THE EFFECT OF *HAEMOPHILUS INFLUENZAE* AND *STREPTOCOCCUS PNEUMONIAE* COINFECTION ON OTITIS MEDIA DISEASE PROGRESSION AND TREATMENT EFFICACY

by

KRISTIN E. D. WEIMER

A Dissertation Submitted to the Graduate Faculty of Wake Forest University Graduate School of Arts and Sciences

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

Microbiology and Immunology

December 2010

Winston-Salem, NC

Approved by:

W. Edward Swords, Ph.D., Advisor

Examining Committee:

Kevin P. High, M.D., Chairman

Sean D. Reid, Ph.D., Co-advisor

Jason M. Grayson, Ph.D.

Charles E. McCall, M.D.
ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Swords. When I started graduate school, we were both nervous about me being able to graduate in the time frame needed for the MD/PhD program, but his guidance and enthusiasm in lab made it possible. He taught me to follow the data and not to be misled or disappointed because we didn’t get our anticipated result. I would not be the scientist I am today without his mentoring. I also want to thank my co-advisor, Dr. Reid, for his help in getting the coinfection project off the ground and for all of his guidance and suggestions along the way.

I also want to thank all of the members of the Swords lab: Rick, Chelsie, Gayle, Bing, Kyle and Toni, for their help throughout my time in lab. I especially want to thank Rick and Chelsie for their willingness to lend a hand with all of my animal experiments and for being there in lab, whether it be for scientific discussions or a much needed distraction. I am also grateful to everyone in the Microbiology and Immunology Department for their support and encouragement over the years and all the members of my committee for their suggestions and input on this project.

I would also like to thank my undergraduate research advisor, Dr. Timothy Warren. I had never thought about research, much less making it a career of it, until he brought me into his lab as a freshman and encouraged me to start my own project. His enthusiasm and patience when teaching me in the lab instilled a love of research that will stay with me the rest of my life.

I also want to thank my family for their love and support. Mom and Dad – from an early age you taught me to push myself and that if I wasn’t trying my best, it wasn’t
worth doing. You guys have supported and encouraged me every step of the way, even though it meant more and more years of school, and I wouldn’t be where I am today without you. And my sister, Jessica – we don’t always see eye to eye on things, but you have been nothing but supportive of me and you and Amy have provided much needed relief and entertainment from the constant grind of school.

And last, but not least, I would like to thank my husband, Eric. You were always one step ahead of me in graduate school, and I’ve loved having your experience to guide me through. Your love and support have meant more than you know. You taught me what it means to be to be a scientist and I’m lucky to have you. And, because Eric thought I wouldn’t, I want to thank our cats, Gracie and Smudge, for their unconditional love and affection over the years, for keeping me company while I studied and for listening to me practice all of my presentations.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER I: Coinfection with <em>Haemophilus influenzae</em> Promotes Pneumococcal Biofilm Formation during Experimental Otitis Media and Impedes the Progression of Pneumococcal Disease</td>
<td>35</td>
</tr>
<tr>
<td>Published in the <em>Journal of Infectious Disease</em>, October 2010.</td>
<td></td>
</tr>
<tr>
<td>CHAPTER II: Divergent Mechanisms for Passive Pneumococcal Resistance to β-lactam Antibiotics in the Presence of <em>Haemophilus influenzae</em></td>
<td>66</td>
</tr>
<tr>
<td>Accepted to the <em>Journal of Infectious Disease</em>, October 2010.</td>
<td></td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>94</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>107</td>
</tr>
<tr>
<td>REFERENCE LIST</td>
<td>112</td>
</tr>
<tr>
<td>SCHOLASTIC VITAE</td>
<td>152</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spectrum of otitis media disease [294].</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>Comparison of the Eustachian tube between adults and infants [313].</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>Bacterial competition during experimental otitis media coinfections.</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>Persistent polymicrobial infection during experimental otitis media coinfections</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>Duration of stable polymicrobial infection</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Imaging of polymicrobial biofilms</td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>This is an image from Video 1</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>Temporal variation of coinfections</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td><em>Haemophilus influenzae</em> (Hi) promotes pneumococcal biofilm formation in vitro</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>Coinfection with <em>Haemophilus influenzae</em> (Hi) promotes persistence of pneumococcal translucent colony variants</td>
<td>62</td>
</tr>
<tr>
<td>11</td>
<td>COMSTAT analysis of NTHi in <em>vitro</em> biofilms</td>
<td>74</td>
</tr>
<tr>
<td>12</td>
<td>Antibiotic susceptibility of NTHi in <em>vitro</em> biofilms</td>
<td>79</td>
</tr>
<tr>
<td>13</td>
<td>NTHi amoxicillin resistance in chinchilla model of otitis media</td>
<td>82</td>
</tr>
<tr>
<td>14</td>
<td>NTHi passive protection of <em>S. pneumoniae in vitro</em></td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>NTHi passive protection of <em>S. pneumoniae</em> in chinchilla model of otitis media</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td><strong>Supplemental Figure 1.</strong> Colony count and systemic disease data from animals infected with different doses of <em>S. pneumoniae</em>.</td>
<td>108</td>
</tr>
</tbody>
</table>
Supplemental Figure 2. Images of histone and elastase staining of biofilms removed from the middle ears of experimentally infected chinchillas.
LIST OF TABLES

Table 1. Carriage rates of the major opportunistic pathogens isolated from the nasopharynx. .................................................................2

Table 2. Factors that increase the risk of nasopharyngeal colonization by opportunistic pathogens. ............................................................5

Table 3. Quantification of systemic disease .............................................................................43

Table 4. Minimal inhibitory concentration of amoxicillin.......................................................76
ABBREVIATIONS

\[ \begin{align*}
H. influenzae, Hi, NTHi & \quad \text{Haemophilus influenzae} \\
S. pneumoniae, Sp, pneumococcus & \quad \text{Streptococcus pneumoniae} \\
M. catarrhalis & \quad \text{Moraxella catarrhalis} \\
S. aureus & \quad \text{Staphylococcus aureus} \\
\text{IgA} & \quad \text{immunoglobulin A} \\
\text{IgG} & \quad \text{immunoglobulin G} \\
\text{UspA} & \quad \text{M. catarrhalis ubiquitous protein A} \\
\text{PspA} & \quad \text{pneumococcal surface protein A} \\
\text{PsaA} & \quad \text{pneumococcal surface adhesin A} \\
\text{Ply} & \quad \text{pneumolysin} \\
\text{COPD} & \quad \text{chronic obstructive pulmonary disease} \\
\text{OM} & \quad \text{otitis media} \\
\text{meso-DAP} & \quad \gamma\text{-D-glutamyl-meso-diaminopimelic acid} \\
\text{Nod1} & \quad \text{nucleotide-binding oligomerization domain} \\
\text{OapA} & \quad \text{H. influenzae opacity-associated protein A} \\
\text{HMW1/HMW2} & \quad \text{H. influenzae high molecular weight adhesins} \\
\text{Hia} & \quad \text{H. influenzae adhesin} \\
\text{Hap} & \quad \text{H. influenzae adhesin} \\
\text{P2} & \quad \text{H. influenzae outer membrane protein 2} \\
\text{P5} & \quad \text{H. influenzae outer membrane protein 5}
\end{align*} \]
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEACAM1</td>
<td>carcinoembryonic antigen family of cell adhesin molecules</td>
<td></td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
<td></td>
</tr>
<tr>
<td>ChoP</td>
<td>phosphorylcholine</td>
<td></td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
<td></td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain enhancer of activated B cells</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
<td></td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
<td></td>
</tr>
<tr>
<td>iga</td>
<td><em>H. influenzae</em> IgA protease</td>
<td></td>
</tr>
<tr>
<td>LL-37</td>
<td>cathelicidin antimicrobial peptide</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>PHiD-CV</td>
<td>10-valent pneumococcal <em>H. influenzae</em> protein D conjugate vaccine</td>
<td></td>
</tr>
<tr>
<td>U.S.</td>
<td>United States</td>
<td></td>
</tr>
<tr>
<td>PCV7</td>
<td>heptavalent pneumococcal conjugate vaccine</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td><em>H. influenzae</em> outer membrane protein 6</td>
<td></td>
</tr>
<tr>
<td>OMP26</td>
<td><em>H. influenzae</em> outer membrane protein 26</td>
<td></td>
</tr>
<tr>
<td>NanA</td>
<td><em>S. pneumoniae</em> neuraminidase</td>
<td></td>
</tr>
<tr>
<td>BgaA</td>
<td><em>S. pneumoniae</em> β-galactosidase</td>
<td></td>
</tr>
<tr>
<td>StrH</td>
<td><em>S. pneumoniae</em> β-N-acetylglucosaminidase</td>
<td></td>
</tr>
<tr>
<td>CbpA/PspC</td>
<td><em>S. pneumoniae</em> choline binding protein A</td>
<td></td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>EndA</td>
<td><em>S. pneumoniae</em> endonuclease</td>
<td></td>
</tr>
<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
<td></td>
</tr>
<tr>
<td>CSP</td>
<td><em>S. pneumoniae</em> competence-stimulating peptide</td>
<td></td>
</tr>
<tr>
<td>LytA</td>
<td><em>S. pneumoniae</em> N-acetylmuramoyl-L-alanine amidase</td>
<td></td>
</tr>
<tr>
<td>LytB</td>
<td><em>S. pneumoniae</em> endo-β-N-acetylglucosaminidase</td>
<td></td>
</tr>
<tr>
<td>PcpA</td>
<td><em>S. pneumoniae</em> choline binding protein</td>
<td></td>
</tr>
<tr>
<td>PCV13</td>
<td>13-valent pneumococcal conjugate vaccine</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
<td></td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
<td></td>
</tr>
<tr>
<td>INF-γ</td>
<td>interferon-gamma</td>
<td></td>
</tr>
<tr>
<td>MARCO</td>
<td>macrophage class A scavenger receptor</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>acute otitis media</td>
<td></td>
</tr>
<tr>
<td>MEE</td>
<td>middle ear effusion</td>
<td></td>
</tr>
<tr>
<td>BLNAR</td>
<td>β-lactamase negative, ampicillin resistant</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
<td></td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
<td></td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
<td></td>
</tr>
<tr>
<td>µm</td>
<td>micron</td>
<td></td>
</tr>
</tbody>
</table>

x
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>TSA</td>
<td>trypticase soy agar</td>
</tr>
<tr>
<td>sBHI</td>
<td>supplemented brain heart infusion</td>
</tr>
<tr>
<td>TEM</td>
<td>triethylene melamine</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>C. difficile</td>
<td><em>Clostridium difficile</em></td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
</tbody>
</table>
ABSTRACT

Otitis media is a major public health problem in pediatrics worldwide. It is the number one reason for pediatric office visits, new antibiotic prescriptions and surgery in children. The two most commonly isolated pathogens from otitis media infection are Haemophilus influenzae and Streptococcus pneumoniae. Once established, these bacteria can cause persistent and/or recurrent infections and are often refractory to antibiotic therapy. One hypothesis to explain this persistence is the presence of biofilm communities, which are inherently resistant to host clearance mechanisms and antibiotics. Biofilm formation by otopathogens has been demonstrated in vitro, in a chinchilla model of experimental otitis media and on human patient samples.

Current data indicate that most otitis media infections involve simultaneous infection with multiple organisms, and thus there is a pressing need for a better understanding of the impact of polymicrobial infection on the establishment, progression and severity of otitis media disease. In this study, we used a well established chinchilla model of experimental otitis media to test the impact of coinfection with H. influenzae and S. pneumoniae. Following transbullar infection, stable biofilm communities containing H. influenzae and S. pneumoniae were formed and persisted for up to 21 days postinfection. Biofilms removed from coinfected animals contained both bacterial species. Notably, the incidence of fatal systemic pneumococcal infection was dramatically reduced in the coinfected animals as compared to animals infected with pneumococcus alone. Moreover, the percentage of translucent pneumococcal colonies recovered from coinfected animals was significantly increased as compared to animals
infected with pneumococci alone, in which the population gradually shifted to a predominantly opaque population. This finding is notable in that the phase variation from translucent (low capsule levels) to opaque (high capsule levels) colony type is a key step in the establishment of systemic pneumococcal infection. Comparison of biofilm formation by \textit{S. pneumoniae} alone and in the presence of \textit{H. influenzae} using \textit{in vitro} biofilm models recapitulated these findings, as pneumococcal biofilm formation was significantly increased in the presence of \textit{H. influenzae}. Therefore, we conclude that coinfection with \textit{H. influenzae} moderates pneumococcal infection by promoting the persistence of translucent pneumococcal variants within surface-attached biofilm communities. These results have great significance in understanding the clinical course of pneumococcal infection, which in patients is usually a localized infection as opposed to the septic infections observed in most animal models.

We next wanted to determine the impact of coinfection with \textit{H. influenzae} and \textit{S. pneumoniae} on treatment efficacy in otitis media. While many strains of bacteria recovered from otitis media have genes or mutations that provide antibiotic resistance, these do not explain all the antibiotic resistance seen. Thus, other types of resistance have been postulated, such as biofilm formation and passive protection of susceptible isolates by resistant bacterial species. In this study, the roles of biofilm formation and $\beta$-lactamase production in \textit{H. influenzae} antibiotic resistance and passive protection were investigated using an isogenic mutant of \textit{H. influenzae} deficient in the production of $\beta$-lactamase, NTHi 86-028 \textit{bla}. NTHi 86-028NP \textit{bla} was resistant to amoxicillin killing in biofilm studies \textit{in vitro}; however, it was cleared by amoxicillin treatment \textit{in vivo}, while NTHi 86-028NP was unaffected in either system. NTHi 86-028NP provided passive
protection for pneumococcus *in vivo* in both the effusion fluid and bullar homogenate. Interestingly, while NTHi 86-028NP *bla* and pneumococcus were both killed by amoxicillin when present in the middle ear alone, both bacterial species were recovered from coinfected animals after treatment with amoxicillin. *H. influenzae* and *S. pneumoniae* form a much larger biofilm together than either species does on its own. We hypothesize that the enhanced biofilm formation is providing protection for both bacterial species against amoxicillin killing. Based on these studies, we conclude that *H. influenzae* provides passive protection for *S. pneumoniae* *in vivo* through two distinct mechanisms: production of β-lactamase and formation of biofilm communities.
INTRODUCTION

Nasopharyngeal Colonization

Although sterile in utero, colonization of the nasopharynx begins rapidly at birth [1]. The nasopharyngeal flora are established within the first year of life and encompass a wide variety of organisms, including commensal bacteria as well as potential pathogens, such as nontypeable Haemophilus influenzae (H. influenzae), Streptococcus pneumoniae (S. pneumoniae), Moraxella catarrhalis (M. catarrhalis) and Staphylococcus aureus (S. aureus) (Table 1) [2-6]. The mean age of first acquisition of H. influenzae, S. pneumoniae and/or M. catarrhalis is 6 months, with a range of 1-30 months [2-9]. By one year of age the majority of children will be colonized with one of these respiratory pathogens [3, 5, 7, 8, 10-13]. S. aureus is isolated much less frequently, 0–30% [14, 15]. While colonization frequently occurs asymptotically, it is the first step in the pathogenesis of upper and lower respiratory tract infections and the frequency of colonization is directly related to the frequency of acute otitis media in children [3, 16].

There are three basic patterns of nasal carriage that a bacterium can follow: rapid elimination of the initial strain, chronic colonization with the initial strain or elimination of the initial strain with subsequent colonization by a different strain. For H. influenzae, 50% of children colonized within the first year of life are only colonized by one strain, the rest are sequentially colonized by as many as seven strains (almost always new) [6, 7, 17-20]. Adults are generally colonized by one strain at a time; however, many children are colonized with multiple strains at one time [17, 21]. The majority of children
Table 1. Carriage rates of the major opportunistic pathogens isolated from the nasopharynx.
<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Carriage rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em></td>
<td>6 – 88</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>4 – 88</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>4 – 82</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0 – 30</td>
</tr>
</tbody>
</table>
are colonized by each strain for less than 2 months [17]. An adaptive immune response does develop to *H. influenzae* during colonization. All children have detectable mucosal antibody (IgA) to an *H. influenzae* outer membrane protein after colonization [22]. Antibodies against highly conserved proteins can eliminate colonization and prevent further colonization by heterologous strains; however, *H. influenzae* can undergo significant phase variation and antigenic shifts that limit antibody effectiveness [17, 23, 24]. Serum antibody (IgG) also develops and can prevent airway infections like otitis media, but it is ineffective against nasopharyngeal colonization [23]. Colonization with *M. catarrhalis* follows a very similar pattern, where most children are colonized with and clear 3-4 different strains in the first year of life [16]. Serum and mucosal antibody develop against a *M. catarrhalis* surface protein (UspA); however, mucosal antibody production is very low in the first two years [25]. *S. pneumoniae* colonization of the nasopharynx is much more variable than that of *H. influenzae* or *M. catarrhalis*. Some children are never colonized, while others are colonized by up to four strains in the first year [5, 6, 18, 23]. Duration of colonization of pneumococcus is between 1-4 months in children and 2-4 weeks in adults [5, 6, 26]. Nasopharyngeal colonization by pneumococcus stimulates both serum and mucosal antibodies to multiple pneumococcal surface proteins (PspA, PsaA and Ply) that are thought to be capable of clearing and preventing colonization by homologous strains [23, 25, 27, 28]. Again, mucosal antibody levels remain low until around two years of age [25].

There are many factors that influence the likelihood of being colonized by one of the respiratory pathogens and they differ between adults and children (Table 2 and [29]). In general, colonization by these bacteria increases through the first 2-3 years of age and
Table 2. Factors that increase the risk of nasopharyngeal colonization by opportunistic pathogens.
<table>
<thead>
<tr>
<th>Children</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 2</td>
<td>Age ≥ 65</td>
</tr>
<tr>
<td>Attending day-care</td>
<td>Exposure to children at home or work</td>
</tr>
<tr>
<td>Large family size</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Frequent contact with other children</td>
<td>Obesity</td>
</tr>
<tr>
<td>Low socio-economic status</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>Parent that smokes</td>
<td>Smoking</td>
</tr>
<tr>
<td>Sleeping in prone position</td>
<td>High alcohol intake</td>
</tr>
<tr>
<td>Viral infection</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>Allergies</td>
<td>Acute sinusitis</td>
</tr>
<tr>
<td>Winter months</td>
<td>Winter months</td>
</tr>
</tbody>
</table>
peaks, gradually decreases until 15-16 years of age, and then increases very gradually in adults over 65 [30-34]. This is not surprising, given that mucosal antibody production is low for all of these pathogens until after 2-3 years of age [25]. All four pathogens can be isolated from the nasopharynx year round, but there is a peak around mid-winter [35].

For children, attending day-care, having a large family, and/or frequent contact with other children all increase the frequency of colonization through increased exposure to other colonized individuals [5, 7, 10, 13, 31, 36-44]. In addition, low socio-economic status and sleeping in the prone position increase the frequency of colonization [8, 38, 45, 46]. Having a parent that smokes, a concurrent viral infection or allergies increase the frequency of colonization by a bacterial pathogen by damaging the nasopharyngeal mucosa, making it easier for bacteria to attach and, in some cases, impairing the innate immune response, which decreases bacterial clearance [3, 12, 13, 18, 33, 45]. Immunizations and a history of antibiotic use change the pattern of colonization by eliminating certain bacterial species, making room for others that would otherwise not be able to compete [2, 47-50]. In adults, factors that increase the risk of colonization include: exposure to children at home or at work, chronic obstructive pulmonary disease (COPD), cystic fibrosis, smoking, high alcohol intake, obesity, immunosuppression and concurrent acute sinusitis [30].

Another important factor in colonization is the bacterial species already present in the nasopharynx. Bacteria co-colonizing in the nasopharynx can have a synergistic, antagonistic or neutral relationship [51]. Synergists will increase the colonization of each other, while antagonists will prevent the other species from establishing colonization. Commensal α-hemolytic streptococci provide a natural barrier against colonization by
opportunistic pathogens, inhibiting the colonization of other streptococcal species, like group A streptococcus and \textit{S. pneumoniae}, as well as \textit{H. influenzae} and \textit{M. catarrhalis} [52-57]. Interestingly, a prophylactic nasal spray of \(\alpha\)-hemolytic streptococci decreased the incidence of streptococcal pharyngotonsillitis and acute and chronic otitis media (OM) in randomized, placebo controlled trials [58, 59]. There are also data suggesting that bacterial opportunistic pathogens interact with and compete with each other in the nasopharynx. A recent epidemiologic study analyzed nasopharyngeal swabs to determine which opportunistic pathogens had positive a association (found together more often than expected) and which had a negative association (found together less often than expected) with each other [60]. This study found that \textit{H. influenzae} had a negative colonization association with \textit{S. pneumoniae}, \textit{M. catarrhalis} and \textit{S. aureus} [60]. In addition, \textit{S. pneumoniae} had a negative association with \textit{M. catarrhalis} [60]. However, when \textit{H. influenzae} and \textit{M. catarrhalis} co-colonized together, they had a positive association with \textit{S. pneumoniae} [60]. One of the largest negative associations was seen with \textit{S. pneumoniae} and \textit{S. aureus}, which decreased the odds of colonization by the other by 40\% [60]. The changes in pathogens recovered from the nasopharynx documented following the introduction of a vaccine against \textit{S. pneumoniae} support the epidemiologic competition data. Following a decrease in \textit{S. pneumoniae} nasopharyngeal colonization after vaccination, colonization by \textit{H. influenzae} and \textit{S. aureus} both increased significantly [14, 61-63].

The mechanism behind bacterial competition and interference in the nasopharynx is not well understood, and the mechanisms behind synergistic relationships are even less well understood. Commensal and colonizing bacteria can occupy all of the binding sites
on the epithelial surface, thereby preventing other bacteria from being able to attach [64]. There can also be competition for the limited nutrients available [65]. In addition, some bacterial species produce enzymes or small molecules that can inhibit the growth or even kill other bacterial species [54, 66-69]. It has been well documented that some streptococci, including α-hemolytic streptococci and *S. pneumoniae*, produce bacteriocins that kill other bacterial species or even closely related species of streptococci, but not the species that produced it [54, 67]. Some pneumococcal strains also produce bacteriocins that kill other strains of *S. pneumoniae* [70]. In addition, it has been shown *in vitro* that hydrogen peroxide production by streptococci can interfere with *S. aureus* and *H. influenzae* growth; however, this may not occur *in vivo* [55, 68, 69, 71]. Studies have also shown that *H. influenzae* can enhance the innate immune response against *S. pneumoniae*, which allows *H. influenzae* to persist in the nasopharynx while *S. pneumoniae* is cleared [71-73]. This occurs through signalling of the γ-D-glutamyl-meso-diaminopimelic acid (meso-DAP) fragment of *H. influenzae* peptidoglycan through the Nod1 receptor, which leads to enhanced complement-mediated opsonophagocytic killing of *S. pneumoniae* [71, 72]. As mentioned previously, examples of bacterial-bacterial synergism are rare and poorly understood. Potential mechanisms include: protection from an immune response or antibiotic killing, enhanced biofilm formation, and providing a site for attachment [74-76].

**Nontypeable *Haemophilus influenzae***

*Haemophilus influenzae* is a Gram negative, non-motile coccobacillus. The species is defined by its nutritional requirements of β-nicotinamide adenine dinucleotide...
and heme, both of which must be supplied for laboratory growth [77-79]. *H. influenzae* can be divided into two types of strains, encapsulated and unencapsulated [24]. Encapsulated *H. influenzae* encompass six serotypes (a-f) with antigenically distinct polysaccharide capsules and cause more invasive disease, usually due to serotype b, than unencapsulated *H. influenzae* [80, 81]. Unencapsulated *H. influenzae* strains are referred to as ‘nontypeable’ because they are nonreactive with the typing sera raised against any of the six capsular polysaccharides of encapsulated *H. influenzae* [80]. Disease caused by encapsulated *H. influenzae* strains has been largely eliminated after the introduction of the type-b polysaccharide-protein conjugate vaccine in 1990 [81]. This vaccine has been so effective because no strain replacement by non-serotype b strains has occurred [82-84]. Researchers do not know why strain replacement occurs after vaccination with some bacteria, like *S. pneumoniae*, but not with others. One study proposed a mathematical model which states that strain replacement occurs proportional to the frequency of carriage of the vaccine strain before the introduction of the vaccine, which was very low for *H. influenzae* serotype b and very high for most pneumococcal strains [85]. Nontypeable *H. influenzae* is most frequently associated with asymptomatic colonization, but it also causes a significant portion of OM, sinusitis, community-acquired pneumonia and COPD exacerbations [86-88].

The first step in *H. influenzae* colonization and pathogenesis is attachment to the epithelial or mucosal surfaces of the nasopharynx or respiratory tract. *H. influenzae* contains many different adhesins that aid in this process. Some strains have an *hif* locus that encodes for fimbriae/pili [89, 90]. Pili promote adherence of *H. influenzae* to oropharyngeal epithelial cells, nasopharyngeal and nasal tissue, in addition to respiratory
mucus [91-94]. Because of their attachment specificity, pili are thought to be involved in the initial steps of colonization [24, 90]. The highly conserved outer membrane lipoprotein OapA is also thought to be involved in initial attachment in the nasopharynx [95]. The majority of H. influenzae strains also contain hmw1 and hmw2 loci that encode high molecular weight adhesins [96]. Despite a high level of homology, HMW1 and HMW2 have different cellular binding specificities. HWM1 interacts with sialic acid in the α-2,3 configuration, while the target of HMW2 is unknown [97, 98]. Binding of HMW1 and HMW2 to cell types found in the upper respiratory tract is very weak, so these adhesins are thought to play a role after initial attachment in colonization in the lower respiratory tract [24, 97, 98]. H. influenzae strains that do not contain hmw loci, almost always have the adhesin Hia, which interacts with many different epithelial cell types of the respiratory tract [99-101]. Another adhesin, Hap, promotes adherence to and invasion of epithelial cells and is also thought to be involved in bacterial aggregation and microcolony formation [102, 103]. Outer membrane proteins P2 and P5 have been shown to interact with mucin, which might play a role in colonizing individuals with impaired mucociliary clearance [98, 104]. P5 is also known to bind to host CEACAM1, a carcinoembryonic antigen family of cell adhesion molecules [105, 106]. Finally, lipooligosaccharides (LOS) on the surface of H. influenzae that have been modified with phosphorylcholine (ChoP) adhere to epithelial cells [107]. Confocal microscopy studies have also shown that LOS ChoP interact with platelet-activating factor (PAF) receptor on the surface of host cells, which increases cell invasion [108].

Many of surface components of H. influenzae can trigger a proinflammatory immune response through recognition of pathogen-associated molecular patterns
(PAMPs), which leads to activation of NF-κB and subsequent production of TNF-α, IL-1β, IL-8, IL-6 and mucin [24, 109, 110]. The major PAMP responsible for eliciting a proinflammatory response to *H. influenzae* is endotoxin, which is recognized by Toll-like receptor 4 (TLR4) [110]. Functional TLR4 is required for efficient clearance of *H. influenzae* in mice [110, 111]. TLR2 is also activated by surface components of *H. influenzae*, but TLR2-deficient mice do not have a severe defect in clearance of *H. influenzae* [109, 112].

In order to efficiently colonize the respiratory tract, *H. influenzae* must escape or dampen the host immune response. One such host response is the production of mucins which can bind different adhesins of *H. influenzae*, like fimbriae/pili, and facilitate clearance [93, 94]. However, many of the adhesins mentioned previously (fimbriae/pili, HWW1, HMW2 and ChoP) have phase variable expression that can be turned on and off [113-115]. This allows strains that are not expressing a particular adhesin to escape mucin binding and, therefore, clearance [24, 94, 116]. This antigenic variation also leads to hundreds of different variants of *H. influenzae* based on surface LOS structure or adhesin expression, allowing the one most suited for the current environment to persist and escape clearance [24]. Another mechanism of immune evasion is the secretion of an IgA protease [24]. IgA is the dominant antibody in the upper respiratory tract and it has been demonstrated that children with atopic conditions have higher levels of cleaved IgA1 in the nasopharynx [117, 118]. Greater than 95% of *H. influenzae* strains contain the gene (*iga*) for IgA1 protease and there are over 30 different antigenic varieties identified [119, 120]. A second IgA protease gene, *igaB*, is found much more frequently in sputum and otitis media isolates than in carriage isolates [121]. Host lysozyme and
lactoferrin do not inhibit the growth of *H. influenzae*; however, lactoferrin can cleave the IgA protease of *H. influenzae* [122, 123]. Other immune evasion mechanisms involve surface modifications of *H. influenzae*. Sialylation of LOS significantly increases resistance to serum killing by preventing the activation of complement [124, 125]. Susceptibility to the antimicrobial peptide LL-37 is reduced by ChoP expression on LOS [126]. In addition, ChoP addition to LOS decreases the potency of LOS in signalling through TLR4 [127]. Interestingly, sensitivity to serum killing is increased with ChoP expression because it binds C-reactive protein, which activates complement [107, 128]. ChoP can also bind to PAF receptor, which has an anti-inflammatory effect through negative regulation of TLR2, TLR4 and TLR9 [129]. Finally, susceptibility to human β-defensin-2 increases with a decrease in acylation of the lipid A portion of LOS [130].

Another component of *H. influenzae* persistence, both in the nasopharynx and in other areas of the respiratory tract like the middle ear, is the formation of biofilm communities [131-133]. Biofilms are surface associated, structured, sessile communities of bacteria encased within an extracellular matrix [134]. This matrix contains both host and bacterial components and the composition varies among different bacterial species [133, 135-139]. Biofilms are inherently more resistant to host defenses and antibiotics than planktonic bacteria and are, therefore, very difficult to eradicate [140-142]. Numerous studies have shown that *H. influenzae* forms biofilms *in vitro*, in a chinchilla model of experimental OM and on patient mucosa samples [127, 131, 135, 136, 143-145]. In addition, *H. influenzae* biofilm formation has been linked to increased bacterial persistence [133].
In addition to being involved in attachment and immune evasion, numerous studies have demonstrated that surface modifications of LOS are important for *H. influenzae* biofilm formation. First, ChoP addition to LOS increases under biofilm growth conditions compared with planktonic growth and mutations that increase (*licON*) or eliminate (*licD*) ChoP expression on the surface of *H. influenzae* increase or decrease, respectively, biofilm formation both *in vitro* and in the chinchilla model of OM [127, 146]. In addition, an *H. influenzae* strain lacking ChoP (*licD*) has a decreased bacterial load in the chinchilla middle ear, but causes more inflammation, indicating that biofilm formation might be another mechanism of immune escape [146, 147]. Multiple studies have also shown that sialic acid addition to LOS is important for biofilm formation. Mutation of the sialic acid activator gene (*siaB*) or the sialyltransferase genes (*siaA* and *lsgB*) reduces virulence and biofilm formation of *H. influenzae* in the chinchilla and gerbil models of OM [136, 145, 148]. *H. influenzae* biofilms removed from the middle ears of experimentally infected chinchillas also contain double-stranded DNA and type IV pilin protein, as well as the host components neutrophil elastase and histone [133, 136]. Another study has shown that the *H. influenzae* biofilm matrix *in vitro* contains the adhesins Hap, HMW1 and HMW2 [149].

A vaccine against nontypeable *H. influenzae* and *S. pneumoniae*, PHiD-CV, was released in 2009 and has been licensed for use in forty countries, but is not approved for use in the U.S. [150]. The vaccine contains ten capsular polysaccharide serotypes of pneumococcus, eight conjugated to protein D of *H. influenzae*, two conjugated to tetanus or diptheria toxoid [150-152]. PHiD-CV has shown some ability to decrease the incidence of *H. influenzae* OM and is as immunogenic for pneumococcus as the
heptavalent pneumococcal conjugate vaccine (PCV7), but the pneumococcal serotypes included are more prevalent outside the U.S. [150, 152-155]. Other vaccine targets for *H. influenzae* are also being explored, with the hope of reducing both nasal carriage and otitis media. Three *H. influenzae* outer membrane proteins, protein D, P6 and OMP26, have shown the most promise as vaccine candidates [156-158].

**Streptococcus pneumoniae**

*Streptococcus pneumoniae* is a Gram positive, encapsulated, α-hemolytic coccoid that usually grows in pairs or short chains. Like *H. influenzae*, *S. pneumoniae* frequently colonizes the nasopharynx asymptomatically [15, 31]. Unlike *H. influenzae*, pneumococcus is a common cause of both invasive and non-invasive disease. It is one of the leading causes of OM, pneumonia, meningitis and septicemia worldwide [159]. The World Health Organization estimates that over 1.6 million people die from pneumococcal diseases each year, mostly in developing countries [160].

There have been over 90 pneumococcal serotypes identified based on different capsular polysaccharide compositions and serotype can predict the disease manifestation [161, 162]. For example, a small subset of serotypes account for the majority of nasopharyngeal isolates. These include serotypes 3, 6A, 6B, 9V, 14, 18C, 19A, 19F and 23F and less commonly, 10, 11, 13, 15, 33 and 35 [35, 162-166]. Serotypes 1, 5 and 46 are rarely found in the nasopharynx, but cause a high proportion of invasive disease [6, 48, 164, 167, 168]. All strains are carried before they cause disease, so it is thought that there is some difference in length or density of colonization [161, 167]. Serotypes also differ in the likelihood of developing antibiotic resistance, which is related to the duration
of nasopharyngeal colonization, the likelihood of requiring hospitalization, the age of the host and the type of disease caused [6, 48, 161, 164, 167, 168].

In addition to serotype differences, *S. pneumoniae* also phase varies between two distinct colony phenotypes, the opaque and translucent, which differ in their ability to colonize the nasopharynx, protein expression and type of disease they cause [169-174]. Phenotypic variants are visible when colonies are viewed on clear media under oblique transmitted light [175]. The frequency of phase variation differs highly between isolates, ranging from $10^{-2}$ to $10^{-7}$ per generation [175]. Opaque colonies express more capsular polysaccharide and less teichoic acid, while translucent colonies express more teichoic acid and less capsular polysaccharide [169, 171, 172]. Without selective pressure, pneumococcal populations are a mixture of both phenotypes [172, 176]. The expression of capsule decreases entrapment within mucus in the airway and prevents opsonophagocytosis in the blood stream, making it ideal for systemic infection [169, 177]. However, high levels of capsule inhibit adherence to the epithelial surfaces [177]. Translucent colonies are more adherent and colonize the nasopharynx more effectively than opaque colonies because the thinner capsule allows more of the surface adhesins to be exposed [170, 173, 178, 179]. However, the thinner capsule makes translucent colonies much more susceptible to opsonophagocytosis [169, 170].

The mechanism behind pneumococcal phase variation has been studied for many years, but it still unclear how this transition occurs and what initiates the switch [175, 180-182]. Colony phenotype is not altered by environmental growth conditions *in vitro* or capsular type, nor does it correlate with differences in chain length, as has been observed in other streptococcal species [175, 183-185]. Electron micrographs of
translucent colonies suggest that these colonies are undergoing autolysis, while opaque colonies under the same conditions are not [186]. Additional studies demonstrated that the frequency of phase variation was increased by the presence of a box stem-loop element that could be transferred between strains and that spontaneous duplications within the capsule genes were present in opaque colonies variants; however, neither of these elements is present consistently for a given phenotype [175, 180].

As with most opportunistic pathogens, the first step in pneumococcal disease is colonization [61, 162]. *S. pneumoniae* has three surface-associated exoglycosidases, neuraminidase (NanA), a $\beta$-galactosidase (BgaA) and a $\beta$-$N$-acetylglucosaminidase (StrH) that can remove the terminal sugars found on the epithelial surface, thereby uncovering potential sites for adherence [187]. *S. pneumoniae* also has adhesins that aid in attachment to and colonization of the nasopharynx. ChoP is incorporated into cell-wall associated acids and lipoteichoic acids on the surface [188]. Pneumococcal ChoP binds to and activates signalling through PAF receptor, which is widely distributed on host tissues, including epithelial surfaces of the nasopharynx [188]. As with *H. influenzae*, this increases attachment and is thought to be important for attachment in the airway [107, 188, 189]. Another adhesin, CbpA (or PspC), is covalently anchored to ChoP on the surface and can also bind secretory IgA [190, 191]. *S. pneumoniae* also has a surface-attached hyaluronidase that breaks down the hyaluronan-containing portion of connective tissue, allowing for pneumococcal penetration through tissue [192].

Once pneumococcus has colonized or invaded tissue, it has numerous mechanisms to prevent clearance by the immune system. During the first few days of a pneumococcal infection there is a substantial influx of neutrophils, but these are generally
unable to clear the infection. First, the polysaccharide capsule prevents complement from binding and protects pneumococcus from opsonophagocytosis in non-immune hosts [193]. This plays a key role in the ability of *S. pneumoniae* to disseminate and cause systemic disease [180, 194-200]. In addition, CbpA binds Factor H, thereby preventing pneumococcal opsonization [201, 202]. PspA, another choline-binding protein on the surface, prevents fixation of C3 on the surface, which also inhibits complement mediated opsonophagocytosis [203]. In addition, PspA binds lactoferrin and decreases pneumococcal sensitivity to killing by the antimicrobial peptide apolactoferrin [204]. Pneumococci also produce a cell-associated endonuclease (EndA) that allows them degrade the DNA component of neutrophil extracellular traps (NETs) *in vitro* [205]. One of the major virulence factors of *S. pneumoniae* is the pore forming toxin pneumolysin, which indiscriminately forms pores in choles trol membranes, killing host cells [161]. Pneumolysin also inhibits the ciliary beating on respiratory epithelial cells and brain ependyma and inhibits the respiratory burst in phagocytes [161, 206].

Another factor that contributes to pneumococcal virulence and persistence is the formation of biofilm communities. *S. pneumoniae* biofilm formation has been demonstrated *in vitro*, in a chinchilla model of experimental OM, and on patient middle ear mucosa samples [131, 137, 207-209]. In pneumococcus, nasopharyngeal colonization and biofilm formation have been linked to quorum sensing and bacterial competence [207, 210]. Both are increased with the addition of competence-stimulating peptide (CSP) [210, 211]. Little is known about the composition of pneumococcal biofilms. There is some evidence that the extracellular matrix contains an exopolysaccharide of some kind, but its identity and structure are unknown [212]. In addition, double-stranded
DNA and choline have been identified as significant portions of the extracellular matrix, although DNase I treatment did not degrade pneumococcal biofilms in another study [137, 213, 214]. Other factors have also been identified that increase or decrease pneumococcal biofilm formation. In addition to inhibiting pneumococcal attachment, capsule interferes with biofilm formation and it has been shown that capsule production is downregulated during a biofilm mode of growth [137, 176, 213, 215, 216]. Translucent colonies are associated with increased adherence to abiotic surfaces and biofilm formation [179, 210, 215-218]. The addition of sialic acid and presence of neuraminidase, which cleaves sialic acid off host molecules, have both been shown to increase pneumococcal biofilm formation and nasopharyngeal colonization in multiple models [209, 219]. In addition, deletion of several different choline-binding proteins (LytA, LytB, LytC, CbpA, PcpA, PspA) decreased biofilm formation in vitro [137, 213].

An interesting study demonstrated that pneumococci have two distinct expression patterns during an infection, one present in bacteremia and sepsis and another during infection of tissues like the lung and meninges [210]. The gene expression and phenotypic profile during sepsis/bacteremia resembles that of planktonic bacteria in vitro, while the expression profile of pneumococci from a tissue infection resembles that of bacteria from a biofilm in vitro [210]. The switch between these two phases, which also alters virulence, occurs via sensing of CSP [210]. Addition of CSP induces biofilm formation in vitro and increases pneumococcal virulence in a mouse pneumonia model [210]. On the other hand, a pneumococcal mutant in the CSP receptor does not form biofilms and has reduced virulence in a mouse pneumonia model [210]. CSP has the
opposite effect on systemic infections [210]. This study provides a potential explanation for how pneumococcus can switch from a localized to systemic infection.

Antibodies to capsular polysaccharide antigens provide serotype specific protection [160]. There are currently two pneumococcal vaccines available that utilize this: a 23-valent pneumococcal polysaccharide vaccine, intended for adults and the elderly, and a 13-valent pneumococcal conjugate vaccine (PCV13), which just replaced the heptavalent pneumococcal conjugate vaccine (PCV7) and is intended for children ≤ 5 years of age [220-222]. Although PCV7 was very effective against disease caused by pneumococcal serotypes included in the vaccine, invasive disease and OM caused by strains not included in the vaccine and other bacterial species increased [62, 223-225]. The overall incidence of invasive pneumococcal disease still decreased by 45%; however, despite the fact that \textit{S. pneumoniae} accounted for over 40% of OM cases, the incidence of OM only decreased by 6% [62, 225]. The PCV13 vaccine includes the serotypes in PCV7 (4, 6B, 9V, 14, 18C, 19F and 23F) as well those that increased in frequency after the introduction of PCV7 (1, 3, 5, 6A, 7F, 19A) [161, 221, 224]. Very little data is available about the effectiveness of PCV13 in reducing OM, or whether or not significant strain replacement occurs.

**Opportunistic Airway Infections**

Because of its central location and proximity to the upper and lower respiratory tracts, the nasopharynx serves as an important reservoir for opportunistic airway infections [29, 226]. These occur when opportunistic pathogens leave the nasopharynx and colonize another location in the respiratory tract, leading to many types of infections
including: OM, sinusitis, community-acquired pneumonia, and COPD exacerbations [227, 228]. Numerous studies have shown that the bacteria isolated from these opportunistic airway infections originate in the nasopharynx [9, 229-231]. *S. pneumoniae* and *H. influenzae* are the two most frequently isolated bacteria from pneumonia, COPD exacerbations and OM [22, 62, 232-235].

Opportunistic airway infections are a significant cause of morbidity and mortality and produce a substantial economic burden [236-238]. Pneumonia is one of the leading causes of death worldwide, and is responsible for over 4.6 million doctors’ visits annually and treatment costs over 8.4 billion dollars in the U.S. annually [239-242]. Patients with moderate to severe COPD experience an average of two acute exacerbations a year and the cost in the U.S. is estimated to be over 32 billion dollars annually [238, 243]. Sinusitis is also very common, with 1-2 out of every 10 people affected at any given time [244]. Many of these infections are chronic or recurrent in nature and involve biofilm formation, which contribute to treatment difficulties and the high economic burden [131, 132, 141, 143, 144, 245, 246].

The transition from asymptomatic carrier to infection is not well understood; however, there are some key factors that are known to increase the likelihood of developing an opportunistic airway infection. These include various abnormalities in host defenses, like COPD, allergies or viral infection, and anatomical defects.

COPD encompasses chronic bronchitis, emphysema and bronchiectasis. Characteristics of a COPD lung include: hypersecretion of mucus, impaired mucociliary clearance and increased inflammation [234, 247-251]. Opportunistic pathogens like *H. influenzae* and *S. pneumoniae* can bind to the damaged epithelium and mucus, and
mucociliary clearance is unable to eliminate the bacteria [24, 91-94, 97-101, 104, 188-191]. This allows colonization of the lower respiratory tract and can lead to bacterial exacerbations [32, 252-254]. Some studies have shown a correlation between patients that have allergies and an increased incidence of sinusitis, but this correlation is not well understood [255, 256].

Bacterial infections frequently develop concurrently or immediately following a viral infection. For example, in the 1918 and 1957 influenza pandemics, bacterial pneumonia, almost always due to \textit{S. pneumoniae}, increased and contributed significantly to mortality [257-260]. Numerous studies have also shown a correlation between viral infection and subsequent bacterial infection, like OM [261-267]. Researchers found serological evidence of a viral infection in 50\% and 60\% of \textit{M. catarrhalis} and \textit{H. influenzae} respiratory infections, respectively [268, 269]. There are multiple explanations for how this could occur. First, viruses can damage the epithelium or cilia of the respiratory tract, which is known to impair bacterial clearance and increase bacterial attachment [270-273]. In addition, studies have shown that viral particles inserted into the cell membrane (glycoprotein G, neuraminidase, hemagglutinin) or virus-induced upregulation of pre-existing receptors (CD14, CD15, CD18, PAF, Fimbriae-associated receptors) during replication can serve as sites for bacterial attachment [188, 274-280]. For example, respiratory syncytial virus (RSV) infection of pneumocyte type II cells increases the expression of the receptors CEACAM1 and PAF, both of which are known receptors for and increase the attachment of \textit{H. influenzae} [274]. In the case of influenza virus and \textit{S. pneumoniae}, it was found that viral neuraminidase strips the sialic acid off lung tissue, allowing for increased attachment and colonization by
pneumococcus [281]. There are also studies indicating that a viral infection alters the ability of the immune system to respond to a bacterial infection. Influenza virus is known to impair neutrophil function [282-285]. Influenza virus induced INF-γ expression also downregulates the expression of the phagocytic receptor MARCO on alveolar macrophages, inhibiting their ability to kill \textit{S. pneumoniae} [286]. Other studies have shown a change in systemic immune or inflammatory parameters following viral infection that alter the ability to clear bacteria [287-292].

**Otitis Media**

OM develops when bacteria spread from the nasopharynx to the middle ear and the frequency of nasopharyngeal colonization by a respiratory pathogen directly correlates with the incidence of OM [3]. The three most commonly isolated bacteria from OM infections are \textit{H. influenzae}, \textit{S. pneumoniae} and \textit{M. catarrhalis} [22, 62, 232]. The two most common, \textit{H. influenzae} and \textit{S. pneumoniae}, are isolated from ~75% of infections [62, 232].

OM encompasses a spectrum of diseases in the middle ear, beginning with acute otitis media (AOM) and extending to recurrent OM and OM with effusion, also known as chronic OM (Figure 1) [293]. OM infections are problematic because they are frequently recurrent or chronic in nature, despite medical treatment [294-296]. A diagnosis of AOM is determined by the presence of middle ear effusion (MEE) in conjunction with one or more signs or symptoms of inflammation in the middle ear (otalgia, otorrhea, fever, irritability), while chronic OM is defined as MEE without symptoms of an acute infection [297]. Chronic OM can develop on its own or as a progression of AOM [294].
Figure 1. Spectrum of otitis media disease [293].
Treatment of OM includes antibiotic therapy, pain management and, in children with recurrent and/or chronic infection, the placement of tympanostomy tubes [293, 298, 299]. The most common complication is conductive hearing loss, which is caused by effusion that remains in the middle ear for weeks to months, even after the infection has been treated [300]. Because these infections occur during the most important years of language development, children are at an increased risk for developing poor verbal skills, decreased language and speech discrimination abilities, decreased vocabulary and other developmental problems [300-302]. Other complications of OM infection include: perforation of the tympanic membrane, mastoiditis, petrositis, meningitis, subdural empyema and brain abscess [294, 301].

OM is one of the leading public health problems in pediatrics, with 50-85% of children experiencing at least one episode by 3 years of age and 40% of older children having six or more total episodes [303, 304]. In addition, it is the most common reason for pediatric office visits, new antibiotic prescriptions and surgical intervention in children [298, 305-307]. The annual cost associated with OM is estimated to be over 5 billion dollars [305, 308].

Many risk factors have been identified in the development of OM, the most important of which is dysfunction of the Eustachian tube. The Eustachian tube is a narrow, ciliated tube that connects the nasopharynx to the middle ear cavity and is important for drainage, ventilation and protection of the middle ear cavity [227]. Infants and young children are especially prone to OM because their Eustachian tubes are shorter, floppier and more horizontal than those of adults, which prevent them from functioning properly (Figure 2) [309-311]. Allergies and viral infection can also lead to
Figure 2. Comparison of the Eustachian tube between adults and infants [312].
Eustachian tube dysfunction and make a person more susceptible to OM [301]. In particular, infection with RSV, rhinovirus, influenza virus and adenovirus have all been shown to predispose a child to developing OM [262, 263]. As children get older and their Eustachian tubes develop fully, the incidence of OM decreases dramatically [303, 313]. Other risk factors shown to have a positive association with the development of OM are very similar to those that increase the risk for nasopharyngeal colonization and include: day-care attendance, parental smoking, pacifier use, crowded living conditions, male gender and a positive family history [303, 313-315]. Breast feeding for longer than three months has been shown to have a negative association with OM and to induce higher levels of antibody against *H. influenzae* [314, 316, 317].

The first-line treatment for OM is amoxicillin because of its favorable pharmacodynamic/pharmacokinetic profile [299]. Second and third-line treatments include amoxicillin plus the β-lactamase inhibitor clavulanate and various cephalosporins [299]. As mentioned previously, OM infections are problematic because they are very difficult to treat and pediatric patients often have multiple infections in a year due to treatment failure, infections that reoccur at the conclusion of antibiotic treatment or development of chronic infections [294-296, 318-320]. In some high risk populations, treatment failure is as high as 50% [321]. Many OM pathogens contain genetic determinants of antibiotic resistance. Approximately 60% of *H. influenzae* strains and essentially all *M. catarrhalis* strains produce a β-lactamase [322-324]. In addition, some strains of *H. influenzae* are resistant β-lactams through a different mechanism. These are called β-lactamase negative, ampicillin resistant strains (BLNAR), and the resistance is due to mutations of the penicillin binding proteins [324, 325]. β-lactam resistance among
pneumococcal strains is much less common, although some strains (~20%) have developed resistance through mutation of the penicillin binding protein [322].

Although antibiotic resistance is becoming increasingly common, it does not explain all of the cases of treatment failure seen in OM [326, 327]. One hypothesis to explain this and the chronic nature of many infections is the formation of biofilm communities [132, 328]. Biofilms are inherently more resistant to host defenses and antibiotics than planktonic bacteria and are, therefore, very difficult to eradicate [140-142]. It has been shown that secreted antibodies are unable to penetrate biofilms due to binding by matrix material in multiple bacterial species [329]. In addition, *Pseudomonas aeruginosa* biofilms secrete catalase that prevents penetration of hydrogen peroxide [330]. Antibiotics are less effective against biofilms because of decreased penetration of the antibiotic, an accumulation of β-lactamase within biofilms and a decreased metabolic rate of bacteria within biofilms, which renders many antibiotics ineffective [331-339]. Many chronic infections are now known to involve biofilm formation and it is also clear that OM is a biofilm disease [340]. It has been shown in our laboratory and others that *H. influenzae* and *S. pneumoniae* cause OM with biofilm formation in a chinchilla model of experimental OM, and biofilm formation has been linked to increased bacterial persistence in OM for *H. influenzae* [133, 208, 341]. In addition, multiple studies have found evidence of biofilms on middle ear mucosa samples from children with chronic OM and in tympanostomy tubes removed from children [131, 143, 246, 342].

**Polymicrobial infection**
As epidemiologic sampling methods have improved, it has become clear that the majority of opportunistic airway infections are polymicrobial infections involving different combinations of multiple bacterial species [60, 343-345]. Polymicrobial communities were first recognized to play an important role in colonization and infection outcome in dental caries. Many different bacterial species colonize the oral cavity together in biofilm communities and, as in the nasopharynx, these interactions can be synergistic, antagonistic or neutral [346-352]. Species of streptococci are the first to attach, followed shortly by species of Actinomyces, Veillonella and Neisseria [353-355]. These bacteria are the initial colonizers because they have multiple adhesins allowing them to attach to saliva and the surface of the oral cavity [76, 356-363]. They can then provide a surface for attachment for other species and also potentially provide needed nutrients within the biofilm [76, 354, 363-370]. Porphyromonas gingivalis, a common cause of periodontal disease, is rarely found in the early binding communities on teeth, but has been shown to have multiple adhesins that attach to species of streptococci, which are early colonizers [76, 371-374]. Bacterial species within these communities do not always cooperate. As in the nasopharynx, many species can produce bacteriocins and hydrogen peroxide that interfere with the survival of other bacteria [76]. In addition, some species decrease expression of adhesins after contact with another bacterial species, preventing attachment of the incoming bacteria [375]. Dental plaque and periodontal disease are the result of a complex interaction between all of these bacterial species and groups of certain bacteria colonizing together are known to increase the risk for periodontal disease [366, 368, 369]. Treatment of periodontal disease is now focused on altering the bacterial community dynamics to favor the less pathogenic bacterial species.
Work is also being done to engineer probiotics for the oral cavity that contain harmless commensals that are better able to prevent colonization by the more pathogenic species [76, 376, 377].

In OM, polymicrobial infections play an important role in both acute and chronic OM and are thought to be the result of polymicrobial interactions in the nasopharynx [56, 60]. Because they colonize the same niche and are the two leading causes of OM, understanding the dynamics of *H. influenzae* and *S. pneumoniae* coinfection is very important for prevention and treatment of OM infections. As mentioned previously, *H. influenzae* and *S. pneumoniae* have a negative colonization association in the nasopharynx [60]. Most OM epidemiologic studies do not test for the presence of more than one bacterial species; however, the few that have indicate that *H. influenzae* and *S. pneumoniae* do coinfect the middle ear. Multiple tympanocentesis studies of children with AOM show that 15-20% patients are infected with both *H. influenzae* and *S. pneumoniae* [378, 379]. Samples were taken from patients within the first 1-3 days of symptoms and before treatment, so they likely represent the initial infecting population. The presence/absence and identity of bacterial species was determined using culture methods [378, 379]. Numerous studies have shown that bacterial species can be detected by PCR in middle ear effusion samples that are not detected in culture studies, so the percentages likely under represent the incidence of coinfection [345, 380-382]. Another study of patients with chronic OM showed by PCR that ~30% of patients were infected with *H. influenzae* and *S. pneumoniae* [345]. Bacterial populations in chronic OM patients are affected by which antibiotics the patients have received, so they do not represent the initial infecting population. However, these studies do show that infection
with multiple bacterial species is a problem throughout OM infection. Despite these data, most experimental OM studies are conducted with pure cultures of a single bacterial species and very little is known about polymicrobial OM infections and how they differ from single species infections.

The implications of polymicrobial infection on bacterial attachment and persistence have already been discussed for the nasopharynx and oral cavity. Very little research has been done on polymicrobial infections in the field of OM; however, there is evidence to suggest that the same type of interactions can take place between otopathogens. *In vitro* studies have shown that *S. pneumoniae* can remove the sialic acid from the surface of *H. influenzae*, and sialic acid increases pneumococcal biofilm formation [73, 209, 219]. More research needs to be done to determine if this happens *in vivo* and to discover other mechanisms of bacterial cooperation and antagonism between these two bacterial species and others.

One of the most important consequences of polymicrobial infections is the impact they have on treatment efficacy. Passive protection of susceptible bacteria by resistant bacterial species, or indirect pathogenesis, has been hypothesized to occur when bacteria colonize the same niche together [74, 383, 384]. For example, a β-lactamase secreting bacterium could protect a β-lactam susceptible bacterium against antibiotic killing by inactivating the antibiotic. Previous studies have shown that a β-lactamase producing strain of *M. catarrhalis* can passively protect pneumococcus from antibiotic killing in a mouse pneumonia model and in biofilms *in vitro* [385, 386]. This protection was abolished if the β-lactamase was inhibited with clavulanate [385, 386]. Other studies have shown that a β-lactamase producing strain of *H. influenzae* does not provide passive
protection for pneumococcus in a mouse model of pneumonia or in a rat model of experimental OM [387, 388]. Neither of these models incorporated biofilm formation, which could be important for ensuring close association of the bacteria. Because OM is a biofilm disease, further studies are needed to determine if *H. influenzae* provides protection for pneumococcus in an animal model where biofilm formation is known to occur. For treatment purposes, it is also important to distinguish which mechanisms of resistance are contributing to treatment failure in both polymicrobial and single species OM: biofilm formation, β-lactamase production, or both.

**Statement of Research Purpose**

The research presented here focuses on OM infection with *H. influenzae* and *S. pneumoniae*. We first wanted to determine if these two bacterial species coinfect in the chinchilla model of experimental OM and determine the effect that coinfection has on bacterial persistence and disease progression. Next, we wanted to determine the effect that coinfection with *H. influenzae* and *S. pneumoniae* has on treatment of OM. Specifically, does *H. influenzae* provide passive protection for *S. pneumoniae* during OM? If so, is this protection due to β-lactamase production, biofilm formation or both?
Chapter I:

Coinfection with *Haemophilus influenzae* Promotes Pneumococcal Biofilm Formation during Experimental Otitis Media and Impedes the Progression of Pneumococcal Disease

K.E.D. Weimer, C.E. Armbruster, R.A. Juneau, W. Hong, B. Pang, W.E. Swords

The following manuscript was published in *The Journal of Infectious Disease*, volume 202, issue 7, pages 1068-1075, 2010 and is reprinted with permission. Stylistic variations are due to the requirement of the journal. K.E.D. Weimer performed the experiments and prepared the manuscript. Dr. W. Edward Swords acted in an advisory and editorial capacity. Copyright © University of Chicago Press, 2010.
INTRODUCTION

Otitis media (OM) is an extremely common pediatric infection, affecting the majority of all children [389]. Otitis media arises as a consequence of impaired drainage of the middle ear chamber, which facilitates colonization by bacterial opportunists residing in the nasopharynx, including nontypeable Haemophilus influenzae and Streptococcus pneumoniae (pneumococcus) [390]. Otitis media often presents as a chronic or recurrent infection in which bacteria persist within biofilm communities [391]. Biofilms have been observed on middle ear mucosal tissues from patients undergoing tympanostomy [131], and it is now clear that biofilms promote bacterial persistence during chronic/recurrent otitis media infections [133].

Recent epidemiologic studies have shown that the majority of cases of otitis media and other opportunistic airway infections involve simultaneous infection with multiple pathogens [60]. Prior work using murine models indicates that H. influenzae components can prime enhanced innate responses that can help contain pneumococcal infection [71, 72, 392]. In this study, we used the chinchilla animal infection model for otitis media to test the impact of coinfection with H. influenzae and pneumococcus on bacterial clearance/persistence and otitis media disease. The results clearly show that coinfection with H. influenzae promotes pneumococcal biofilm formation and persistence in localized infections.
MATERIALS AND METHODS

Bacterial strains and growth conditions. *Streptococcus pneumoniae* TIGR4 is a well-studied clinical isolate for which a complete genomic sequence is available [393], and we have recently demonstrated that it forms biofilms during experimental otitis media infections [208]. Pneumococci were grown on Todd-Hewitt yeast extract agar (Difco) with 4 µg/mL of gentamicin (Sigma) added, brain heart infusion agar (Difco) with 5% sheep’s blood and gentamicin, or trypticase soy agar (BD) with 315 U/mL of catalase (Worthington) added, as indicated. Nontypeable *Haemophilus influenzae* 86-028NP is an otitis media isolate that has been fully sequenced [394] and is known to cause otitis media featuring biofilms in the chinchilla infection model [133, 135, 136, 146, 147, 395]. Bacteria were grown on brain heart infusion agar supplemented with hemin (ICN Biochemicals) and nicotinamide adenine dinucleotide (Sigma) and 3 µg/mL of vancomycin (Sigma).

Chinchilla infections. Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher’s chinchilla ranch and were allowed to acclimate to the vivarium for 1 week prior to infection. All animals were examined by otoscopy prior to infection, and none had any clinical signs of middle ear infection or other overt disease. The chinchilla infection protocols were performed essentially as described elsewhere [133, 146, 147, 208]. *S. pneumoniae* TIGR4 and/or nontypeable *H. influenzae* 86-028NP were diluted using sterile phosphate-buffered saline (PBS), and the bacterial density was confirmed by plate count. Chinchillas (3-5 animals/group/time point) were anesthetized with isoflurane and were inoculated via transbular injection with 0.1 mL of bacterial
suspension containing either *S. pneumoniae*, *H. influenzae* or both bacterial species, as indicated. Infectious doses ranged from $1 \times 10^2$ to $1 \times 10^5$ colony-forming units (CFU), as indicated in the individual experiments. Groups of animals were euthanized at 3, 7, 14, or 21 days postinfection. Animals exhibiting overt symptoms of systemic disease were euthanized. Blood was collected at euthanasia and was plated to determine the presence of bacteremia. After euthanasia, the superior bullae were opened to expose the middle ear cavity as described elsewhere [146], and the presence of visible biofilm was assessed. If present, middle ear effusion fluids were collected. The middle ear cavity was then lavaged with 1 mL of sterile PBS. Effusion and lavage fluids were serially diluted and assessed by plate count. For animals that received both bacteria, fluid was plated on 2 separate plates, 1 selective for *H. influenzae* (supplemented brain infusion plus vancomycin) and 1 selective for *S. pneumoniae* (Todd-Hewitt yeast extract plus gentamicin or TSB plus catalase). Middle ear bullae were aseptically removed and homogenized using a Power Gen 1000 homogenizer (Fisher Scientific); the bullar homogenates were plated to assess tissue-associated bacterial load. Representative bullae were fixed in 4% paraformaldehyde for microscopy studies. All of the chinchilla infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

**Microscopy.** Biofilms were excised from the middle ear chamber, were rinsed with PBS, were then placed in Cryomolds (Sakura Finetek) with OCT compound (Sakura Finetek), and were frozen at -70° C. Serial 5-µm sections were cut with Accu-Edge Low Profile Blade (Feather Safety Razor) at -20° C, were placed on adhesive slides, and were stored
at –70°C. For immunofluorescent staining, slides were brought to room temperature, were washed with PBS, were blocked with 1% bovine serum albumin (Sigma), and were then stained with monoclonal antibody specific for pneumococcal type 4 capsule (kindly provided by Dr. Moon Nahm, UAB Bacterial Pathogen Reference Laboratory) and rabbit anti-

*H. influenzae* antiserum [147] as primary antibodies. Donkey anti-rabbit immunoglobulin G AlexaFluor 488 (Invitrogen) or goat anti-rabbit immunoglobulin G Texas Red (Invitrogen) were used as secondary antibodies. Stained slides were mounted with Prolong Gold anti-fade reagent (Invitrogen). Samples were visualized using a Zeiss LSM510 confocal laser-scanning microscope.

**Static Biofilms.** Assessment of biofilm formation in static cultures was performed essentially as described elsewhere [208, 396]. *H. influenzae* 86-028NP (~1 x 10^7 CFU) was plated in each well of a 24-well plate in a total volume of 1.5 mL of supplemented brain heart infusion or Morse’s minimal media [397]. After 24 h, supernatants were removed and replaced with 1.5 mL of supplemented Morse’s minimal media + 10% horse serum. *S. pneumoniae* TIGR4 (~1 x 10^7 CFU) was added to the coinfection and *S. pneumoniae* wells. After 48 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping and were resuspended in 0.2 mL of sterile PBS; this suspension was then diluted and plated for bacterial counts.
RESULTS

Competitive infections. The impact of *H. influenzae*-pneumococcal coinfection on bacterial persistence and otitis media disease was assessed using infectious doses previously established for monospecies infections with each organism [147, 208]. Groups of chinchillas were inoculated with either \(~1 \times 10^3\) CFU of *H. influenzae*, \(~150\) CFU of *S. pneumoniae*, or the same infectious doses of both species. On day 3, equivalent bacterial counts for both bacterial species were obtained from both middle ear fluids (Figure 3A) and bullar homogenates (Figure 3B). However, by day 7 postinfection, no pneumococci were recovered from the coinfect ed animals (Figure 3). Notably, although a significant percentage of animals infected with pneumococci developed systemic disease, no systemic disease was apparent in the coinfected animals (Table 3). To determine whether polymicrobial biofilms were formed and whether there was a difference in biofilm formation in animals infected with *H. influenzae*, pneumococcus, or both, the numbers of ears containing biofilm were enumerated, and biofilms were removed and analyzed by confocal laser scanning microscopy. There was no difference in the number of ears with biofilms in coinfection versus single infection animals, although animals infected with *S. pneumoniae* alone tended to form small biofilms, whereas coinfect ed animals formed large biofilms that filled the majority of the middle ear space (data not shown). Based on these data, we conclude that in the conditions of this experiment, *H. influenzae* and pneumococcus can establish a competitive relationship during otitis media infection. Moreover, coinfection appeared to moderate the progression of pneumococcal disease to fatal systemic infection (Table 3).
Figure 3. Bacterial competition during experimental otitis media coinfections. Total recovered colony-forming units (CFU) from the middle ears of infected animals on days 3 and 7 postinfection. All animals received inocula of $\sim 1 \times 10^3$ CFU of *Haemophilus influenzae* (Hi) and/or $\sim 150$ CFU of *Streptococcus pneumoniae* (Sp) as indicated. Each data point represents 1 ear. The dashed line indicates the limit of detection; the short solid and dashed lines represent the mean CFU for *H. influenzae* and *S. pneumoniae* for each group, respectively.
Table 3. Quantification of systemic disease. The number of animals that developed systemic disease in each experimental group was determined by plate counts of blood. The numbers in the parentheses in the experimental group column indicate the inoculating dose of *S. pneumoniae*. CFU, colony-forming units; Hi, *Haemophilus influenzae*; Sp, *Streptococcus pneumoniae*.
<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Portion that develop systemic disease</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp plus Hi (1 x 10^4 CFU)</td>
<td>0/12</td>
<td>3</td>
</tr>
<tr>
<td>Sp alone (1 x 10^2 CFU)</td>
<td>2/8</td>
<td>3</td>
</tr>
<tr>
<td>Sp plus Hi (1 x 10^4 CFU)</td>
<td>2/7</td>
<td>4</td>
</tr>
<tr>
<td>Sp plus Hi (1 x 10^3 CFU)</td>
<td>1/6</td>
<td>4</td>
</tr>
<tr>
<td>Sp plus Hi (1 x 10^4 CFU)</td>
<td>12/26</td>
<td>5</td>
</tr>
<tr>
<td>Sp, followed by Hi (1 x 10^4 CFU)</td>
<td>8/10</td>
<td>8A</td>
</tr>
<tr>
<td>Hi, followed by Sp (1 x 10^4 CFU)</td>
<td>0/10</td>
<td>8B</td>
</tr>
</tbody>
</table>
**Stable coinfections.** Although the above results clearly showed that *H. influenzae* could outcompete pneumococci in some infection conditions, it should be noted that the infectious doses used may have afforded a competitive advantage to *H. influenzae* simply because of greater numbers of these bacteria. Therefore, groups of animals were infected with either 1:1 or 10:1 ratios of *H. influenzae* to pneumococcus. No animals were given *S. pneumoniae* alone, because at these doses, the majority of animals would be expected to rapidly develop systemic disease (data not shown). Notably, at 3 and 7 days postinfection, *H. influenzae* and *S. pneumoniae* were present in equivalent numbers in the middle ears of mixed infection animals from both the 1:1 ratio and 10:1 ratio groups (Figure 4). At these inoculating doses, there was a marked difference in the prevalence of visible biofilm in the infection groups; 89% of the ears of mixed infection animals (16 of 18) contained biofilm, whereas biofilms were observed in 6 (50%) of 12 ears from animals infected with either species alone. In addition, biofilms from coinfectected animals were much larger and generally filled the entire middle ear chamber (data not shown). Confocal images of biofilms from mixed infection animals were very similar to those seen in the previous experiment, where both bacterial species are present in close proximity to one another.

With the results of these infection studies in hand, we asked how long a stable coinfection was maintained. Groups of chinchillas were infected with *H. influenzae* alone or with a 10:1 ratio of *H. influenzae* to *S. pneumoniae* and were euthanized at 7, 14, and 21 days postinfection. As in the preceding experiment, equivalent numbers of *H. influenzae* and *S. pneumoniae* were recovered from the middle ears of single- and mixed-infection animals at 7 days postinfection (Figure 5). On day 14, *S. pneumoniae* was not
Figure 4. Persistent polymicrobial infection during experimental otitis media coinfections. Symbols represent total recovered colony-forming units (CFU) of each species recovered from the middle ears of animals. All animals received \( \sim 1 \times 10^4 \) CFU of *Haemophilus influenzae* (Hi); coinfected animals also received \( \sim 1 \times 10^3 \) CFU (10:1 ratio) or \( \sim 1 \times 10^4 \) CFU (1:1 ratio) of *Streptococcus pneumoniae* (Sp). Each point represents 1 ear. The *dashed line* indicates the limit of detection; the *short solid* and *dashed lines* represent the mean CFU for *H. influenzae* and *S. pneumoniae* for each group, respectively.
Figure 5. Duration of stable polymicrobial infection. Symbols represent total recovered colony-forming units (CFU) from the middle ears of infected animals 7, 14, and 21 days postinfection. Coinfected animals received $\sim 1 \times 10^4$ CFU of *Haemophilus influenzae* (Hi) and $\sim 1 \times 10^3$ CFU of *S. pneumoniae* (Sp), whereas another group of animals received $\sim 10^4$ CFU of *H. influenzae*. Each point represents 1 ear. Dark and light colored symbols represent the right and left ears of 1 animal with respect to both bacterial species. The dashed line indicates the limit of detection; the short solid and dashed lines represent the mean CFU for *H. influenzae* and *S. pneumoniae* for each group, respectively.
recovered from the middle ears of every animal except one, where it was found in both ears. Interestingly, the only animal with *S. pneumoniae* recovered from its ears had the lowest levels of *H. influenzae* (light and dark blue circles and squares, respectively, Figure 5). In contrast to day 14, on day 21, the majority of coinfected animals had higher numbers of *S. pneumoniae* than *H. influenzae*. The most likely explanation for this is that, although *S. pneumoniae* was undetectable in the effusion, it was able to persist within biofilm communities or at a second site within the upper airway at day 14, and reestablished an infection when the conditions were more favorable.

There were equivalent numbers of ears with biofilm recovered from single infection and coinfected animals on days 7 and 14; however, on day 21, 4 of 6 ears from animals with mixed infection contained biofilm, whereas no biofilms were observed in animals infected with a single species (data not shown). Figure 6 shows the amount of biofilm recovered within the middle ear chamber of animals at 14 and 21 days postinfection (Figure 6A-6C). Cryosections of biofilms excised from the middle ear chamber were stained with mouse monoclonal anti-serotype 4 capsular antibody to detect *S. pneumoniae* or a polyclonal rabbit anti-*Haemophilus* antibody and were analyzed by confocal laser scanning microscopy. Confocal laser scanning microscopy images from coinfected animals showed the presence of both bacterial species at 7, 14, and 21 days postinfection (Figure 6D-6F). Three-dimensional images of polymicrobial biofilms recovered from infected chinchillas at 7, 14, and 21 days postinfection were constructed from stacked Z-series images collected by confocal laser scanning microscopy (Figure 7; see also Videos 1 [day 7], 2 [day 14], and 3 [day 21], which are available in the online version of the *Journal*).
Figure 6. Imaging of polymicrobial biofilms. A-C, Gross images of sectioned bullae removed from mock (A) and coinfected animals 14 (B) and 21 (C) days postinfection. D-F, Confocal laser scanning microscopy images of biofilms removed from mixed infection animals 7, 14, and 21 days postinfection. Green is staining for *Haemophilus influenzae*, and red is staining the capsule of *Streptococcus pneumoniae*. Yellow indicates colocalization of the 2 bacterial species.
Figure 7. This is an image from Video 1. Videos 1, 2, and 3 are available in the online version of the *Journal of Infectious Disease*. 
**Temporal variation of inoculation has no effect on bacterial persistence.** We next wanted to determine if a preexisting infection with one of the bacterial species would prevent colonization with the other bacterial species or alter the type of coinfection. To address this question, 2 sets of experiments were performed. In the first experiment, 3 groups of chinchillas were infected. The first received *H. influenzae* on day 0 and PBS on day 7, the second received *H. influenzae* on day 0 and *S. pneumoniae* on day 7, and the last received PBS on day 0 and *S. pneumoniae* on day 7. All animals were euthanized 14 days after the initial infection. One animal (dark and light green symbols; Figure 8A) had no recovery of *S. pneumoniae* and had high levels of *H. influenzae*, similar to results seen in Figure 3. At the other end of the spectrum, 1 animal (light and dark orange symbols; Figure 8A) had no *H. influenzae* in either ear but had high numbers of *S. pneumoniae*. The other 3 coinfected animals had equivalent numbers of both bacterial species in each ear. Although the outcome varied with each animal, the experiment clearly shows that a preexisting *H. influenzae* infection does not prevent *S. pneumoniae* from establishing an infection. However, no systemic disease was observed in animals with preexisting *H. influenzae* infection (Table 3).

All ears from coinfected animals contained biofilm (10 of 10), compared with 4 of 8 ears from *S. pneumoniae*-infected animals and 6 of 8 ears from *H. influenzae*-infected animals. As observed in other experiments, biofilms from coinfected animals consistently appeared larger than those from animals infected with either species alone. In the second set of experiments, we wanted to determine whether a preexisting *S. pneumoniae* infection could prevent *H. influenzae* from establishing an infection. The same experimental groups were used, except the timing of inoculation of *H. influenzae*
**Figure 8. Temporal variation of coinfections.** Counts were obtained from middle ear fluids or bullar homogenates 14 days after initial infection. *A*, Animals received either *Haemophilus influenzae* (Hi) or phosphate-buffered saline (PBS) on day 0 and *Streptococcus pneumoniae* (Sp) or PBS on day 7. *B*, Animals received either *S. pneumoniae* or PBS on day 0 and *H. influenzae* or PBS on day 7. Each point represents 1 ear. Dark and light colored symbols represent the right and left ears of 1 animal with respect to both bacterial species. The dashed line indicates the limit of detection; the short solid and dashed lines represent the mean colony-forming units (CFU) for *H. influenzae* and *S. pneumoniae* for each group, respectively.
and *S. pneumoniae* was reversed. Groups of animals were given either *S. pneumoniae* or PBS on day 0. The majority of animals infected with *S. pneumoniae* developed systemic disease and were, thus, euthanized before day 7 (Table 3). The 2 surviving animals that received *S. pneumoniae* and the 4 animals given PBS on day 0 were inoculated with *H. influenzae* on day 7. All animals were euthanized on day 14. The 2 coinfect ed animals established stable coinfections containing equivalent numbers of *H. influenzae* and *S. pneumoniae* in their middle ears (Figure 8B), showing that a preexisting *S. pneumoniae* infection does not prevent *H. influenzae* colonization. All four ears from coinfect ed animals contained visible biofilms, and immunofluorescence showed the presence of both bacterial species (data not shown).

**The presence of *H. influenzae* increases pneumococcal biofilm formation in vitro.** One observation made during the in vivo studies was that *S. pneumoniae* formed biofilms with a greater frequency and of a larger size in the presence of *H. influenzae* than when alone. Thus, the impact of *H. influenzae* on pneumococcal biofilm formation was assessed using an in vitro static biofilm assay. The results show significantly greater numbers of *S. pneumoniae* in surface-attached communities containing *H. influenzae* than in those containing pneumococcus alone (Figure 9A). Of note, these results were more dramatic when performed in chemically defined minimal media (Figure 9B).

**Coinfection increases the translucent colony type within pneumococcal populations in vivo.** Pneumococci undergo phase-variation between 2 distinct colony phenotypes; opaque colonies produce greater amounts of capsular polysaccharide and are associated with systemic infections, whereas translucent colonies have less capsular polysaccharide
Figure 9. *Haemophilus influenzae* (Hi) promotes pneumococcal biofilm formation in vitro. Total recovered *Streptococcus pneumoniae* colony-forming units (CFU) from 48 h static biofilms in supplemented brain heart infusion (*A*) or minimal media (*B*). The black bars represent the *S. pneumoniae* recovered from coinfection with *H. influenzae* and the white bars represent the *S. pneumoniae* recovered from *S. pneumoniae* alone static biofilms. Bars represent the mean ± the standard error of the mean from 4 independent wells from 1 representative experiment. Statistical significance was assessed using a Mann-Whitney nonparametric *t* test. **P < .005.**
and more choline-containing cell wall teichoic acid on their surfaces. Notably, the translucent populations predominate during the early stages of infection and within the tissue-associated populations that presumably include biofilms. Thus, we hypothesized that *H. influenzae* may influence pneumococcal colony phenotype. If this were the case, we would expect to see a higher percentage of translucent colonies in *S. pneumoniae* removed from mixed infection animals than in animals that received *S. pneumoniae* alone. To address this hypothesis, animals were infected with *H. influenzae* alone, *S. pneumoniae*, or both organisms. The colony type within the pneumococcal inocula was determined to be ~40% translucent. Animals were euthanized 7 days postinfection, and colony counts and opacity from both the effusion (planktonic bacteria) and homogenized bullae (surface-associated bacteria) were assessed. All infection groups established stable middle ear infections with equivalent numbers of both bacterial species in both the effusion and homogenized bullae (Figures 10A and 10B). In the effusion, the percentage of translucent colonies decreases to ~20% for both *S. pneumoniae* alone and *S. pneumoniae* plus *H. influenzae* animals (Figure 10C). However, in the bullae, the percentage of translucent colonies increased significantly to ~50% in mixed infection animals and decreased to ~20% in animals infected with *S. pneumoniae* alone animals (Figure 10C, p = .015).
Figure 10. Coinfection with *Haemophilus influenzae* (Hi) promotes persistence of pneumococcal translucent colony variants. Total recovered colony-forming units (CFU) from the middle ears (*A*) and homogenized bullae (*B*) of mixed and single infection animals 7 days postinfection. Animals were given \(\sim 1 \times 10^4\) CFU of *H. influenzae* alone or \(\sim 1 \times 10^4\) CFU of *H. influenzae* and \(\sim 1 \times 10^4\) CFU of *Streptococcus pneumoniae* (Sp). *S. pneumoniae* alone animals only received 100 CFU. Each point represents 1 ear. The *dashed line* indicates the limit of detection; the *short solid* and *dashed lines* represent the mean CFU for *H. influenzae* and *S. pneumoniae* for each group, respectively. *C*, The percentage of translucent *S. pneumoniae* colonies from each infection group from the effusion and bullae. Bars are the mean ± standard error of the mean from 6 ears. Statistical significance was assessed using a Mann-Whitney nonparametric *t* test. *P = .015.*
DISCUSSION

Although it is clear that the majority of otitis media infections involve multiple species, most of our current knowledge regarding the bacterial pathogenesis of otitis media has been derived from infections using pure cultures of single organisms. Because *H. influenzae* and pneumococcus collectively account for the majority of otitis media infections [390], it is of particular importance to understand how these species influence one another. Other work has provided clear evidence for modulation of pneumococcal disease by *H. influenzae*, most notably by priming enhanced host innate responses to clear the pneumococcal infection [71, 72, 392]. The results of our work clearly establish that preceding or concurrent infection with *H. influenzae* moderates the progression and severity of pneumococcal disease in chinchillas. Additional studies with phagocytes in vitro showed no difference in pneumococcal killing in accordance with coinfection (data not shown).

Our data also clearly show that the presence of *H. influenzae* significantly increases the percentage of translucent pneumococcal colonies, compared with the populations recovered from animals that received pneumococcus alone. Translucent pneumococcal variants have a decreased propensity toward systemic infections [398] and instead are more adherent to abiotic and host cell surfaces [399]. Work from a number of groups has established that biofilm communities contain predominantly translucent colony variants [400-404]. Notably, the *H. influenzae* biofilm matrix consists of sialylated lipooligosaccharides [136, 396, 405]. Recent work has clearly established that pneumococcal neuraminidases promote biofilm formation [406], a result that is
recapitulated in the presence of free sialic acid [407]. The observed enhancement of pneumococcal biofilm formation by *H. influenzae* was unaffected in neuraminidase mutant strains or *H. influenzae siaB* mutants lacking sialylated matrix [396], which would seem inconsistent with a specific role for sialic acid in this phenotype. It may be that *H. influenzae* plays a more generic role, such as enhanced retention of pneumococci on a surface with an established biofilm matrix scaffold. Based on our results, we conclude that enhancement of pneumococcal persistence within the biofilm phase on mucosal surfaces can delay or even ablate the progression to systemic disease. It is important to note that in contrast to the systemic infections observed in most animal model systems, the majority of pneumococcal infections in patients are localized mucosal infections. It is clear from many studies that particular host immunodeficiencies (most notably, complement deficiencies) can predispose to systemic infection with pneumococci and other bacteria [408, 409]. In light of the results of this study, it may be important to consider the possibility that systemic pneumococcal infection results not only from host susceptibility but also from infection and colonization by a bacterial population in which pneumococci predominate. It is equally important to consider the possibility that elimination of one or more components of the nasopharyngeal microbiota by vaccination or therapy may have unforeseen sequelae, not only in terms of opening host niches for colonization but also, potentially, by changing the course of infection by opportunists within this population.
CHAPTER II:

Divergent Mechanism for Passive Pneumococcal Resistance to β-lactam Antibiotics in the Presence of *Haemophilus influenzae*


The following manuscript was accepted for publication to the *The Journal of Infectious Disease* in October 2010 and is reprinted with permission. Stylistic variations are due to the requirement of the journal. K.E.D. Weimer performed the experiments and prepared the manuscript. Dr. W. Edward Swords acted in an advisory and editorial capacity. Copyright © University of Chicago Press, 2010.
INTRODUCTION

Otitis media (OM) is one of the leading public health problems in pediatrics, with most children experiencing at least one episode by 3 years of age and 40% of older children having six or more total episodes [303]. Common causes of OM include nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* (pneumococcus) and *Moraxella catarrhalis*; NTHi and pneumococcus account for ~75% of infections [62]. In recent years, epidemiologic studies have shown that coinfections with multiple bacterial species are an important part of both acute and chronic OM, particularly coinfections with NTHi and pneumococcus [345, 378, 379].

As with many upper airway infections, treatment failure and antibiotic resistant organisms are common problems in OM, with failure rates as high as 50% in some populations [318-321]. The majority of strains of both NTHi and *M. catarrhalis* produce a β-lactamase [322-324]. β-lactam resistance among pneumococcal strains is much less common, although some strains (~20%) have developed resistance through mutation of the penicillin binding protein [322].

Common theories proposed to explain OM treatment failure involving antibiotic susceptible bacterial strains are the formation of biofilm communities and passive protection by other bacterial pathogens that produce a β-lactamase [74, 328, 383, 384]. Bacteria in biofilms are known to be more resistant to antibiotic killing than planktonic bacteria [142, 144, 339, 410]. Previous studies have also shown *Moraxella catarrhalis* can passively protect pneumococcus from antibiotic killing in a mouse pneumonia model.
and in biofilms in vitro, this protection is abolished if the β-lactamase is inhibited [385, 386].

In this study, we used a β-lactamase producing strain of NTHi and an isogenic mutant deficient in β-lactamase production to distinguish between the roles of biofilm formation and β-lactamase production in NTHi antibiotic resistance. In addition, these strains were utilized to determine if NTHi provides passive protection for pneumococcus, and, if so, the mechanism behind this protection.
MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Nontypeable *Haemophilus influenzae* 86-028NP (NTHi 86-028NP) is an OM isolate that has been fully sequenced and is known to cause OM featuring biofilms in the chinchilla infection model [133, 135, 136, 146, 147, 395]. Bacteria were grown on brain heart infusion agar supplemented with hemin (ICN Biochemicals) and nicotinamide adenine dinucleotide (Sigma) and 3 µg/mL of vancomycin (Sigma). *Streptococcus pneumoniae* TIGR4 is a well-studied clinical isolate for which a complete genomic sequence is available, and which we have recently shown to form biofilms during experimental OM infection [208, 393]. Pneumococci were grown on trypticase soy agar (TSA, BD) with 5% sheep’s blood (Hemostat Laboratories) or in supplemented BHI (sBHI) media with 10% horse serum.

**Generation of β-lactamase deficient mutant (NTHi 86-028NP bla).** A ~2-kb DNA fragment containing the β-lactamase triethylene melamine (TEM) open reading frame (NTHi 2055) was amplified from 86-208NP genomic DNA using βlac forward (TGG TTA CGC TCG GGT CTC AA) and βlac reverse (ATG GCA CAA GTT ACA CGA TTC AA) primers with an annealing temperature of 55.9°C and extend time of 2.5 min. The fragment was ligated into pCR2.1 (Invitrogen) and transformed into *E. coli* according to the manufacturers’ instructions to generate pCR-βlac. pCR-βlac was digested with HincII, a unique site within the β-lactamase gene, then dephosphorylated with Calf Intestinal Phosphatase (New England Biolabs). This fragment was then ligated with a chloramphenicol resistance cassette [411] cut with SmaI and transformed into *E. coli*, generating pCR-βlacCm. pCR-βlacCm was linearized with NotI and introduced
into NTHi 86-028NP via natural transformation, as described previously, to generate NTHi 86-028NP bla::Cm (NTHi 86-028NP bla) [146, 147]. Colonies that grew on sBHI containing chloramphenicol (1.5 µg/mL) were screened using primers flanking the site of insertion, forward (GAT GCT GAA GAT CAG TTG GG) and reverse (GTA TGG CTT CAT TCA GCT CC) with an annealing temperature of 52°C and extend time of 2.5 min to find (86-028NP)βlacCm.

**Amoxicillin susceptibility.** Bacteria were harvested from overnight sBHI plates, resuspend in phosphate buffered saline (PBS) and diluted to the desired concentration. To determine planktonic MICs, ~10^6 CFU of NTHi 86-028NP or NTHi 86-028NP bla were resuspended in 5 mL of sBHI with varying concentrations of amoxicillin (Sigma). Cultures were grown for 16-20 h at 37°C, shaking at 150 rpm. Turbidity of the media was used to determine bacterial growth and survival. To determine biofilm susceptibility to amoxicillin, ~10^7 CFU of NTHi 86-028NP or NTHi 86-028NP bla were plated in 1.5 mL of sBHI in 24-well plates (Falcon) and incubated at 37°C and 5% CO₂. After 24 h, the supernatant was removed from each well and replaced with 1.5 mL of sBHI containing various concentrations of amoxicillin (Sigma). After 48 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping, resuspended in 0.2 ml of sterile PBS, diluted and plated for bacterial counts.

**Live/dead staining.** NTHi 86-028NP and NTHi 86-028NP bla biofilms were grown in sBHI in Tab-TekII cover glass slides (Nunc). After 24 h, biofilms were washed with sterile PBS and stained using BacLight LIVE/DEAD staining according to the protocol (Molecular Probes). Briefly, biofilms were incubated with 1 mL of PBS that contained a
mixture of SYTO 9 and propidium iodide for 15 min, then washed 3 times and PBS. Samples were visualized using a Zeiss LSM510 confocal scanning laser microscope. Z-series images were exported into MATLAB (Version 5.1), and COMSTAT analysis were performed using the Image Processing Toolbox as previously described [412, 413].

**Biofilm protection assay.** NTHi 86-028NP or NTHi 86-028NP bla (~10^7 CFU) were plated in each well of a 24-well plate (Falcon) in a total volume of 1.5 mL of sBHI and incubated at 37°C and 5% CO₂. After 24 h, supernatants were removed and replaced with 1.5 mL of sBHI + 10% horse serum. *S. pneumoniae* TIGR4 (~10^7 CFU) was added to coinfection and *S. pneumoniae* alone wells at this time. After 48 h, the supernatant was removed and replaced with sBHI with or without amoxicillin. After 72 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping, resuspended in 0.2 mL PBS, diluted and plated for bacterial counts.

**Chinchilla infections.** Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher’s chinchilla ranch (LaRue, OH) and allowed to acclimate to the vivarium for 1 week prior to infection. All animals were examined by otoscopy prior to infection, and none had any clinical signs of middle ear infection or other overt disease. The chinchilla infection protocols were performed essentially as described previously [133, 146, 147, 208]. On day 0, NTHi 86-028NP and NTHi 86-028NP bla were harvested from a plate and a freezer stock of known CFU/mL of *S. pneumoniae* TIGR4 was thawed, both were diluted using sterile PBS, and bacterial density was confirmed by plate-count. Chinchillas (3-5 animals/group/time point) were anesthetized with isofluorane and inoculated via transbullar injection with 100 µL of bacterial suspension
containing NTHi 86-028NP, NTHi 86-028NP bla, S. pneumoniae TIGR4, NTHi 86-028NP and S. pneumoniae TIGR4 or NTHi 86-028NP bla and S. pneumoniae TIGR4, as indicated in the text. Infectious doses ranged from $10^2$-10$^4$ CFU, as indicated for each experiment. On days 4, 5 and 6, groups of animals were injected with either 20 µg of amoxicillin (100 µL of 200 µg/mL, Sigma) or 100 µL of sterile PBS directly in the middle ear. Oral administration of the antibiotic was contraindicated due to the risk of developing a Clostridium difficile infection [414]. All animals were euthanized 7 days postinfection. Animals exhibiting overt symptoms of systemic disease were euthanized prior to day 7. After euthanasia, the superior bullae were opened to expose the middle ear cavity as previously described [146], and the presence of visible biofilm formation was assessed. If present, middle ear effusion fluids were collected. The middle ear cavity was lavaged with 1 mL of sterile PBS. Effusion and lavage fluids were combined, serially diluted and assessed by plate-count. For animals that received both bacteria, fluid was plated on two separate plates, 1 selective for NTHi (sBHI + vancomycin) and 1 selective for S. pneumoniae (TSA + sheeps blood + gentamicin). Middle ear bullae were aseptically removed and homogenized using a PowerGen 1000 homogenizer (Fisher Scientific); the bullar homogenates were plated to assess tissue-associated bacterial load. All of the chinchilla infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.
RESULTS

*Generation and confirmation of NTHi β-lactamase mutant.* To determine the role that β-lactamase secretion plays in NTHi antibiotic resistance, an isogenic β-lactamase deficient mutant of NTHi 86-028NP was generated (NTHi 86-028NP *bla*) by insertional disruption of the NTHi 2055 gene (NCBI Reference Sequence NC_007146) with a chloramphenicol resistance cassette. There was no difference in growth of NTHi 86-028NP and NTHi 86-028NP *bla* (data not shown). There was also no difference in *in vitro* biofilm formation between the two strains in terms of biomass, average thickness, maximum thickness and surface to biovolume ratio, as determined by COMSTAT analysis of confocal laser scanning microscopy Z-series of 24 h biofilms (Figure 11). To confirm the β-lactamase negative phenotype of the mutant, the minimal inhibitory concentration of amoxicillin (a β-lactam antibiotic) for both NTHi 86-028NP and NTHi 86-028NP *bla* was determined. The MIC for the NTHi 86-028NP was ≤ 32.0 µg/mL of amoxicillin (Table 4). The MIC of NTHi 86-028NP *bla* was ≤ 1.0 µg/mL, 32 fold less than the parental strain (Table 4). The inactivation of the β-lactamase gene rendered an amoxicillin resistant strain of NTHi amoxicillin susceptible, confirming the phenotype of NTHi 86-208NP *bla*.

*Role of β-lactamase in NTHi biofilm antibiotic resistance in vitro.* To determine the role that β-lactamase production plays in NTHi antibiotic resistance in biofilm, the susceptibility of NTHi 86-028NP and NTHi 86-028NP *bla* biofilms to amoxicillin killing was determined. Various concentrations of amoxicillin were added for 24 h to preformed, 24 h NTHi biofilms of each strain (0 to 2 mg/mL). NTHi 86-028NP was
Figure 11. COMSTAT analysis of NTHi in vitro biofilms. NTHi 86-028NP (white bars) and NTHi 86-028NP bla (black bars) 24 h biofilms were washed, stained with a Live/dead stain, visualized using confocal laser scanning microscopy and then analyzed using COMSTAT for A. average thickness, B. maximum thickness, C. biomass and D. surface to biovolume ratio. The bars represent the mean ± the standard error of the mean of 8 replicates. Statistical significance was assessed by Mann-Whitney nonparametric analysis.
A. Average Thickness

B. Maximum Thickness

C. Biomass

D. Surface to Biovolume Ratio
Table 4. Minimal inhibitory concentration of amoxicillin. The MIC of amoxicillin was determined for NTHi 86-028NP and NTHi 86-028NP bla in planktonic culture. +/- indicate the presence/absence of growth which was determined looking at the turbidity of the culture.
<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
<th>16.0</th>
<th>32.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NTHi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>86-028NP</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><strong>NTHi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>86-028NP blal</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
resistant to killing at all concentrations of amoxicillin tested, with no decline in recovered CFU at any concentration (Figure 12). Interestingly, while NTHi 86-028NP bla was much more susceptible than NTHi 86-028NP to amoxicillin killing in planktonic culture (MIC 32-fold less), it was resistant to amoxicillin killing in biofilm at all of the concentrations tested (Figure 12). There was a significant decrease in the recovered CFU of NTHi 86-028NP bla from biofilms compared to the parental strain at all antibiotic concentrations tested; however, this difference was less than 1-log and there were still ~10^8 CFU recovered at all concentrations tested. While slightly less resistant than the parental strain, NTHi 86-028NP bla biofilms are very resistant to amoxicillin killing in vitro. Based on these data, the production of a β-lactamase is necessary for resistance of NTHi to amoxicillin in planktonic culture, but it does not appear to be required for biofilm resistance in vitro.

Role of β-lactamase in NTHi amoxicillin resistance in chinchilla model of otitis media.

While the previous experiment showed β-lactamase production was not necessary for biofilm resistance to amoxicillin in vitro, we wished to confirm this result in vivo using the chinchilla model of experimental OM. It has been previously established by our lab and others that NTHi establishes a chronic infection in the middle ears of chinchillas, with biofilm formation occurring by day 3 in most animals [133, 147]. The infection/treatment timeline used in this experiment was set up to as closely mimic the course that pediatric patients frequently experience as possible, in keeping with the limitations of the animal model. On day 0, ~10^3 CFU of NTHi 86-028NP or NTHi 86-028NP bla were inoculated directly into the middle ears of chinchillas. The infection was
**Figure 12. Antibiotic susceptibility of NTHi in vitro biofilms.** Varying concentrations of amoxicillin (0 to 2 mg/mL) were added to preformed NTHi 86-028NP (white bars) and NTHi 86-028NP bla (black bars) biofilms. The amounts of amoxicillin on the x-axis indicated concentration per mL added to each well. After 24 h, biofilms were scraped, serially diluted and plated for bacterial counts. Bars represent the mean ± standard error of the mean. Graph represents data combined from three experiments (8-9 replicates total). Statistical significance was assessed by Mann-Whitney nonparametric analysis. *P < 0.05, **P < 0.005, ***P < 0.0005.
Recovered Biofilm CFU

**NTHi 86-028NP**

**NTHi 86-028NP bla**
allowed to establish for 3 days. On days 4, 5 and 6 postinfection, 20 µg of amoxicillin or sterile PBS were injected directly into the middle ear. The concentration of amoxicillin in the middle ear following oral antibiotic treatment in children is 1-8 µg/mL [319]. The dose in our experiment is a higher concentration than these bacteria would normally experience, allowing for a stringent test of NTHi resistance. On day 7, all of the animals were euthanized and the effusion and bullae were harvested and plated for bacterial counts.

In animals infected with NTHi 86-028NP, there was no difference in recovered CFU from animals treated with amoxicillin or PBS in either the effusion (planktonic bacteria) or bullar homogenate (tissue-associated/biofilm bacteria) (Figure 13). This confirms what was seen in vitro, where a β-lactamase producing strain of NTHi was resistant to amoxicillin killing. However, in animals infected with the β-lactamase deficient mutant of NTHi, there were no detectable bacteria in either the effusion or the bullar homogenate of the group treated with amoxicillin, while the group treated with PBS had bacterial counts equivalent to those of animals infected with the parental strain (Figure 13). These results are contrary to what was seen in vitro and demonstrate that β-lactamase production is required for NTHi resistance to amoxicillin in the chinchilla model of OM. Biofilm formation in the PBS treated animals was within the normal range seen at this time postinfection for animals infected with either the parental strain (5/6) or the β-lactamase deficient mutant (3/6) of NTHi. Either biofilm formation by NTHi is not protective in this model, or the biofilms formed by NTHi 86-028NP bla were not sufficient to provide protection against this concentration of amoxicillin.
Figure 13. *NTHi* amoxicillin resistance in chinchilla model of otitis media.

Chinchillas were infected with either ~$10^3$ of *NTHi* 86-028NP or *NTHi* 86-028NP *bla* on day 0. On days 4, 5 and 6, animals were treated with either 20 µg of amoxicillin (white squares) or sterile PBS (black circles) directly into the middle ear. All animals were harvested on day 7 postinfection. The left half of the graph represents counts from the effusion (planktonic bacteria), the right half is from bullar homogenates (tissue-associated/biofilm bacteria). The long dashed line represents the limit of detection. The short solid lines represent the mean CFU for each group.
Effusion Bullar Homogenate

Total Recovered CFU

- + PBS
- + amoxicillin

NTHi 86-028NP NTHi 86-028NP bla NTHi 86-028NP NTHi 86-028NP bla
**NTHi passive protection of *S. pneumoniae* in vitro.** Because NTHi and *S. pneumoniae* are frequently found together in the middle ear, we wanted to determine if NTHi could provide passive protection for pneumococcus against amoxicillin killing. *S. pneumoniae* is extremely susceptible to killing by β-lactam antibiotics [415]. Both *S. pneumoniae* planktonic and biofilm bacteria were completely killed by as little as 0.064 µg/mL of amoxicillin *in vitro* (Table 4 and Figure 14). To determine if NTHi provides passive protection for *S. pneumoniae* within a biofilm *in vitro*, biofilms containing NTHi 86-028NP and *S. pneumoniae* were treated with amoxicillin. NTHi 86-028NP completely protected pneumococcus from amoxicillin killing, with no difference in recovered pneumococcal CFU in wells treated with amoxicillin and media alone (Figure 14). This passive protection extended to at least 500 µg/mL of amoxicillin (data not shown). Interestingly, although NTHi 86-028NP *bla* viability was not affected by treatment with amoxicillin and its biofilms have a similar structure to that of the parental strain (Figure 11), NTHi 86-028NP *bla* provided no protection for pneumococcus with as little as 0.064 µg/mL of amoxicillin (Figure 14). These data clearly demonstrate that NTHi provides passive protection for pneumococcus *in vitro* within a biofilm through the production of a β-lactamase.

**NTHi passive protection of *S. pneumoniae* in the chinchilla model of experimental otitis media.** To determine if NTHi provides protection against amoxicillin killing for pneumococcus *in vivo*, and if this protection was dependent on the production of β-lactamase, a coinfection was done using the chinchilla model. It has been previously established by our laboratory that NTHi and pneumococcus establish a chronic
Figure 14. **NTHi passive protection of *S. pneumoniae* in vitro.** Media alone or 0.064 µg/mL of amoxicillin were added to preformed biofilms containing *S. pneumoniae* (Sp) alone (white bars), *S. pneumoniae* and NTHi 86-028NP (grey bars with black lines) or *S. pneumoniae* and NTHi 86-028NP bla (black bars). 24 h after the addition of antibiotics, the biofilms were scraped, serially diluted and plated for *S. pneumoniae* bacterial counts. Bars are the mean ± the standard error of the mean of quadruplicate wells. Graph is representative of three independent experiments. * P < 0.05
Total Recovered CFU

- Sp Alone
- Sp + NTHi 86-028NP
- Sp + NTHi 86-028NP bla

None
Amoxicillin
coinfection in this model with the formation of very large biofilm communities (larger than with either bacterial species alone) that contain both bacterial species [416]. Chinchillas were infected with either NTHi 86-028NP and pneumococcus, NTHi 86-028NP bla and pneumococcus or pneumococcus alone directly into the middle ear. The same infection/treatment timeline and doses were used as in the previous chinchilla experiment (Figure 13). Briefly, animals were infected on day 0, treated with 20 µg of amoxicillin or PBS on days 4, 5 and 6 and euthanized and processed on day 7.

In the animals infected with pneumococcus alone, ~10^8 CFU were recovered from both the effusion (planktonic bacteria) and bullar homogenates (biofilm/surface-associated bacteria) of PBS treated animals (Figure 15A and 15B). In contrast, there were no detectable bacteria in the effusion and recovered CFU were right at the limit of detection in bullar homogenates of pneumococcus alone animals treated with amoxicillin (Figure 15A and 15B). This confirms that the dose of amoxicillin given essentially clears the infection in animals given pneumococcus alone.

In animals that were coinfected with NTHi 86-028NP and pneumococcus and treated with PBS, equivalent numbers of both bacterial species were recovered from both the effusion and bullar homogenate, as expected (Figure 15A and 15B). Interestingly, in animals in this group that were treated with amoxicillin, pneumococcus was recovered from all 4 effusions and 3/4 bullar homogenates, with a mean recovered CFU of ~10^7 (Figure 15A and 15B). The recovered CFU of NTHi was equivalent to that of PBS treated animals (Figure 15A and 15B). These results are similar to what was seen in vitro (Figure 14) and clearly demonstrate that NTHi passively protects pneumococcus in the chinchilla OM model.
Figure 15. **NTHi passive protection of *S. pneumoniae* in chinchilla model of otitis media.** Chinchillas were infected with either \( \sim 10^3 \) of NTHi 86-028NP and \( \sim 10^3 \) of *S. pneumoniae* (NTHi 86-028NP + Sp), \( \sim 10^3 \) of NTHi 86-028NP bla and \( \sim 10^3 \) of *S. pneumoniae* (NTHi 86-028NP bla + Sp) or \( 10^2 \) CFU of *S. pneumoniae* alone (Sp alone) on day 0. On days 4, 5 and 6, animals were treated with either 20 µg of amoxicillin (+ Amox) or sterile PBS (+ PBS) directly into the middle ear. All animals were harvested on day 7 postinfection. The effusion (A.) and bullar homogenate (B.) were serially diluted and plated for bacterial counts. The white circles represent the recovered CFU for NTHi; the black circles represent the recovered CFU for *S. pneumoniae*. The long dashed line represents the limit of detection. The short solid lines represent the mean CFU for NTHi in each group and the short dashed lines represent the mean CFU for pneumococcus in each group.
In animals that were coinfectected with the β-lactamase deficient mutant of NTHi and pneumococcus and treated with PBS, equivalent numbers of both species were recovered from the effusion and bullar homogenate, as seen with the parental strain (Figure 15A and 15B). In the animals in this group that were treated with amoxicillin, no pneumococcus was detected in the effusion; however, NTHi 86-028NP bla was recovered from the effusion in 5/6 ears, with a mean CFU of ~10^7 (Figure 15A and 15B). In addition, NTHi 86-028NP bla was recovered from the bullar homogenate of 6/6 ears and pneumococcus was recovered from the bullar homogenate of 5/6 ears in the amoxicillin treated animals (Figure 15A and 15B). This is in stark contrast to what was seen in Figure 11, where animals infected with NTHi 86-028NP bla alone and treated with amoxicillin had no detectable bacteria in either the effusion or bullar homogenate. This is also contrary to what was seen in the in vitro passive protection assay (Figure 14), where NTHi 86-028NP bla provided no protection for pneumococcus. These data indicate that, while β-lactamase production may be necessary for passive protection in vitro, it is not required for NTHi passive protection of pneumococcus against amoxicillin killing in vivo. In addition, these studies indicate that the presence of pneumococcus in the middle ear is affording some sort of protection for NTHi, even though pneumococcus is very susceptible to amoxicillin killing.
DISCUSSION

As antibiotic treatment failure becomes increasingly common, it is important to understand bacterial mechanisms of antibiotic resistance so that this knowledge can be applied to improve treatment. It is known that biofilm formation and the production of a β-lactamase both contribute to NTHi antibiotic resistance; however, the contribution of each during an infection and the role in passive protection of other bacterial species had not been elucidated.

We have shown that, in vitro, NTHi 86-028NP and NTHi 86-028NP bla biofilms are both resistant to amoxicillin killing at all concentrations tested, 0 to 2 mg/mL (Figure 12). Despite this, only NTHi 86-028NP was able to passively protect pneumococcus from amoxicillin killing in biofilms in vitro (Figure 14). When the antibiotic resistance of NTHi 86-028NP was tested in the chinchilla model, equal numbers of CFU were recovered from the effusion (planktonic bacteria) and bullar homogenate (biofilm/tissue-associated bacteria) regardless of whether the animal was treated with PBS or amoxicillin (Figure 13). However, NTHi 86-028NP bla was cleared from both the effusion and bullar homogenate of every animal. There are two possible explanations for this. The first is that NTHi 86-028NP bla did not form sufficient biofilms to protect against the continuous, large doses of amoxicillin given. If this were the case, NTHi 86-028NP bla might persist if a lower antibiotic concentration were given, or if biofilm formation was increased. The other possibility is that biofilm formation is not protective in vivo. Taken together, these data indicate that, while the production of β-lactamase is not required for
NTHi antibiotic resistance *in vitro*, it is necessary for *in vivo* NTHi resistance and *in vitro* passive protection of pneumococcus.

In this study, we also demonstrated that NTHi provides passive protection for pneumococcus in the chinchilla model (Figure 15). When coinfected with NTHi 86-028NP, pneumococcus was recovered from both the bullar homogenate and effusion of the majority of animals. Interestingly, when coinfected with NTHi 86-028NP *bla*, both NTHi and pneumococcus were recovered from the bullar homogenate, but only NTHi was recovered from the effusion (Figure 15). These data point towards two separate mechanisms of NTHi protection of pneumococcus from amoxicillin killing *in vivo*. The first is through the production of β-lactamase, which provides strong protection in both the effusion and bullar homogenate. The second mechanism is through the formation of biofilm communities. As mentioned previously, NTHi and pneumococcus form a much larger biofilm together than either bacterium does on its own [416]. This larger mixed biofilm appears to have provided protection against amoxicillin killing for both pneumococcus and NTHi 86-028NP *bla*, both of which were cleared when inoculated on their own and treated with amoxicillin. In contrast, prior work has shown a lack of protection by a β-lactamase producing strain of NTHi in a rat experimental OM model [388]. Potential differences in the two studies that could explain the differences seen are the route/dose of amoxicillin and the animal model used. In the previous study, amoxicillin was given in the water and reached an average serum concentration of ~4 µg/mL. While that is more clinically relevant than our method of delivery, giving the antibiotic directly in the middle ear allowed the concentration in the middle ear to be the known and the kept same for all animals in a group. Additionally, the concentration of
amoxicillin in our study was much higher, 20 µg, and should provide a more stringent test of protection. The most important difference in the studies is that biofilm formation occurs in the majority of chinchillas with OM, while it is unclear if biofilms are formed in the rat model of OM. This could explain the differences in protection seen in the two studies, and would indicate an even larger role for biofilm formation in passive protection.
CONCLUSIONS

Epidemiologic studies have established that most upper airway infections, including OM, are coinfections involving multiple bacterial species [345, 378, 379]. However, most studies in bacterial pathogenesis have been performed using pure cultures of a single bacterial species. There is a need to extend these studies to polymicrobial infections to better understand the interactions that are taking place in infections in patients and how these affect disease and treatment. Because *H. influenzae* and *S. pneumoniae* are the leading causes of OM, as well as sinusitis, community-acquired pneumonia and COPD exacerbations, my research focused on coinfection with these two bacteria [22, 62, 232-235]. The goal of my research was to define the impact of coinfection with *H. influenzae* and *S. pneumoniae* on bacterial clearance/persistance in OM. In addition, the roles of two different mechanisms of antibiotic resistance, biofilm formation and β-lactamase production, and the impact of coinfection with *H. influenzae* and *S. pneumoniae* on these was examined.

**Coinfection Studies**

When chinchillas are infected in the middle ear with *S. pneumoniae* alone, even at very low doses, the disease can quickly progress to bacteremia/systemic disease and lead to death (Table 3). As the inoculating dose of *S. pneumoniae* increases, the number of animals that develop systemic disease also increases (Supplemental Figure 1). However, when *S. pneumoniae* was inoculated either with or after *H. influenzae*, pneumococcal progression to systemic disease was limited (Figures 3-5, 8 and 10). With a low
inoculating dose of *S. pneumoniae*, pneumococcus was cleared from the middle ear in the presence of *H. influenzae* (Figure 3). At a higher inoculating dose of *S. pneumoniae*, *H. influenzae* and pneumococcus established a chronic coinfection with the formation of large biofilm communities (Figures 2, 3 and 10). Some coinfected animals did develop systemic disease, but the percentage was much lower than that of animals infected with a high dose of *S. pneumoniae* alone (Table 3). We also found that the presence of *H. influenzae* in the middle ear increased the percentage of translucent pneumococcal colonies recovered from homogenized bullae, which represent tissue-associated and biofilm bacteria (Figure 10C). Translucent colonies (less capsule) are more efficient at colonizing mucosal surfaces and forming biofilms, while opaque colonies (more capsule) are resistant to opsonophagocytosis and are associated with systemic disease [169-171, 176, 178, 210, 215-218]. Having a higher percentage of the translucent colony variant of pneumococcus provides an explanation for the decrease in pneumococcal systemic disease and increase in pneumococcal biofilm formation observed in these studies in the presence of *H. influenzae*.

Importantly, in these studies it was noted that biofilms formed in coinfected animals were much larger, filling the entire middle ear space, than biofilms formed in animals infected with either bacterial species alone (Figure 6). This appears to be a synergistic increase in biomass, rather than just additive. Biofilms removed from coinfected animals contained equivalent amounts of both bacterial species in close association with one another (Figure 6B). We also demonstrated that *H. influenzae* increases pneumococcal biofilm formation *in vitro* (Figure 9).
It is unclear what accounts for the increase in biofilm formation that is seen both in vitro and in vivo; however, there appear to be two components: increased bacterial numbers and increased extracellular matrix material. Approximately $10^7$ CFU each of $S.\ pneumoniae$ and $H.\ influenzae$ were recovered from the homogenized bullae (tissue-associated/biofilm bacteria) of coinfected animals (Figure 10B). This is equivalent to what was recovered from the homogenized bullae of animals infected with either species alone (Figure 10B). The additive effect of $H.\ influenzae$ and $S.\ pneumoniae$ present in the coinfection biofilms (roughly double the CFU) likely accounts for some, but not all of the increase in biofilm mass seen (Figure 10B).

The presence of $H.\ influenzae$ increases the number of pneumococci present within a biofilm. This was clearly shown in an in vitro assay, where ~10–fold more pneumococci were recovered from biofilms in the presence of $H.\ influenzae$ than in $S.\ pneumoniae$ alone biofilms (Figure 9A). $H.\ influenzae$ could also increase $S.\ pneumoniae$ CFU in biofilms in vivo. While there was no a difference in the CFU of pneumococci recovered from homogenized bullae in single versus coinfected animals, all of the recovered CFU might not be persisting within biofilms (Figure 10B). Equivalent numbers of pneumococci were recovered from the homogenized bullae of animals infected with $S.\ pneumoniae$ alone regardless of the presence of observable biofilms within the middle ear. Previous studies have shown that $S.\ pneumoniae$ can persist intracellularly, including within the middle ear mucosa, and infect bone [417-422]. It is possible that pneumococcus is persisting intracellularly within the bullar tissue and bone of the animals infected with pneumococcus alone. Although the pneumococcal CFU in the bullae does not increase in the presence of $H.\ influenzae$, we hypothesize that more $S.
*pneumoniae* is persisting within biofilm communities, rather than intracellularly, in the coinfected animals (Figure 10B). This data is supported by the fact that *S. pneumoniae* was identified in all of the biofilms formed in coinfected animals (Figure 6B).

The majority of the increase in biofilm biomass seen in the coinfected animals appears to comes from an increase in the biofilm matrix material, either bacterial or host in origin. However, it is unclear which component or components are increasing and why. Previous studies have shown by confocal microscopy that biofilms removed from chinchillas infected with *H. influenzae* contain the host NET components neutrophil elastase and histone [133]. Interestingly, biofilms removed from animals infected with *H. influenzae* and *S. pneumoniae* or *S. pneumoniae* alone only contain histone (Supplemental Figure 2). *S. pneumoniae* produces a neutrophil elastase inhibitor that could be inactivating neutrophil elastase [423-426]. In addition, an endonuclease produced by pneumococcus has been shown to degrade NETs [205]. It is possible that *S. pneumoniae* is degrading components of NETs within the biofilm communities. Although NETs do not kill *H. influenzae* within a biofilm, they could be limiting or preventing the secretion of some extracellular matrix material [133]. In the absence of these components, *H. influenzae* could secrete more extracellular matrix material, partially explaining the increase in biomass seen in coinfected animals.

Another possible explanation is that the increase in biofilm matrix material observed is due to an increase in the influx of host components. Previous studies have shown that coinfection with *H. influenzae* and *S. pneumoniae*, both *in vitro* and *in vivo*, induces a synergistic increase in NF-κB activation and production of IL-8, which is a potent neutrophil chemoattractant [427]. If the production of IL-8 is increased in the
middle ears of coinfected chinchillas, thereby increasing neutrophil influx into the middle ear, this could lead to increased neutrophils and NETs within coinfected biofilms. Neutrophils are a large component of *H. influenzae* and *S. pneumoniae* in vivo biofilms and a substantial increase in neutrophil numbers could increase biofilm biomass [133, 208].

**Implications of Coinfection Studies**

There is a strong push to develop vaccines that eliminate disease not only by overt pathogens, but also by bacteria of the normal flora, like *H. influenzae*, that can cause disease in an impaired host. While it has been well established that disruption of normal flora in the intestinal tract can cause serious disease by other bacterial species, like *C. difficile*, the studies presented here indicate that there could be deleterious effects with the elimination of nasopharyngeal colonizers as well [428, 429].

First, we have shown that the presence of *H. influenzae*, either by prior infection or coinfection with *S. pneumoniae*, alters the type of pneumococcal disease seen from invasive to a more localized infection. Therefore, if *H. influenzae* colonization were eliminated or reduced with the use of a vaccine, there is a strong possibility that the incidence of systemic pneumococcal disease seen in patients could also increase.

In addition, with the elimination or reduction of *H. influenzae* colonization and OM disease, it is reasonable to assume that the incidence of pneumococcal OM would increase. There is a strong precedent for this, as OM caused by both *H. influenzae* and *S. aureus* increased after the introduction of PCV7 [232, 430, 431]. In addition, we have shown that *H. influenzae* outcompetes *S. pneumoniae* in the chinchilla model of
experimental OM, so without *H. influenzae* present, *S. pneumoniae* would be better able to establish itself in OM (Figure 3).

It is well known that OM caused by *S. pneumoniae* is a more serious disease than OM caused by *H. influenzae*. Pneumococcal OM is associated with a higher fever and severe pain in pediatric patients and there is an increased risk for serious complications like meningitis, bacteremia and mastoiditis [432-437]. While the vast majority of children recover from pneumococcal OM with no serious complications, this could change with the elimination of *H. influenzae*. The data presented here demonstrate that the presence of *H. influenzae* plays a role in limiting the spread of pneumococcus from the middle ear and the same could be true in pediatric patients. It is possible that the severe complications seen in patients with pneumococcal OM occur when pneumococcus is the sole pathogen in the middle ear, without *H. influenzae* or another bacterial species present to impede pneumococcal spread. If this were the case, elimination of *H. influenzae* colonization and its presence in OM disease could increase the incidence not only of pneumococcal OM, but also of severe complications from OM.

While vaccines are very effective at eliminating many different diseases, it is unlikely that a vaccine, or multiple vaccines, will ever be able to completely eliminate colonization by opportunistic pathogens in the nasopharynx. When the colonization of one bacterial species is decreased, others quickly replace it [232, 430, 431]. A safer and more effective means of prevention of disease caused by opportunistic pathogens might be to increase colonization of harmless commensals of the nasopharynx that interfere with colonization of otopathogens. A nasopharyngeal spray of commensal α-hemolytic streptococci has shown efficacy in preventing colonization by opportunistic pathogens,
OM disease and pharyngotonsillitis in clinical trials in children [58, 59]. In addition, for the prevention of periodontal disease, a strain of *Streptococcus mutans* has been engineered that does not contribute to dental caries, but it can permanently colonize the oral cavity, replacing disease-causing strains [376]. If employed in the nasopharynx, this tactic could decrease colonization by all opportunistic pathogens, not just one species, and therefore would not disturb the balance in favor of one over another.

**Antibiotic Studies**

We demonstrated that *H. influenzae* provides passive protection for *S. pneumoniae* against amoxicillin killing both *in vitro* and in the chinchilla model of experimental OM (Figures 14 and 15). An isogenic mutant of NTHi 86-028NP deficient in the production of β-lactamase, NTHi 86-028NP *bla*, was used to distinguish between the roles of β-lactamase production and biofilm formation in this protection. Initial antibiotic resistance studies with *H. influenzae* alone found that production of β-lactamase was not required for resistance to amoxicillin in biofilms *in vitro*; however, β-lactamase was required for amoxicillin resistance in the chinchilla model of OM (Figures 12 and 13). *In vitro* studies also indicated that *H. influenzae* passive protection of *S. pneumoniae* required the production of β-lactamase; however, this was not the case *in vivo* (Figures 14 and 15). *S. pneumoniae* was recovered from the bullar homogenates of all but one ear from animals coinfecte[d with NTHi 86-028 *bla* and *S. pneumoniae* and treated with amoxicillin (Figure 15B). This result was very surprising, given that NTHi 86-028NP *bla* was completely cleared from the middle ear when animals were infected with it alone and treated with amoxicillin (Figure 13). These data demonstrate that a
coinfection between *H. influenzae* and *S. pneumoniae* increases antibiotic resistance for both bacterial species. Based on the observation from our previous studies that biofilms formed in coinfected animals are much larger than those formed in animals infected with either species alone, we hypothesize that the enhanced biofilm formation is providing protection against antibiotic killing. It could be just a shear increase in biofilm size that is providing protection for *S. pneumoniae* and *H. influenzae* in coinfection biofilms, or there could be a structural difference between single and multi-species biofilms that is providing protection.

While both NTHi 86-028NP bla and pneumococcus were recovered from the homogenized bullae from the majority of animals, only NTHi 86-028NP bla was recovered from the effusion fluids of coinfected animals treated with amoxicillin (Figure 15). Biofilm communities involve a constant turnover of bacteria that are attaching and breaking off the surface [340, 438]. One possible explanation is that NTHi 86-028NP bla and pneumococcus both detached from the biofilm throughout the infection and after the final treatment with amoxicillin, but residual amoxicillin in the middle ear killed pneumococcus, while NTHi 86-028NP bla was resistant. The MIC of *S. pneumoniae* to amoxicillin is much lower than that of NTHi 86-028NP bla, < 0.064 and < 1.0 respectively (Table 4). Another possibility is that only pneumococcus present within the interior of the biofilm survived antibiotic treatment, so all the bacteria on the surface of the biofilm, and those able to break off, were *H. influenzae*. This observation supports the idea that biofilm formation is providing protection from amoxicillin killing in animals infected with NTHi 86-028NP bla and *S. pneumoniae*. In the animals infected with the parental strain of NTHi 86-028NP and *S. pneumoniae*, both bacterial strains persisted in
the effusion fluid, presumably due to inactivation of amoxicillin by the secreted β-lactamase (Figure 15).

**Implications of Antibiotic Studies**

These data point to two distinct mechanisms of *H. influenzae* antibiotic resistance and passive protection of *S. pneumoniae*: β-lactamase production and biofilm formation. These results have important implications for the treatment of OM and shed new light on the mechanisms behind treatment failure. Amoxicillin is the most commonly prescribed antibiotic for the treatment of OM [299]. If these studies are indicative of what is occurring in the human middle ear, amoxicillin will only be effective in treating a select few cases of OM. It is inactivated by β-lactamase, which ~60% of *H. influenzae* strains and essentially all of *M. catarrhalis* strains produce [322-324]. The β-lactamase of both of these bacterial species also provides protection for *S. pneumoniae* during coinfection (Figure 15 and [385, 386]). In addition, when β-lactamase is not present, sufficient biofilm formation can provide protection for bacteria and this protection can extend to multiple bacterial species (Figure 15).

As with most upper airway infections, treatment failure and antibiotic resistance are common in OM, with failure rates for amoxicillin as high as 50% in some populations [318-321]. There is a need to find alternative antibiotics that are more efficacious against OM and other polymicrobial or biofilm infections. The most efficacious antibiotic for treating a biofilm infection may not be the antibiotic with the highest MIC *in vitro*. In addition to being safe for use in children and being able to diffuse into the middle ear, there are a couple of criteria that potential antibiotics should meet. First, candidate
antibiotics should be resistant to inactivation by the β-lactamase produced by *H. influenzae* and *M. catarrhalis*, as this is able to provide protection against amoxicillin killing for the bacteria that produce it, as well as others colonizing the same location. Second, candidate antibiotics should be able to kill biofilm bacteria. This includes being able to penetrate a biofilm, as well as being able to kill bacteria in a biofilm mode of growth [140, 340, 439, 440]. Biofilm communities contain a heterogeneous bacterial population that varies with biofilm depth and can have very low replication rates and altered metabolic activity, which affect antibiotic efficacy [140, 331, 333, 340, 439-441].

The majority of β-lactam antibiotics do not meet these criteria, despite having favorable pharmacokinetic and safety profiles in children [299]. While β-lactams can be administered with the β-lactamase inhibitor clavulanate and some classes of β-lactams are resistant to *H. influenzae* and *M. catarrhalis* β-lactamase inactivation, the majority of β-lactam antibiotics are not effective against slow growing bacteria [336, 442-444]. Therefore, β-lactam antibiotics, like amoxicillin, are not a good choice for treatment of infections involving biofilm formation, regardless of whether the infecting bacteria produce a β-lactamase. Some extended spectrum β-lactams, like cephalosporins, have shown some efficacy against slow growing bacteria and biofilms of some bacterial species, but not against others, and should be further tested for their efficacy *in vivo* and against biofilms of *H. influenzae* and *S. pneumoniae* [439, 440, 445, 446].

Although more effective against rapidly growing bacteria, other classes of antibiotics, including macrolides, aminoglycosides and fluoroquinolones, are capable of killing slow growing bacteria [439, 445]. There are a lot of conflicting data in the literature concerning these classes of antibiotics in terms of which can penetrate biofilms
and kill biofilm bacteria, and which cannot. Several studies have shown that aminoglycoside antibiotics do not penetrate the biofilms of *P. aeruginosa*, *S. aureus* or *Staphylococcus epidermidis*, while other studies have found the opposite result for the same species [447-451]. An additional study showed that aminoglycoside antibiotics actually increase biofilm formation by *P. aeruginosa* and *E. coli* by increasing expression of cyclic di-guanosine monophosphate, which regulates cell surface adhesiveness in these bacteria [452]. Azithromycin, a macrolide antibiotic, has shown some efficacy against *H. influenzae* biofilms, but not against *S. pneumoniae* biofilms [213, 453, 454]. Sulfamethoxazole and trimethoprim have shown very little efficacy against bacteria within a biofilm [440, 446]. Finally, numerous studies have shown that fluoroquinolones can penetrate biofilms and are efficacious against biofilm bacteria, including those of *S. pneumoniae* and *H. influenzae*; however, these antibiotics are not recommended for use in most children because of the risk of joint and cartilage damage [339, 446, 454-458].

All of studies mentioned above were performed *in vitro* using different biofilm and testing models and, if anything, highlight the need for *in vivo* testing of antibiotic efficacy, especially for biofilms, where host components, like neutrophils and NETs, are known to be involved [133, 208]. It is also clear that antibiotic efficacy against biofilms differs for different bacterial species and needs to be tested for each. For treatment of OM, antibiotics that meet the initial criteria (safe for use in children, diffusion into the middle ear and capable of killing non-replicating bacteria) could be tested in the chinchilla model used in these studies to determine their efficacy against biofilm infection *in vivo*. Other model systems should be used to test antibiotic efficacy for other types of infections. New more effective drugs could also be designed based on the
properties of antibiotics that effectively kill biofilm bacteria, but that are unsafe for use in children in their current form, like fluoroquinolones.

**Future Directions**

The studies presented here provide insight into how *H. influenzae* and *S. pneumoniae* interact during a coinfection and the impact that this has on disease progression, disease severity and treatment efficacy. However, *H. influenzae* and *S. pneumoniae* only account for a subset of polymicrobial infections. Future studies should focus on determining the interactions between other bacterial species and the effect that these have on disease progression and treatment. For OM, the next step would be to study the interaction between *M. catarrhalis* and *S. pneumoniae* to determine if coinfection with *M. catarrhalis* alters the disease progression of pneumococcus, or if that is specific to *H. influenzae*. The results of these studies would also provide further insight into how *H. influenzae* influences *S. pneumoniae* colony phenotype and disease progression. In addition, polymicrobial infections containing *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* should be done to determine how the presence of all three bacterial species impacts OM disease. In the nasopharynx, it was found that all three bacterial species together (*H. influenzae*, *S. pneumoniae* and *M. catarrhalis*) had a different relationship than any two of the bacterial species together without the third [60]. The presence of *M. catarrhalis* could enhance the effect that *H. influenzae* has on *S. pneumoniae* or prevent it.

Another line of study should focus on testing the efficacy of different antibiotics for treating polymicrobial OM using the chinchilla model. Specific classes of antibiotics
to be tested were discusses above. In the studies presented here, antibiotic was injected
directly into the bullae because of the risk of chinchillas developing *C. difficile* infection
with enteric antibiotic exposure. Future studies should focus on finding a more clinically
relevant means of antibiotic delivery. For example, chinchillas might tolerate oral β-
lactam antibiotics if another antibiotic, like vancomycin, is given to prevent *C. difficile*
infection.
APPENDIX
Supplemental Figure 1. Colony count and systemic disease data from animals infected with different doses of *S. pneumoniae*. Chinchillas were infected with either 40 or 400 CFU of *S. pneumoniae* TIGR4 (indicated on the x-axis) and euthanized 3 or 7 days postinfection. The filled circles represent the recovered CFU from animals infected with 40 CFU; the empty circles represent the recovered CFU from animals infected with 400 CFU. The dashed line represents the limit of detection. The red X’s along the limit of detection line represent the number of animals that developed systemic disease and required euthanasia between days 0 and 3 (left side of center line) or days 4 and 7 (right side of center line) for each infection group.
**Supplemental Figure 2. Images of histone and elastase staining of biofilms removed from the middle ears of experimentally infected chinchillas.** These biofilms were removed from animals from the experiments in Figure 9. Panels A and D are from an animal inoculated with PBS on day 0 and Sp on day 7, red is staining for *S. pneumoniae*. Panels B and E are from an animal infected with Hi on day 0 and PBS on day 7, red is staining for *H. influenzae*. Panes C and F are from an animal infected with Hi on day 0 and Sp on day 7, red is *S. pneumoniae* in panel C and *H. influenzae* in panel F. Green is staining for elastase in panels A-C and histone in panels D-F. Yellow indicates colocalization of two components.
REFERENCE LIST


112


40. Boost MV, O'Donoghue MM and Dooley JS. Prevalence of carriage of antimicrobial resistant strains of *Streptococcus pneumoniae* in primary school children in Hong Kong. Epidemiol Infect 2001;127:49-55


116


63. Brook I, Gober AE. Frequency of recovery of pathogens from the nasopharynx of children with acute maxillary sinusitis before and after the introduction of vaccination with the 7-valent pneumococcal vaccine. International Journal of Pediatric Otorhinolaryngology 2007;71:575-579


65. Brook I. The Role of Bacterial Interference in Otitis, Sinusitis and Tonsillitis. Otolaryngology - Head and Neck Surgery 2005;133:139-146


70. Dawid S, Roche AM and Weiser JN. The blp Bacteriocins of *Streptococcus pneumoniae* Mediate Intraspecies Competition both In Vitro and In Vivo. Infect. Immun. 2007;75:443-451


73. Shakhnovich EA, King SJ and Weiser JN. Neuraminidase expressed by *Streptococcus pneumoniae* desialylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: a paradigm for interbacterial competition among pathogens of the human respiratory tract. Infect Immun 2002;70:7161-4

74. Brook I. The concept of indirect pathogenicity by beta-lactamase production, especially in ear, nose and throat infection. J Antimicrob Chemother 1989;24 Suppl B:63-72

75. Armbruster CE, Hong W, Pang B, et al. Indirect Pathogenicity of *Haemophilus influenzae* and *Moraxella catarrhalis* in Polymicrobial Otitis Media Occurs via Interspecies Quorum Signaling. MBio 2010;1


118


86. Brunton S. Current face of acute otitis media: Microbiology and prevalence resulting from widespread use of heptavalent pneumococcal conjugate vaccine. Clinical Therapeutics 2006;28:118-123


95. Weiser JN, Chong ST, Greenberg D and Fong W. Identification and characterization of a cell envelope protein of Haemophilus influenzae contributing to phase variation in colony opacity and nasopharyngeal colonization. Molecular Microbiology 1995;17:555-564


98. Geme JWS. Molecular and cellular determinants of non-typeable Haemophilus influenzae adherence and invasion. Cellular Microbiology 2002;4:191-200


100. St Geme IJW, Grass S. Secretion of the Haemophilus influenzae HMW1 and HMW2 adhesins involves a periplasmic intermediate and requires the HMWB and HMWC proteins. Molecular Microbiology 1998;27:617-630


121


114. Weiser JN, Pan N. Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. Molecular Microbiology 1998;30:767-775


148. Bouchet V, Hood DW, Li J, et al. Host-derived sialic acid is incorporated into *Haemophilus influenzae* lipopolysaccharide and is a major virulence factor in experimental otitis media. Proc Natl Acad Sci U S A 2003;100:8898-903


158. Kyd JM, Cripps AW, Novotny LA and Bakaletz LO. Efficacy of the 26-kilodalton outer membrane protein and two P5 fimbrin-derived immunogens to induce clearance of nontypeable Haemophilus influenzae from the rat middle ear and lungs as well as from the chinchilla middle ear and nasopharynx. Infect Immun 2003;71:4691-9


165. Syrogiannopoulos GA, Katopodis George D, Grivea Ioanna N and Beratis Nicholas G. Antimicrobial Use and Serotype Distribution of Nasopharyngeal Streptococcus pneumoniae Isolates Recovered from Greek Children Younger than 2 Years Old. Clinical Infectious Diseases 2002;35:1174-1182


170. Kim JO, Weiser JN. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J Infect Dis 1998;177:368-77


175. Saluja SK, Weiser JN. The genetic basis of colony opacity in *Streptococcus pneumoniae*: evidence for the effect of box elements on the frequency of phenotypic variation. Molecular Microbiology 1995;16:215-227


180. Waite RD, Penfold DW, Struthers JK and Dowson CG. Spontaneous sequence duplications within capsule genes cap8E and tts control phase variation in *Streptococcus pneumoniae* serotypes 8 and 37. Microbiology 2003;149:497-504


187. King SJ, Hippe KR and Weiser JN. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. Molecular Microbiology 2006;59:961-974


206. Hirst RA, Kadioglu A, O'Callaghan C and Andrew PW. The role of pneumolysin in pneumococcal pneumonia and meningitis. Clinical & Experimental Immunology 2004;138:195-201


221. Licensure of a 13-valent pneumococcal conjugate vaccine (PCV13) and recommendations for use among children - Advisory Committee on Immunization Practices (ACIP), 2010. MMWR Morb Mortal Wkly Rep;59:258-61


237. Pfister MA, Ehrhardt AF and Jones RN. Frequency of pathogen occurrence and antimicrobial susceptibility among community-acquired respiratory tract infections in the respiratory surveillance program study: microbiology from the medical office practice environment. Am J Med 2001;111:4S-12S


133


270. Hament JM, Kimpen JL, Fleer A and Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunology & Medical Microbiology 1999;26:189-195


344. Murphy TF, Bakaletz LO and Smeesters PR. Microbial interactions in the respiratory tract. Pediatr Infect Dis J 2009;28:S121-6


142


143


384. Wardle JK. *Branhamella catarrhalis* as an indirect pathogen. Drugs 1986;31 Suppl 3:93-6


401. Allegrucci M, Sauer K. Formation of *Streptococcus pneumoniae* non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. J Bacteriol 2008;190:6330-6339


SCHOLASTIC VITAE

NAME: Kristin E. D. Weimer

ADDRESS:

Department of Microbiology and Immunology
Wake Forest University Health Sciences
Medical Center Boulevard
Winston-Salem, North Carolina 27157-1064
Telephone - (336) 716-1697
Email – kweimer@wfubmc.edu

EDUCATION:

2002-2006 Georgetown University
Washington, DC
B.S. (Honors degree in Chemistry)
Cum Laude

2006-present Wake Forest University School of Medicine
Winston Salem, NC
M.D/Ph.D. Program (Microbiology & Immunology)
"The Effect of Haemophilus influenzae and Streptococcus pneumoniae Coinfection on Otitis Media Disease Progression and Treatment Efficacy"
W. Edward Swords, Ph.D., Advisor
Sean Reid, Ph.D., Co-advisor

PROFESSIONAL MEMBERSHIPS:

American Society for Microbiology
American Association of Immunologists
North Carolina Medical Association
American Medical Association
American College of Physicians
BIBLIOGRAPHY:


