts of viable bacteria, as seen in Table 2.1.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Non-exposed Plate LOG(CFU/ml)</th>
<th>NGI Exposed Plate LOG(CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-6}$</td>
<td>8.38</td>
<td>8.46</td>
</tr>
</tbody>
</table>

Table 2.1. Average colony forming units per milliliter (LOG(CFU/ml)) for exposed and non-exposed bacterial cultures
The difference in bacteria colonies was so small it was determined that there was no significant difference in bacterial count when comparing the exposed and non-exposed bacterial cultures.

2.2.3. Conclusion

Airflow to exposed bacterial cultures contained in the LVLIS in the NGI did not affect bacterial viability and there was not a significant reduction in bacterial count.

2.3 Characterization and analysis of ceftazidime solution in normal and LVLIS collection cups within the NGI

Preliminary studies characterized ceftazidime in the normal and modified NGI. Briefly, ceftazidime was quantified by nebulizing a known concentration of drug (10 mg/ml) through the NGI at 15 L/min for 10 min. The modified stage was stage 4 and forty-two milliliters of saline was placed into the LVLIS. Comparison of the deposition of ceftazidime in the normal and modified NGI did not show statistically different results. Therefore, the deposition was similar in both collection cups (Appendix B). The aerodynamic parameters that were obtained from both collection cups were also not statistically significant (Appendix B).

The actual amount of ceftazidime that was deposited on each stage was determined in the above experiments. It was determined that around 32 µg/ml of ceftazidime was deposited onto stage 4 when it contained normal saline as a liquid impaction surface.43
2.4 Nebulization of ceftazidime solution in LVLIS modified NGI containing *E. coli* K12

Preliminary experiments showed bacterial viability within the modified NGI, unaltered deposition and aerodynamic parameters, and consistent deposition of a known concentration of antibiotic within the modified NGI. To validate this model as an *in vitro* aerosol testing device, bacterial cultures needed to be incorporated into the LVLIS NGI with an antibiotic solution to show adequate bacterial culture killing via the antibiotic solution.

2.4.1 Materials and methods

Ceftazidime for Injection, containing one gram of ceftazidime was obtained from Sandoz Pharmaceuticals, Inc. (Princeton, NJ, USA). Sterile water for injection, obtained from Hospira, Inc. (Lake Forest, IL, USA) was used to reconstitute the antibiotic. MHB and MHA were obtained from Fisher Scientific (Pittsburg, PA, USA). Sterile bacterial petri plates were obtained from Fisher Scientific (Pittsburg, PA, USA). *E. coli* K12 was provided by Dr. Nancy Hanson’s laboratory. The Next Generation Impactor was obtained from MSP Corporation (Shoreview, MN, USA). Calibration of airflow in the NGI was carried out using the TSI 4000 series airflow meter, obtained from TSI (Shoreview, MN, USA). LVLIS that held the bacterial cultures were custom made from Benson Machine Works, Inc. (Omaha, NE, USA). Disposable flint glass test tubes, O.D. x L: 16 x 100 mm, were obtained from Fisher Scientific (Pittsburg, PA, USA). Sterile disposable serological pipets were obtained from Fisher Scientific (Pittsburg, PA, USA). Propylene test tube disposable closures (Kim-Kap) with an O.D. of 16 mm were obtained from
Fisher Scientific (Pittsburg, PA, USA). Aeroneb Pro nebulizer was obtained from Aerogen, Ltd. (Galway, Ireland) and was used for aerosolizing the antibiotic formulations through the NGI.

MHB, MHA and saline tubers were prepared as described in section 2.2.1. The NGI was assembled according to section 2.2.1 and the same airflow meter and procedure was used as in section 2.2.1.

A culture of *E. coli* K12 was used as the bacteria inoculum as in section 2.2.1. Ceftazidime solution was prepared by reconstituting the vial of ceftazidime powder for injection by adding ten milliliters of sterile water for injection. Ten milliliters of the reconstituted solution was added to a 100 milliliter volumetric flask and diluted with distilled water to a final concentration of 10 mg/ml.

Forty-two milliliters of inoculated MHB was added to stage 4 in the NGI, which had the normal collection cup replaced with a LVLIS. Five milliliters of ceftazidime solution was nebulized through the NGI for 10 minutes. At the end of the NGI run samples were collected, diluted, plated, and analyzed as stated in section 2.2.1.

### 2.4.2 Results and discussion

The MHA plates that corresponded to dilution $10^{-1}$ showed no bacterial growth, however all subsequent dilutions had bacteria growth. This can be explained by the fact that the antibiotic was diluted out during the saline dilutions along with the bacteria cells. The concentration of antibiotic in dilution $10^{-1}$ was higher than the MIC and therefore killed the bacteria. However, since the antibiotic was getting diluted out along with the bacteria in all of the subsequent saline dilutions, the concentration of ceftazidime present in the
other dilutions was less than the MIC for *E. coli* K12. Additionally, ceftazidime is a beta-lactam antibiotic and therefore its mechanism of action relies upon the amount of time the antibiotic is exposed to the bacteria, in addition to the concentration of the antibiotic. Therefore complete killing of *E. coli* K12 was not seen due to the fact that the antibiotic was only exposed to the culture for 15 minutes and the amount of ceftazidime present in all dilutions was not above the MIC.

**2.4.3 Conclusion**

While there was a reduction in bacteria count for dilution $10^{-1}$, there was not a complete inhibition of growth of all colonies. This was due to the fact that the antibiotic was diluted out along with the bacterial cultures and the bacterial cultures were not exposed to the antibiotic within the NGI long enough to show killing.

**2.5 Incorporation of an antibiotic binding resin into the LVLIS modified NGI**

Due to the limitations described in section 2.4, there was a need to extend the length of time the bacteria cultures were exposed to the antibiotic. Not only would extending the exposure period enhance the killing of the bacteria cultures, but it would add to the robustness of the model and allow it to more closely mimic an *in vitro* model. However, there was a need to be able to remove the antibiotic from the culture so that it was not diluted along with the bacteria cultures and to ensure that killing of the bacteria was only associated with the specified time of exposure. If the antibiotic was not removed from the system it would have remained in the cultures and become incorporated in the MHA plates which are incubated for 24 hours, hence allowing at least a 24 hour exposure to the
antibiotic which is not desired, not clinically relevant. Therefore to remove residual
antibiotic, Amberlite® (Sigma-Aldrich, St. Louis, MO, USA) beads were chosen as the
binding resin for the antibiotic. This resin has been used and shown to be effective. 27
Amberlite® beads XAD4 have a surface area of 725 m²/g and a pore diameter 40 Å.
These binding resins are a nonionic macroreticular resin that adsorbs and releases ionic
species through hydrophobic and polar interactions. 28

2.5.1. Materials and methods
The same method to incorporate E. coli K12 into the LVLIS modified NGI was used as
in section 2.4.1.
Two-tenths of a gram of Amberlite® beads were placed into individual test tubes,
capped, and autoclaved to ensure sterility.
Ceftazidime solution was prepared and utilized according to section 2.4.1.
Forty-two milliliters of inoculated MHB was added to stage 4 in the NGI, which had the
normal collection cup replaced with a LVLIS. Five milliliters of ceftazidime solution was
nebulized through the NGI for 10 minutes.
Three milliliters of the MHB/E. coli inoculum was pipetted into an Amberlite® bead
tube. The solution was mixed with the Amberlite® beads and was allowed to sit for 15
minutes. 27 Next, one milliliter from the Amberlite® bead tube was pipetted into dilution
10⁻¹ and serial dilutions were performed. Samples from each tube were plated, incubated,
and analyzed as stated in section 2.2.1.
A control solution was prepared by placing three milliliters from the MHB/E. coli
inoculum that had ceftazidime nebulized, however Amberlite® beads were not utilized.
before dilutions were performed. The same procedure was followed as above for diluting, plating, incubating, and analyzing the results.

2.5.2 Results and discussion

After completing the experiment in triplicate, the average CFU/ml for the bacterial inoculum utilizing Amberlite® beads and not utilizing Amberlite® beads is found in table 2.2.

Table 2.2. Average colony forming units per milliliter (LOG(CFU/ml))
for utilizing Amberlite® beads and Control

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Amberlite® bead Exposure LOG(CFU/ml)</th>
<th>Not exposed to Amberlite® beads LOG(CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-6}$</td>
<td>8.04</td>
<td>5.52</td>
</tr>
</tbody>
</table>

There was a significant decrease in colonies in the control plate; a three log difference was observed. This is because the Amberlite® beads were not present to remove the antibiotic before the bacterial cultures were incubated for 24 hours. Therefore, Amberlite® beads were able to remove the antibiotic from the bacterial culture system and allow for measure of colonies present. The colony count portrayed exposure to the antibiotic only in the amount of time that it was exposed to the nebulized antibiotic.

2.5.3 Conclusion

Incorporating the Amberlite® bead binding resin into the bacterial cultures ensured that the count of bacterial colonies was only due to exposure of the culture to the antibiotic for the designated amount of time that it was nebulized into the NGI.
2.6. Conclusion

Previous studies showed that modification of the NGI collection cups to LVLIS did not significantly affect the deposition and aerodynamic parameters of the aerosolized drug formulation. Incorporation of bacterial cultures into the modified NGI did not show a loss in cell viability. Additionally, ceftazidime was characterized and the amount deposited on each stage was determined. Finally, incorporating in bacterial cultures as the liquid in the LVLIS collection cups showed killing of the bacteria with nebulization of an antibiotic solution, ceftazidime. However, dilution of the antibiotic with the bacterial cultures and inadequate exposure time did not show complete eradication of the bacterial culture, therefore the parameters of the experiment need to be adjusted to show adequate bacterial culture killing.
Chapter 3: Incorporating clinical strains of bacteria to build a pneumonia model
3 Incorporating clinical strains of bacteria to build a pneumonia model

In chapter two, it was shown that a model incorporating bacterial cultures into the LVLIS could be created and validated as an *in vitro* model of the lung. Now that preliminary studies have demonstrated the feasibility of the model, it is important to show that bacteria that are commonly associated with pneumonia can be incorporated and continue to show adequate killing by nebulizing an antibiotic solution. The robustness of the LVLIS modified NGI as a pneumonia model is dependent upon how adequately the antibiotic formulation kills specific bacterial cultures. Time kill studies were completed with the various bacterial strains. These studies allowed the bacterial cultures to be exposed to the antibiotic for a specified amount of time, which was two-fold in importance. First, the antibiotic chosen was a beta-lactam cephalosporin antibiotic that exhibits time dependent killing. Second, exposure to the antibiotic over time would allow trends to be seen with the amount of killing once exposed to the antibiotic, mimicking typical microbiological methods to test the efficacy of a particular antibiotic with a given strain of bacteria.

3.1 Characterization and choice of an antibiotic solution

Ceftazidime was chosen because it is currently utilized in the treatment of pneumonia, and it has anti-pseudomonal activity. However, in the last few years there has been a drop in its use due to the development of resistance to it in various organisms, but especially *Pseudomonas*. It is also hypothesized that ceftazidime is among a few of the cephalosporins that is more likely to select for mutants with resistance mechanisms,
especially in *E. coli* and *Klebsiella* organisms, and therefore makes it an ideal antibiotic to study for antibiotic resistance.\(^{11}\)

Ceftazidime was also chosen due to a variety of aerosolization studies that have been published and were performed at other institutions.\(^{30,31}\) Additionally, the properties of ceftazidime were conducive to the experiments that were to be performed. Ceftazidime is a third generation cephalosporin in the beta-lactam class of antibiotics. Therefore it has a broad spectrum of activity against both gram negative and gram positive organisms. It has a distinct ultraviolet absorbance that allows for straightforward detection. Once reconstituted, the injectable solution is stable at room temperature for 24 hours and stable seven days if refrigerated, allowing for ease of reconstitution and storage. Finally, ceftazidime was used in preliminary studies to evaluate the deposition patterns and aerodynamic parameters of the nebulized solution in the LVLIS modified NGI.

### 3.2 Determining the MIC for bacterial cultures

E tests were used to determine the ceftazidime MIC for the bacterial cultures that were used in the following experiments. E tests were purchased from bioMerieux (Durham, NC, USA). MHB was inoculated to a MacFarland standard with the desired bacteria strain. A bacterial lawn was then created from the inoculated MHB onto MHA 20 plates (sterile petri plates that contain 20 milliliters of solidified MHA). Once the lawn was formed, an E test was placed in the middle of each petri plate. The plates were inverted and incubated for 18 hours. After 18 hours the plates were read and the MIC of the organism was determined. The MIC is determined by the outer ring of the circle/oval that is formed around the E test. The number on the E test strip that lines up with the outer
ring of the circle of no growth on the petri plate is the MIC for that particular organism, as seen in Figure 3.1.

![Figure 3.1. Photograph of an E test after 18 hours of incubation](image)

E tests are specific for each class of antibiotics and should be completed for each specific organism. The MIC value that is determined via the E test is accurate up to one dilution fold difference; for example, in figure 3.1, the E test reads an MIC of 0.125, the true MIC is in the range of 0.94 to 0.19 µg/ml.

### 3.3 Incorporation of various strains of *E. coli*

Various *E. coli* strains were chosen to be incorporated into the LVLIS modified NGI. As explained above, *E. coli* can be the causative organism for hospital-acquired pneumonia infections in patients, especially when specific resistance mechanisms are present. Table 3.1 contains the *E. coli* strains that were evaluated, along with their corresponding MICs.
Table 3.1 *E. coli* strains incorporated and tested in the LVLIS modified NGI

*Not a clinical strain

<table>
<thead>
<tr>
<th>Type of Strain</th>
<th>Strain</th>
<th>MIC in ceftazidime (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory</td>
<td>K12</td>
<td>0.06</td>
</tr>
<tr>
<td>Clinically relevant, susceptible</td>
<td>ATCC 25922</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>B/r*</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>CUMC 243 CMY-2</td>
<td>0.125</td>
</tr>
<tr>
<td>Clinically relevant, not susceptible</td>
<td>261 DHA (+)</td>
<td>24</td>
</tr>
<tr>
<td>Clinically relevant, ESBL producer</td>
<td>Misc. 238 TEM-10</td>
<td>32</td>
</tr>
</tbody>
</table>

Among the strains evaluated was a laboratory strain, K12, it was chosen based on the low MIC, which would ensure adequate killing of the organism if it was exposed to the amount of antibiotic that is thought to deposit onto stage 4 of the NGI. Additionally, this particular strain does not have any known resistance mechanisms and has been studied thoroughly, allowing it to be an ideal prototype strain. Next, strains that have been obtained from the clinical environment but susceptible to ceftazidime were evaluated. In addition, strains with higher MICs that were obtained from the clinical setting were evaluated. Finally, one ESBL producing strain was evaluated in the model. TEM-10 is the ESBL enzyme present on this strain. This particular strain was chosen because it produced an ESBL which inactivates third generation cephalosporins, such as ceftazidime.
3.3.1. Materials and methods

MHB, MHA, normal saline tubes, and Amberlite® bead tubes were prepared according to section 2.2.1 and 2.5.1, respectively. MHA was inoculated with one of the *E. coli* strains to an optical density of 0.132, as described in section 2.2.1. The NGI was set up and run according to section 2.2.1, with stage 4 being the modified stage with incorporation of the LVLIS collection cup. Ceftazidime was prepared according to section 2.4.1. Five milliliters of ceftazidime was nebulized over ten minutes using the Aerogen nebulizer into the LVLIS modified NGI. After ten minutes had expired, the first sample was collected and this was denoted as time zero. The sample was then placed into an Amberlite® bead tube and the procedure in section 2.5.1 was followed. The sample was diluted, plated, and incubated for 24 hours. Once the sample from the LVLIS cup had been taken, the NGI was closed and the entire machine was placed into a large incubator that was set to 37°C. The next sample was taken two hours after the first, at time point equal to two. The same procedure was used here as was used for the sample collected at time zero. Additionally, samples were taken at time points 4, 6, and 8 hours, with the NGI being placed back into the large incubator after all samples were taken. After twenty-four hours of incubation, the MHA plates were removed and the colonies were counted. Plate counts were obtained and a growth curve was determined correlating LOG(CFU/ml) versus time.
3.3.2. Results and discussion

3.3.2.1 Results and discussion for laboratory strain, *E. coli* K12

Overall there was a significant and almost immediate reduction in colony counts of the *E. coli* K12 strain. Figure 3.2 shows the time kill curve with nebulized ceftazidime and *E. coli* K12. The data represent an average of three independent experiments.

![Figure 3.2](image)

*Figure 3.2. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *E. coli* K12 in LVLIS modified NGI (n=3), with standard error bars*

Complete eradication of the organism was seen when exposed to five milliliters of nebulized ceftazidime. There was a four log killing after two hours and between a 5 and 8 log killing between hours four and six, which is thought to be the optimal amount of exposure time of ceftazidime.
3.3.2.2. Results and discussion for clinically susceptible strains, *E. coli* ATCC 25922, B/r*, and CUMC 243 CMY-2

Overall there was a significant and almost immediate reduction in colony counts of the susceptible strains, however, B/r showed a more significant reduction. B/r is not a clinical isolate, but it is susceptible based on its low MIC. Figure 3.3 shows the time kill curve with nebulized ceftazidime and both *E. coli* ATCC 25922, B/r*, and CUMC 243 CMY-2. The data represent an average of three independent experiments.

![Figure 3.3](image)

Figure 3.3. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and clinically susceptible *E. coli* in LVLIS modified NGI (n=3), with standard error bars

For ATCC 25922, time zero had a colony count of around 7.51 log (CFU/ml), which was decreased to 5.61 log (CFU/ml) by hour four, and decreased even more to 4.83 by hour eight. Overall not as much killing was seen in this strain when compared to the laboratory strain of K12, however over a two log killing was seen.

For B/r time zero had a colony count of around 7.84 (Log (CFU/ml)), which decreased to almost half by hour four. By hour eight the average colony count was 2.41 log (CFU/ml).
Overall a large reduction in colony count was seen over the eight hour time frame. One of the runs for the experiment showed faster killing after four hours, which is denoted by the larger error bar at hour 6.

For strain CUMC 243 CMY-2, time zero had an average colony count of around 8.08 log (CFU/ml), which was decreased to around 5.81 log (CFU/ml) by hour eight. Overall, there was a slight reduction in growth, of just over 2 logs of killing. Overall, the susceptible strains showed more than two logs of killing over the course of eight hours. It was predicted that due to the low MIC of these strains, each strain would show adequate killing. Incorporating these strains into the model showed that clinical isolates could be killed utilizing nebulized ceftazidime.

3.3.2.3. Results and discussion for clinically non-susceptible strains, *E. coli* 261 DHA(+)

Overall there was not a significant reduction in colony counts of *E. coli* 261 DHA(+).

Figure 3.4 shows the time kill curve with nebulized ceftazidime and a non-susceptible *E. coli* strain. The data represent an average of three independent experiments.
For strain 261 DHA(+), time zero had an average colony count of around 7.69 (log (CFU/ml)), which was decreased to only 6.99 (log (CFU/ml)) by hour eight. Overall, even though there was a slight reduction in growth, there was less than a one log difference. Due to the higher MIC and the fact that about 32 µg/ml of ceftazidime is deposited onto the modified stage, noticeable and adequate killing was not seen in this organism.

3.3.2.4. Results and discussion for clinically relevant ESBL producing, Misc. 238 TEM-10

Overall there was not a significant reduction in colony counts of the E. coli Misc. 238 TEM-10 strain. Figure 3.5 shows the time kill curve with nebulized ceftazidime and E. coli Misc. 238 TEM-10. The data represent an average of three independent experiments.
Figure 3.5. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *E. coli* Misc. 238 TEM-10 in LVLIS modified NGI (n=3), with standard error bars

Time zero had an average colony count of around 7.99 (log (CFU/ml)), which was decreased to only 7.73 by hour eight. There was a slight increase in colony count at hour two to 8.08 (log(CFU/ml)). This could be due to the fact that cephalosporins exhibit time dependent killing and perhaps the antibiotic was not present long enough in the system for inhibition of killing to be seen. Overall, even though there was a slight reduction in growth, there was less than a one log difference. Due to the higher MIC with the production of an ESBL, adequate killing was not seen for this organism. However it is important to note that even though significant killing of the organism was not seen, there was not a growth of the organism either, which would be expected if the antibiotic was completely inactivated. Therefore, nebulizing the antibiotic did not necessarily kill the bacteria, but it did inhibit the growth of the organism. This could be due a mechanism called the inoculum effect. The inoculum is the dependency of the susceptibility of a bacteria to an antibiotic upon the inoculum size, or the number of bacteria present. The
data show there was not much of a death or growth of the organism and it is likely due to the inoculum effect, rather than the antibiotic exhibiting bacteriostatic characteristics, rather than bacteriocidal, which is the case for beta-lactam antibiotics. The number of bacteria present in the culture, with the given resistance mechanisms, are too much for the amount of antibiotic present and are therefore overwhelming the antibiotic. The inoculum effect can occur readily with bacteria from the Enterobacteriaceae family.  

**3.3.3. Conclusion**

Both the laboratory and clinically susceptible strains of *E. coli* showed a reduction in bacterial counts over the course of eight hours. Each of the strains exhibited various time-kill curves, with some showing more adequate killing than others, with the susceptible strains showing the largest log difference. As predicted, the non-susceptible strains were not susceptible to ceftazidime. While there was not adequate killing, there was still not much re-growth in the organisms, which denotes that the antibiotic may not have been at a concentration adequate enough to eradicate the organism, but it was still present at a high enough concentration to inhibit growth. Finally, the ESBL producing strain showed a slight reduction in colony count and then a re-growth over the course of eight hours. These time-kill curves suggest that it is possible to correlate the susceptibility and MICs of various strains of *E. coli* to how adequately they will die when they are exposed to a nebulized antibiotic. Put simply, the MICs which typically relate to *in vitro* concentrations can be correlated to nebulized concentrations of antibiotic as well.
3.4. Incorporation of various strains of *Klebsiella pneumoniae*

Various *Klebsiella pneumoniae* strains were chosen to be incorporated into the LVLIS modified NGI. As explained above, *K. pneumoniae* is a common pathogen in HAP pneumonia, additionally it has been shown to carry resistance mechanisms that allow the bacteria to be resistant to all beta lactam antibiotics. To continue to build the robustness of the LVLIS modified NGI pneumonia model, several different clinical *K. pneumoniae* strains were incorporated into the model. Table 3.2 contains all of the *Klebsiella* strains that were incorporated, along with ceftazidime MICs.

Table 3.2 *Klebsiella* strains incorporated and tested in the LVLIS modified NGI

<table>
<thead>
<tr>
<th>Type of Strain</th>
<th>Strain</th>
<th>MIC in ceftazidime (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory*</td>
<td>KP 23</td>
<td>0.06</td>
</tr>
<tr>
<td>Clinically relevant, susceptible</td>
<td>Cu 111</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Cu 129</td>
<td>0.5</td>
</tr>
<tr>
<td>Clinically relevant, not susceptible</td>
<td>249 CMY-2-like</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>225 ACT-1</td>
<td>128</td>
</tr>
<tr>
<td>Clinically relevant, ESBL producer</td>
<td>Misc. 233 TEM-5</td>
<td>32</td>
</tr>
</tbody>
</table>

*This is not a laboratory strain of *Klebsiella*, but is the closest strain available to a lab strain.

Among the strains that were chosen to be incorporated was a ‘laboratory strain’, KP23. This strain is the closest *Klebsiella* strain available that can mimic a laboratory strain. It was obtained in 1973 before third generation cephalosporin and later antibiotics were introduced, therefore it does not have any of the resistance mechanisms that had developed from the use of these antibiotics. Next, strains that have been obtained from
the clinical environment that were known to be susceptible to ceftazidime were evaluated. Then strains with higher MICs that were obtained from the clinical setting that were known to be non-susceptible to ceftazidime were evaluated. Finally, one ESBL, TEM-5, producing strain was evaluated in the model. This particular strain was chosen because ceftazidime should not be active against this strain due to the ESBL enzyme.

3.4.1. Materials and Methods
The same materials and methods were used in the following studies that were utilized in section 3.3.1., except the bacteria cultures were replaced with one of the Klebsiella pneumoniae.

3.4.2. Results and discussion
3.4.2.1. Results and discussion for ‘laboratory strain’, Klebsiella pneumoniae KP23
Overall there was a significant reduction in colony counts of the K. pneumoniae KP23 strain. Figure 3.6 shows the time kill curve with nebulized ceftazidime and K. pneumoniae KP23. The data represent an average of three independent experiments.
Figure 3.6. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *K. pneumoniae* KP23 in LVLIS modified NGI (n=3), with standard error bars

There was a two log killing after two hours and between around a 4-fold log killing after eight hours of exposure to the antibiotic. Even though more killing was seen in the laboratory strain of *E. coli*, when compared with this strain of *Klebsiella*, the killing is significant and the strain is more clinically relevant.

3.4.2.2. Results and discussion for clinically susceptible strains, *Klebsiella pneumoniae* Cu 111 and Cu 129

Overall there was a reduction in colony counts of both clinically susceptible strains. Figure 3.7 shows the time kill curve with nebulized ceftazidime and both *Klebsiella pneumoniae* Cu 111 and 129. The data represent an average of three independent experiments.
Figure 3.7. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and clinically susceptible 
*Klebsiella* in LVLIS modified NGI (n=3), with standard error bars

Time zero had a colony count of around 7.73 (log (CFU/ml)), which was decreased to 
6.15 (log(CFU/ml)) by hour eight. Overall not as much killing was seen in this strain
when compared to the ‘laboratory strain’ of KP23, however almost a two log killing was
seen.

Time zero had a colony count of around 7.73 (log (CFU/ml)), which decreased to nearly 
5.5 logs by four hours, and continued to drop to 3.13 logs by eight hours. Overall a large 
reduction in colony counts were seen over the eight hour time frame.

Comparing the two clinically relevant, susceptible strains, Cu 111 had a more 
pronounced colony reduction than the other strain, which could be correlated with its 
lower MIC.
3.4.2.3. Results and discussion for clinically non-susceptible strains, *Klebsiella pneumoniae* 249 CMY-2-like and 225 ACT-1

Overall there was not a significant reduction in colony counts of both the *Klebsiella* 249 CMY-2-like and 225 ACT-1 strains. Figure 3.8 shows the time kill curve with nebulized ceftazidime and non-susceptible *Klebsiella* strains. The data represent an average of three independent experiments.

![Figure 3.8](image_url)

Figure 3.8. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and non-susceptible *Klebsiella* in LVLIS modified NGI (n=3), with standard error bars

For the 249 CMY-2-like strain, time zero had an average colony count of around 7.94 (log (CFU/ml)), which was decreased to 6.97 (log(CFU/ml)) by eight hours. Overall, only less than or equal to a log difference was observed over the eight hour time course. Due to the higher MIC and the fact that about 32 µg/ml of ceftazidime is deposited onto the modified stage, noticeable and adequate killing was not seen in this organism.

For the 225 ACT-1 strain, time zero had an average colony count of around 8.07 (Log (CFU/ml), which was decreased to around 7.41 (log(CFU/ml)) by four hours. Overall,
there was a slight reduction in growth. The time kill study showed that even with an adequate exposure time, there was a resurgence in growth of the organism. It is known that this strain has five different beta-lactamases, including an AmpC beta-lactamase, which attributes to the inability of ceftazidime to adequately kill it.\textsuperscript{32}

3.4.2.4. Results and discussion of a clinically non-susceptible, ESBL producing strain of \textit{K. pneumoniae}, Misc. 233 TEM-5

Overall there was not a significant reduction in colony counts of the \textit{K. pneumoniae}, Misc. 233 TEM-5 strain. Figure 3.9 shows the time kill curve with nebulized ceftazidime and \textit{K. pneumoniae}, Misc. 233 TEM-5. The data represent an average of three independent experiments.

![Time Kill Curve](image.png)

\textbf{Figure 3.9.} Average LOG (CFU/ml) vs. time of nebulized ceftazidime and \textit{K. pneumoniae}, Misc. 233 TEM-5 in LVLIS modified NGI (n=3), with standard error bars
Time zero had an average colony count of around 7.86 (Log (CFU/ml)), which had increased to 8.11 (log(CFU/ml)) by eight hours. Interestingly, there was a slight increase in colony count at hour two to 8.09 with a slight killing at hour four and then a resurgence in growth afterwards, which was also seen in the E. coli ESBL-producing strain. Overall, even though there was a slight reduction in growth before the increase in growth was seen, there was less than a one log difference. Due to the higher MIC and the fact that this organism produced an ESBL, adequate killing was not seen in this organism. However it is important to note that even though significant killing of the organism was not seen, there was not a significant growth of the organism either, which would be expected if the antibiotic was completely inactivated. Therefore, nebulizing the antibiotic did not necessarily kill the bacteria, but it did inhibit the growth of the organism. Again, the inoculum effect is probably occurring here. To determine if this is what is occurring, decreasing the initial inoculum and performing the same procedure should allow for a more accurate time-kill curve to be determined. By changing the inoculum, the effect of the antibiotic by either inhibiting growth or allowing resurgence should be seen.

3.4.3 Conclusion

Quite similar to the E. coli strains, both the laboratory and clinically susceptible strains of Klebsiella showed a reduction in bacterial counts over the course of eight hours. Each of the strains exhibited various time-kill curves, with some showing more adequate killing than others. However, when compared to the clinically non-susceptible strains, the susceptible strains showed much more killing. As predicted, the non-susceptible strains were not susceptible to ceftazidime. While there was not adequate killing, there was still
not much of a re-growth in the organisms, which denotes that the antibiotic may not have been at a concentration adequate enough to eradicate the organism, but it was still present at a high enough concentration to inhibit growth. Finally, the ESBL producing strain showed a slight reduction in colony count and then a re-growth over the course of eight hours. These time-kill curves suggest that it is possible to correlate the susceptibility and MICs of various strains of *Klebsiella* to how adequately they will die when they are exposed to a nebulized antibiotic. Put simply, the MICs which typically relate to *in vitro* concentrations can correlate to nebulized concentrations of antibiotic as well.\textsuperscript{37,38}

3.5 Incorporation of *Pseudomonas aeruginosa* cultures into the LVLIS modified NGI

*Pseudomonas aeruginosa* was chosen to be incorporated into the LVLIS modified NGI. As explained above, *P. aeruginosa* is a common pathogen in HAP pneumonia and it has been shown to carry numerous resistance mechanisms. Additionally, this bacteria requires careful selection of antimicrobial therapy to ensure that the organism will be killed and to avoid development of resistance. To continue to build the robustness of the LVLIS modified NGI pneumonia model a laboratory strain of *P. aeruginosa* was first explored.

3.5.1 Incorporation of a ‘laboratory strain’ of *P. aeruginosa*, PAO-1, utilizing a time kill study

The next strain chosen to be incorporated in the LVLIS modified NGI was *P. aeruginosa*, PAO-1. This particular strain has ceftazidime MIC of 1 µg/ml, which is considered
susceptible. This organism was chosen based on the fact that it was a laboratory strain with a low MIC and is known to not have any resistance genes. It does however have mechanisms that were discussed previously and include the ability to produce a biofilm and a lipopolysaccharide outer coat.

### 3.5.1. Materials and Methods

The same materials and methods were used as in section 3.3.1., except the bacteria culture was replaced with *P. aeruginosa* PAO-1.

### 3.5.1.2. Results and discussion

Overall there was not a significant reduction in colony counts of the *P. aeruginosa* PAO-1 strain. Figure 3.10 shows the time kill curve with nebulized ceftazidime and *P. aeruginosa* PAO-1 in triplicate.

![Figure 3.10. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and *P. aeruginosa* PAO-1 in LVLIS modified NGI (n=3), with standard error bars](image_url)
Time zero had an average colony count of around 8.26 (Log (CFU/ml)), which was decreased to only 7.71 (log(CFU/ml)) by eight hours. Overall, even though there was a slight reduction in growth, there was less than a one log difference. This was unexpected due to the fact that the MIC of PAO-1 is 1 μg/ml and the amount of ceftazidime getting deposited onto the culture is on average, 32 μg/ml. It is important to note that even though significant killing of the organism was not seen, there was not a growth of the organism either, which would be expected if the antibiotic was completely inactivated. Therefore, nebulizing the antibiotic did not necessarily kill the bacteria, but it did inhibit the growth of the organism. Due to the fact that these results were replicated three times, the reason for insignificant killing of *P. aeruginosa*, when there should have been significant killing, needed to be investigated further.

3.5.2. Aerosolized versus instilled deposition of ceftazidime to *P. aeruginosa* PAO-1

To attempt to determine the flaws in the above experiment a series of experiments were conducted. The first of these experiments was designed to determine if nebulization of the antibiotic into the NGI affected growth of the organism.

3.5.2.1. Materials and methods

MHA, MHB, saline tubes, and Amberlite® bead tubes were all prepared according to section 2.2.1 and 2.5.1. MHB was inoculated with *P. aeruginosa* PAO-1. The NGI was set up and assembled according to section 2.2.1. Forty-two milliliters of the MHB/PAO-1 inoculum was pipetted into modified stage 4 within the NGI. Five milliliters of ceftazidime solution was nebulized into the NGI according to section 2.5.1. Samples
were taken, diluted, plated, incubated, and analyzed according to section 2.2.1. For a control, forty-two milliliters of the MHB/PAO-1 inoculum was pipetted into a LVLIS that was allowed to sit on the countertop (it was not contained within the NGI). One milliliter of ceftazidime (concentration 0.1 mg/ml) was deposited into the modified cup to mimic the amount of ceftazidime that typically gets deposited by the nebulizer. The solution was mixed and allowed to sit for 10 minutes, which is the same amount of time the antibiotic solution is nebulized throughout the NGI. After this, samples were collected, diluted, plated, and analyzed according to the same methods as described above.

**3.5.2.2. Results and discussion**

The known amount of ceftazidime that was deposited into the countertop LVLIS exhibited the same non-killing pattern as the *P. aeruginosa* culture that was placed inside the NGI. Figure 3.11 shows the time kill curve for both the nebulized and controlled delivery of ceftazidime to the *P. aeruginosa* culture.
Figure 3.11. Comparison of nebulized delivery and instilled delivery of the same amount of ceftazidime onto a *P. aeruginosa* PAO-1 culture in the LVLIS

According to figure 3.11, both the nebulized and instilled samples exhibited similar time kill patterns. The difference in the two lines is from the initial inoculum, where the nebulized inoculum was slightly higher throughout the experiment. According to the above data, the amount of ceftazidime getting deposited onto the PAO-1 culture in the LVLIS is similar to what is known and therefore something else is affecting the killing that is not occurring with PAO-1.

### 3.5.2.3. Conclusion

The amount of drug that was getting deposited from the nebulizer is similar to the amount that was deposited in a known, controlled manner. The concentration is not different in either delivery and therefore something else is affecting the killing of the organism.
3.5.3. Time kill study with PAO-1 suspended in normal saline

The studies that determined the amount of ceftazidime that was getting deposited on various stages within the modified LVLIS NGI were completed in normal saline, rather than in MHB, which is what was utilized in the previous experiments. Despite the previous studies that showed adequate killing in both *E. coli* and *Klebsiella* it was necessary to ensure that there was no interaction occurring with PAO-1, the MHB, and nebulized antibiotic. It was hypothesized that there was a potential interaction, due to the fact that MHB has a higher viscosity than saline. The quantification of ceftazidime was initially done in normal saline, rather than in MHB, due to the fact that absorbance was correlated with concentration to determine deposition and the presence of added particles, such as those contained in MHB, would skew the results. Therefore, it was necessary to determine if the presence of MHB was affecting the deposition of ceftazidime and therefore the killing of PAO-1.

3.5.3.1. Materials and Methods

The same materials and methods were used as in section 3.3.1.1., except the bacteria culture was replaced with *P. aeruginosa* PAO-1. Additionally, instead of suspending PAO-1 in MHB, it was inoculated into normal saline with a starting inoculum optical density of 0.5. The NGI was set up and the ceftazidime solution was nebulized as described in section 2.5.1.
3.5.3.2. Results and discussion

Overall there was a significant reduction in colony counts and complete eradication of the \textit{P. aeruginosa} PAO-1 strain after four hours. Figure 3.12 shows the time kill curve with nebulized ceftazidime and \textit{P. aeruginosa} PAO-1, suspended in normal saline. The data represent an average of three independent experiments.

![Figure 3.12. Average LOG (CFU/ml) vs. time of nebulized ceftazidime and \textit{P. aeruginosa} PAO-1 in LVLIS modified NGI, suspended in normal saline (n=3), with standard error bars](image)

Due to the fact that \textit{P. aeruginosa} does not grow as well in normal saline as it does in MHB, the starting inoculum was lower, around $3.04 \times 10^3$. After two hours the PAO-1 colonies were half that of the original inoculum and after four hours there was complete eradication of the organism, denoting significant killing. Due to these results, it is hypothesized some mechanism occurs with the interaction of \textit{P. aeruginosa}, ceftazidime, and MHB. It is noted that in the previous experiment utilizing MHB instead of normal saline as a growth medium, a biofilm formed quite readily, more so than any of the other
strains previously tested. Figure 3.13 shows a photograph of the biofilm of *P. aeruginosa* PAO-1 in the LVLIS.

![Figure 3.13. Photograph of *P. aeruginosa* PAO-1 in modified LVLIS collection cup after nebulization of ceftazidime into MHB/PAO-1 inoculum; production of biofilm](image)

Production of a biofilm, while not unique to *P. aeruginosa*, formed at a much higher rate than the other organisms. As described in the introduction section, production of a biofilm can inhibit antibiotics from penetrating an organism. Presence of this biofilm is only a potential explanation as to why adequate killing was not seen when it should have been observed, however other explanations need to be explored further.

### 3.5.3.3. Conclusion

Nebulizing ceftazidime through the modified LVLIS NGI utilizing an inoculum containing bacteria and normal saline, rather than MHB, showed eradication of the organism as it should have done in the MHB as well due to the fact that the organism’s MIC was around one. Additionally, it was noted that no biofilm was produced when *P. aeruginosa* was grown in normal saline, rather than in MHB.
3.5.4. Time kill study of a decreased inoculum of PAO-1 with nebulized ceftazidime

To determine if any other factors were playing a role in the inadequate killing of PAO-1 a few of the experiment parameters were altered to determine if they played a role. First, a decreased bacteria inoculum was explored. It was thought that the organism was overwhelming the antibiotic and an initial decrease in cell density may show adequate killing, giving time for the antibiotic to work.

3.5.4.1. Materials and methods

The same materials and methods were used as in section 3.3.1.1., except the bacteria culture was replaced with *P. aeruginosa* PAO-1. Additionally, after the MHB was inoculated to an optical density of 0.132, the bacterial suspension was diluted with forty milliliters of sterile MHB to obtain a final inoculum of around $10^6$ cells, which is less than what is normally used, $10^8$.

3.5.4.1 Results and discussion

Decreasing the starting inoculum did not affect the killing of PAO-1. Figure 3.14 shows the time kill data of the inoculum that is normally used ($10^8$) and the decreased inoculum ($10^6$).
Figure 3.14. Comparison of $10^6$ and $10^8$ PAO-1 inoculum of the same amount of ceftazidime onto a *P. aeruginosa* PAO-1 culture in MHB in the LVLIS

Even though the starting bacterial cell counts are different, both inoculums follow the same pattern of time kill curves. This shows that starting inoculum of PAO-1 does not affect the lack of killing observed with nebulized ceftazidime and PAO-1 in the modified LVLIS NGI.

### 3.5.4.3. Conclusion

Altering the starting inoculum of PAO-1 did not affect the time kill curve pattern. The same non-killing was observed with both starting inoculums and therefore starting cell density is not affecting killing.
3.5.5. Time kill study of an increased concentration of ceftazidime with PAO-1

The next study that was performed was to increase the concentration of ceftazidime to determine if this would affect the killing of PAO-1. PAO-1 did not exhibit killing as expected with an MIC of 1 µg/ml and exposure to ceftazidime with a concentration around 32 µg/ml. Therefore, to determine if the concentration of ceftazidime was affecting killing patterns the concentration of ceftazidime that was nebulized through the NGI was increased.

3.5.5.1. Materials and methods

The same materials and methods were used as in section 3.3.1.1., except the bacteria culture was replaced with *P. aeruginosa* PAO-1. Additionally, instead of nebulizing ceftazidime as described in section 2.5.1, the concentration was increased to 100 mg/ml. This increase in concentration was ten-fold higher than what was normally utilized.

3.5.5.2. Results and discussion

Increasing the concentration of ceftazidime that was nebulized throughout the NGI did not affect killing of PAO-1. Figure 3.15 shows the time kill data of the concentration that is normally used (10 mg/ml) and the increased concentration (100 mg/ml).
Figure 3.15. Comparison of 100 mg/ml and 10 mg/ml of ceftazidime solution that was nebulized onto a *P. aeruginosa* PAO-1 culture in the LVLIS

Increasing the concentration ten-fold did not affect the killing of PAO-1 and again, significant killing was not observed even though it was expected with an organism with a ceftazidime MIC of 1 µg/ml. This experiment reinforced the potential explanation of the formation of a biofilm as the reason behind non-killing. *P. aeruginosa*, even specifically PAO-1, has been shown to have increased MICs in the presence of a biofilm. Denoted as biofilm inhibitory concentration (BIC), MICs have shown to increase up to four fold from the presence of a biofilm alone.\textsuperscript{32} PAO-1 has shown to exhibit concentrations in the range of 128 µg/ml and up to 512 µg/ml if a biofilm is formed.\textsuperscript{33} Based on this information, increasing the concentration of ceftazidime ten-fold would still not deposit enough antibiotic onto the culture if PAO-1 has increased MICs in the presence of a biofilm.\textsuperscript{32,33,34}
3.5.5.3. Conclusion

Nebulization of an increased concentration of ceftazidime did not show significant killing of PAO-1. However, this data does reiterate early data that suggests that the formation of a biofilm could be affecting the time kill curve data of PAO-1 and help determine why significant killing is not being observed, even though it should be occurring.

3.5.6. Mimicking a ‘re-dosing’ of ceftazidime utilizing PAO-1

The last experiment to determine why significant killing in PAO-1 was not being observed was to determine if re-dosing the antibiotic after a set amount of time would exhibit significant killing. This experiment was performed to mimic the clinical setting when a patient receives multiple doses of antibiotic to eradicate an infection.

3.5.6.1. Materials and methods

The same materials and methods were used as in section 3.3.1.1., except the bacteria culture was replaced with *P. aeruginosa* PAO-1. Additionally, once the samples were taken after hour eight, a second run of the NGI occurred. Fifteen milliliters of sterile MHB were added back into the LVLIS collection cup at stage 4 to maintain a total of 42 milliliters of liquid when running the NGI, essentially maintaining the critical impaction distance. Next, the NGI was set up and ceftazidime was nebulized as stated in section 2.5.1. Again, samples were obtained at time points, 0, 2, 4, 6, and 8 hours, for a total incubation time of 16 hours for the NGI.
3.5.6.2. Results and discussion

Overall there was not a significant difference in bacteria growth at hour zero compared to hour sixteen, after the second dose of antibiotic. Figure 3.16 shows the time kill data of *P. aeruginosa* PAO-1 after being exposed to a second nebulized dose of ceftazidime after hour eight.

![Graph showing average LOG (CFU/ml) vs. time of nebulized ceftazidime and *P. aeruginosa* PAO-1 in LVLIS modified NGI, after a second dose of ceftazidime was administered after hour eight.](image)

Time zero had an average colony count of around 8.14 (log (CFU/ml)), which was decreased to 7.76 (log(CFU/ml)) by six hours, but after this time point the bacteria started to proliferate again. After exposure to a second dose of antibiotic, the average colony count was decreased down to 7.63 (log(CFU/ml)), which is a lower count than what was obtained in the first dose of antibiotic. However, by hour sixteen (hour eight of the re-dose) the count had increased back to 8.09 (log(CFU/ml)), which is very close to the starting inoculum concentration. A second dose of antibiotic was not enough to observe
significant killing of PAO-1 and the organism grew in the presence of antibiotic; significant killing was not observed. Based on the MIC data, this was an unexpected result.

3.5.6.3. Conclusion
Re-dosing ceftazidime after exposure to a bacteria culture for eight hours did not exhibit significantly killing in PAO-1.

3.6 Conclusion
Unlike other bacteria cultures, *E. coli* and *Klebsiella pneumoniae*, that were studied, *P. aeruginosa* did not show significant killing when expected to, based off of the MIC of the organism. *E. coli* and *Klebsiella pneumonia* exhibited killing of each strain with MICs below the amount of ceftazidime deposited in the NGI and no resistance mechanisms present. However, neither strain showed killing when non-susceptible organisms were exposed to nebulized ceftazidime within the NGI. Upon incorporation of *P. aeruginosa*, other factors were determined to affect the non-killing of the susceptible PAO-1 organism. One potential explanation for the lack of killing is the production of a biofilm by PAO-1 that inhibits ceftazidime from reaching the bacteria in adequate amounts for killing.
Chapter 4: Summary and Future Studies
4 Summary and future studies

4.1 Summary

With bacterial pneumonia remaining one of the leading causes of morbidity and mortality in the United States, the need for improved therapeutic options still exists, especially the development of new antibiotics or drug formulations. Targeted antibiotic therapy utilizing the pulmonary route is a potential option to treat pneumonias. An in vitro model was developed and discussed throughout this Master’s thesis. This model was able to address both the deposition and aerodynamic parameters of a nebulized antibiotic, along with antibiotic efficacy via time-kill studies, concurrently.

Incorporation of various clinically relevant bacteria cultures, to mimic a pneumonia model, were successfully integrated into the LVLIS modified NGI. Laboratory strains of both *E. coli* and *Klebsiella pneumoniae* were incorporated and adequate killing was demonstrated via time-kill data. To build the robustness of the model, clinically susceptible strains of both organisms were incorporated into the NGI and adequate killing was noted. Additionally, clinical strains with specific resistance mechanisms and high MICs were incorporated into the model. These strains did not exhibit killing, when studied via time-kill data, which was expected. These results suggest that the LVLIS modified NGI is a viable model for incorporation of aerodynamic testing parameters with analysis of antimicrobial efficacy using strains of *E. coli* and *Klebsiella pneumoniae*.

Upon incorporation of a laboratory *P. aeruginosa* strain, killing was not observed when it was expected. Upon further investigation, it was proposed that the production of a biofilm in this particular strain provided a resistance mechanism that did not allow for significant killing, even when it was expected, based upon the organisms MIC.
4.2 Future studies

*E. coli* and *Klebsiella pneumonia* were both successfully incorporated into the LVLIS modified NGI with validated susceptibility and resistance patterns using aerosolized ceftazidime solutions. Future studies that utilize these successful results include characterizing other antibiotics with different mechanisms of action, incorporating different classes of bacteria, including gram positive bacteria, and incorporating additional parameters to mimic the lung. To continue to build the robustness of the LVLIS modified NGI model and therefore its use to effectively model various drug formulations, the number of organism and antibiotics studied needs to increase. By expanding the number of drug products to include others classes of antibiotics will make the model will be more useful and allow for a better understanding of the correlation between deposition, aerodynamic parameters, and antibiotic efficacy. The parameters that were utilized in this pneumonia model do not adequately simulate a diseased lung. It is necessary to determine parameters that would more accurately mimic a lung with a pneumonia infection, including but not limited to: decreased airflow rate, liquid composition (simulated lung fluid, increased viscosity to mimic sputum), protein content, and pH.

The experiments that utilized *P. aeruginosa* did not show significant killing, but the mechanism as to why was not clearly defined. Future studies should focus on understanding the resistance mechanism(s) that elucidated resistance in this LVLIS modified NGI model. This could be approached by selecting a strain of *Pseudomonas* that does not produce a biofilm and has a low MIC. Additionally, the *Pseudomonas*
cultures that grew after exposure to nebulized ceftazidime should be analyzed to
determine if any inducible genes were upregulated when exposed to the antibiotic.
References


76
38. Lister PD. Pharmacodynamics of 750 mg and 500 mg doses of levofloxacin against ciprofloxacin-resistant strains of *Streptococcus pneumoniae*. *Diag Micro and Infect Dis* 2002;44:43-49.


Appendix A
Appendix B

Figure B.1. Comparison of deposition in unmodified NGI and LVLIS after theophylline solution nebulization

Table B.1. Average aerodynamic parameters in the unmodified NGI (n=3) and LVLIS (n=3)

<table>
<thead>
<tr>
<th>Aerodynamic Parameters</th>
<th>Unmodified NGI n=3</th>
<th>Modified NGI n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAD (µm)</td>
<td>5.22 ± 1.06</td>
<td>4.81 ± 0.57</td>
</tr>
<tr>
<td>% FPF</td>
<td>48 ± 0.08</td>
<td>51 ± 0.02</td>
</tr>
<tr>
<td>GSD</td>
<td>3.69 ± 1.15</td>
<td>3.75 ± 0.61</td>
</tr>
</tbody>
</table>

Table B.2. Change of height of water after exposure to airflow in the NGI.

Control: LVLIS water placed on desktop
Sample: LVLIS containing water placed in the NGI
Table B.3.: Change in height of MHB after exposure to airflow in the NGI

Control: LVLIS containing MHB placed on desktop

Sample: LVLIS containing MHB placed in the NGI

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Sample</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height loss (mm)</td>
<td>% height loss</td>
<td>Height loss (mm)</td>
<td>% height loss</td>
</tr>
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<td>0%</td>
<td>0.000</td>
<td>0%</td>
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<td>0.414</td>
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</tr>
<tr>
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<td>0.85%</td>
<td>0.581</td>
<td>4.70%</td>
</tr>
</tbody>
</table>