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# Protein Vaccines Against Acute Otitis Media Caused by Nontypeable *Haemophilus influenzae* in a Murine Model

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BS Biochemistry, Nazareth College, Rochester NY, 2015

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry in the School of Chemistry and Materials Science, College of Science Rochester Institute of Technology

May 2018

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Director, M.S. Degree Program Date

SCHOOL OF CHEMISTRY AND MATERIALS SCIENCE
COLLEGE OF SCIENCE
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ROCHESTER, NEW YORK

CERTIFICATE OF APPROVAL

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M.S. DEGREE THESIS

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The M.S. Degree Thesis of Mark Zavorin
has been examined and approved by the thesis
committee as satisfactory for the thesis required for
the M.S. degree in Chemistry.

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# ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi) is one of the major organisms in the upper respiratory nasopharyngeal microbiota. In addition to its role as a commensal in the nasopharynx, NTHi is also the cause of sinusitis, pneumonia, and meningitis. Most relevant to this work, NTHi is one of the major causes of otitis media (OM), an inflammatory disease of the middle ear that affects 65-300 million children globally each year. In the US, acute OM (AOM or ear infection) is the most common reason for prescribed antibiotics. Due to the dramatic increase in antibiotic resistant strains of bacteria, there is currently an urgent need for alternative treatments for NTHi. After decades of studies, several proteins have risen to the top as potential protein vaccine candidates for NTHi. P6, Protein D, and OMP26 are three such NTHi proteins, which have been shown to be immunogenic in young children. We proposed to test all three vaccine candidates as a single trivalent vaccine formulation and as individual protein vaccines for protection against AOM using a mouse model. This work describes our efforts to develop a robust AOM mouse model for the assessment of protein vaccines, as well as our protein vaccine study which employed that model. The results of our study suggest that Protein D, Omp26, and P6 all elicit protection against colonization of NTHi in the ear, and the best protection occurs when all three proteins are contained within a single formulation. We also describe a surprising finding that two of the proteins interact *in vivo*, yielding one of the proteins ineffective at eliciting an antibody response.

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# ABBREVIATIONS

| | |
|---|---|
| Alum | Aluminum Hydroxide |
| AOM | Acute Otitis Media |
| APS | Ammonium Persulfate |
| BHI | Brain Heart Infusion |
| CFU | Colony Forming Unit |
| COM | Chronic Otitis Media |
| COPD | Chronic Obstructive Pulmonary Disorder |
| E. coli | *Escherichia coli* |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ET | Eustachian Tube |
| GN | Gram-negative |
| Hi | *Haemophilus influenzae* |
| Hib | *Haemophilus influenzae* type b |
| HRP | Horseradish Peroxidase |
| IFNγ | Interferon Gamma |
| Ig | Immunoglobulin |
| IM | Intramuscular |
| IN | Intra Nasal |
| IVA | *Influenza A Virus* |
| *M. cat* | *Moraxella catarrhalis* |
| NL-P6 | Non-Lipidated Protein 6 |
| NTHi | non-typeable *Haemophilus influenzae* |
| NP | Nasopharynx |
| OM | Otitis Media |
| OME | Otitis Media with Effusion |
| OMP26 | Outer Membrane Protein 26 |
| PBS | Phosphate Buffered Saline |
| PD | Protein D |
| PDB | Protein Data Bank |
| PF | Protein F |
| PR8 | Influenza A/Puerto Rico/8/1943 Virus |
| RGH-RI | Rochester General Hospital Research Institute |
| RSV | Respiratory Syncytial Virus |
| SDS | Sodium Dodecyl Sulfate |
| Spn | *Streptococcus pneumoniae* |
| TBS | Tris Buffered Saline |
| TEMED | Tetramethylethylenediamine |
| TNTC | Too Numerous to Count |
| URI | Upper Respiratory Infection |
| WHO | World Health Organization |
| WT | Wild Type |

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# INTRODUCTION

## *Haemophilus influenzae*

A microbiota is defined as an ecological community of commensal, symbiotic, and pathogenic microorganisms. The microorganisms colonize in different parts of the body, such as the upper respiratory tract, and can play an important role in maintain health. The nasopharynx (NP) has it is own microbiota, which prevents respiratory pathogens from descending to the lower respiratory tract where they can cause severe infections such pneumonia.[1] Some of the bacterial species of the NP microbiota are opportunistic pathogens, meaning that they can cause severe infections when they cross the barrier that separates the NP from other parts of the human body. One of such organisms is *Haemophilus influenzae (Hi*).

*Hi* is a commensal Gram-negative (GN) bacterium that is found in the NP. There are six generally recognized "typeable" groups of *Hi*: types a, b, c, d, e, and f. These types of *Hi* contain polysaccharide capsules that surround the bacterium. When these bacteria infect their host, the host can respond with the production of specific antibodies that bind to the capsule and can facilitate opsonization/killing of the microorganism.[19] In addition to these encapsulated types of *Hi*, there is also a non-encapsulated type of *Hi*, referred to as NTHi. Although there is no confirmed link between capsule and pathogenesis, type b *Hi* (*Hib*) is the common cause of diseases such as pneumonia, bacteremia, meningitis, epiglottitis, septic arthritis, and osteomyelitis, while NTHi causes less invasive diseases (or similar diseases in children, who are less immunologically developed).[20] More often *Hi* and NTHi can cause chronic obstructive pulmonary disease (COPD) and otitis media (OM) when present in the lungs or middle ear, respectively.[2, 3, 4]

## *Otitis Media*

Otitis media is the medical term for a bacterial middle ear infection that often occurs as a complication of the common cold. OM can be classified into three major classes based on the clinical symptoms: acute otitis media (AOM), otitis media with effusion (OME), and chronic otitis media (COM).[5, 6] AOM is defined as inflammation of the middle ear due to onset of a bacterial infection; AOM presents with rapid symptoms such as fever, irritability, or vomiting and usually has a short duration (<6 weeks). OME is defined as asymptomatic middle ear effusion and can be often associated with a 'plugged ear' feeling, which may resolve on its own. COM is a long-term middle ear infection that lasts longer than 6 weeks.[5, 7] Statistics show that OM is most common in children less than three years old and is the third most common reason for pediatric visits after the common cold and respiratory infections. Specifically, AOM most often occurs within a week or two of a viral infection, such as a cold; the virus allows for the colonization density of NTHi to increase and then travel to the middle ear, where it is responsible for a large portion of the bacterial AOM cases.[4, 8, 9] Studies show that by the age of three, 70% of children have at least one episode of AOM, and out of those children, 30% will develop reoccurring OM and suffer six or more OM episodes by the time they are seven years old.[8, 10, 11] AOM is also the most common reason why doctors prescribe antibiotics.[10] The recent dramatic increase in antibiotic resistant bacteria points to the urgent need for a vaccine against organisms that cause AOM. Currently, there is a highly effective vaccine against *Hib*, which contains part of *Hib*'s polysaccharide capsule. Since NTHi lacks a capsule, the leading vaccine strategy for NTHi focuses on using conserved proteins expressed on the surface of NTHi.

## *Viral Coinfection and AOM*

The occurrence of AOM undergoes seasonal variation. In temperate regions, AOM cases are higher during colder times of the year and lower during the summer months. This corresponds to the occurrence of viral upper respiratory infections (URI). Statistics show that the incidence of AOM is higher in children who more prone to respiratory infections. In many of these children, AOM episodes will decrease rapidly after the age of 2 to 3 years, at which time their respiratory infections also become less prevalent.[9] In a study of 363 children diagnosed with AOM, symptoms of viral URI, such as fever, cough, poor appetite, vomiting, diarrhea, and tiredness, were present in 94% of the AOM patients, suggesting *an important role of the common cold* in the development of AOM.[9]

Viral URI often causes congestion of the NP-mucosa. Congestion in and around the NP opening of the Eustachian tube (ET) that connects the NP to the middle ear can lead to dysfunction of the tube, which is considered the most relevant factor in the development of AOM.[9] As result of the dysfunction, there can be: (i) impairment of pressure equilibration between the NP and the middle ear cavity; (ii) decreased drainage into the NP of secretions produced in the middle ear; (iii) loss of protection of the middle ear from NP secretions; and/or (iv) loss of the protection barrier between NP and the middle ear. The latter phenomenon is most relevant to children with NTHi AOM, as it was found that the bacteria can ascend the ET by growing within the mucus of the ET and reach the middle ear by binding to the middle ear epithelial cells.[9, 12] In addition, the muscular opening function of the ET in less developed than in adults, because the ET is shorter, more flexible, and physiologically more horizontal, resulting in increased susceptibility to the development of AOM. [9, 13]

There are several viruses that have been shown to play roles in the development of AOM, including respiratory syncytial virus (*RSV*), *influenza virus A (*IVA*)*, and *adenovirus*. Interestingly, different bacteria will colonize differently in the NP, depending on the virus that is present. For example, *Streptococcus pneumoniae* (*Spn)* colonization in the middle ear is increased in patients with IVA, a phenomenon that was confirmed by a chinchilla AOM model. When chinchillas were infected with adenovirus, *Spn* did not develop into AOM; however, *Spn* did cause AOM when chinchillas were infected with IVA. In another study using a rat AOM model, NTHi colonization in the middle ear increased within 4 days of RSV infection.[9] *In vitro* studies using RSV have shown a significant increase in the attachment of NTHi to human respiratory epithelial cells. Similarly, increased adherence of NTHi to NP epithelial cells has also been demonstrated in adult volunteers infected with IVA.[9] Specifically, scientists suggest that the virus "activates" the epithelial cell lining in the NP (perhaps via inflammation), which results in increased adherence of bacteria to the cells and development of AOM.[9, 14] In summary, these studies and others point to a clear connection between viral infection and AOM.

## *Complications of OM*

One of the possible severe outcomes of AOM is a hearing loss. During an infection, bacteria travel to the middle ear, which can result in perforation of the tympanic membrane when bacteria/fluid builds-up in the middle ear. In severe or unresolved cases, this can lead to the destruction of the ossicular chain, resulting in deafness.[14] The World Health Organization (WHO) lists chronic ear infections as one of the major acquired causes of hearing loss. The prevalence of AOM-caused deafness is especially high in under-developed countries where appropriate treatments (antibiotics) are not commonly available.

In the past several years, there has been a significant increase in the resistance of bacteria to antibiotics. For example, there are 90 serotypes of *Spn*. In the early 2000's, it was estimated that 7 *Spn* serotypes (~7.8%) caused serious infections and were resistant to the most potent antibiotics; since then, this number has increased to 30% of total *Spn* serotypes (in 2013).[15] NTHi also have strains, which have been shown to be resistant to various classes of antibiotics. NTHi have been shown to have an increased production of β-lactamase enzyme that helps the bacteria to become resistant to β-lactam antibiotics.[16] In addition, even if antibiotics are effective at killing the bacteria, it does not guarantee that all of the bacterial debris are removed from the middle ear.[16] There is evidence to suggest that the presence of bacterial products, such as lipopolysaccharide (LOS), remaining in the middle ear can contribute to the prolonged presence of middle ear fluid and conductive hearing loss in cases of chronic otitis media. Lastly, as mentioned earlier, appropriate antibiotics may not be readily available. All of these potential complications of OM point to the urgent need for a vaccine against the specific bacteria that cause AOM.[16] In the US, the only vaccine against AOM bacteria is Merck's Prevnar-13 conjugate vaccine, which protects against 13 virulent strains of *Spn*.[14, 15, 17, 18] This vaccine has led to a significant decrease in *Spn*-caused AOM cases, while NTHi-caused AOM cases have been steadily rising.[14, 17, 18]

## *Vaccines*

What is a vaccine? A vaccine is a biological product made to produce immunity to a specific disease **(Fig. 1)**. There are multiple ways to make a vaccine; this work focuses on the development of **a protein vaccine** for NTHi. To determine vaccine composition, it is important to understand the roles of bacterial proteins in disease and how the host reacts to those proteins.

For example, AOM is initiated when bacteria attach to NP epithelial cells through adhesion components on the bacterial surface. Expression of adhesins increase during a viral URI co-infection (at least in ~95% of AOM cases), which makes adhesins strong protein vaccine candidates.

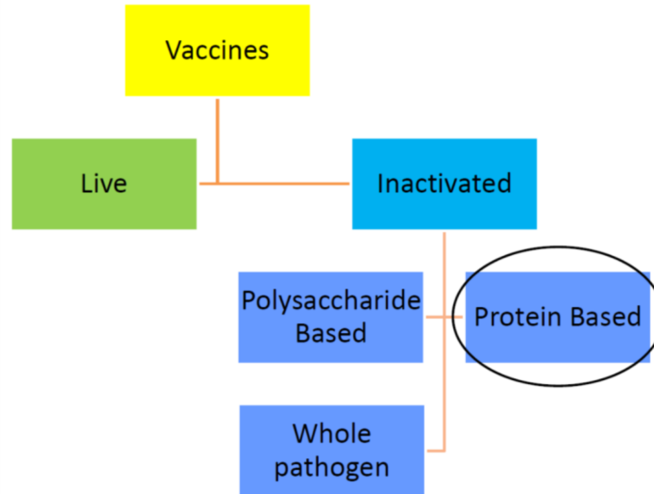



**Figure 1.** Various possibilities of vaccine formulations.

Despite the host innate immune response, some bacteria can manage to survive, which raises the question- what proteins are needed for survival. For example, it is known that invasion of NTHi in the middle ear elicits a strong host proinflammatory immune response. This response can cause damage to epithelial and goblet cells of the middle ear mucosa, thereby facilitating survival of NTHi in that niche.[18] Proteins involved in eliciting that proinflammatory response may be good protein vaccine candidates. Survival of NTHi in the human host also requires multiple metabolic processes by the bacteria to secure necessary nutrients for growth. Critical proteins involved in these processes may also serve as valuable vaccine ingredients.[18, 20, 21]

## *An NTHi Protein Vaccine*

There are several characteristics, which define an ideal protein vaccine candidate. Proteins should be conserved among all/most clinically relevant strains in order to elicit broad protection. To determine if the targets are conserved, it is important to have access to a large collection of bacterial strains.[18] The conservation of the target protein can be determined at several levels: first, whether or not the gene is present in all strains; second, how conserved the protein amino acid sequence is among strains; third, how conserved is the global structure of the protein among strains (i.e., can monoclonal antibodies bind to the protein in all bacterial strains). Even though a protein is identified as conserved across strains, it is also critical to predict the toxicity of the protein. For instance, after introduction of the protein into the system, the protein should not stimulate an unwanted autoimmune response, since the point of the protein is to stimulate production of specific antibodies and help induce memory immunity. If the protein is a toxin, then a genetic modification of the protein or a chemical treatment may be required, such as what was done for the pertussis toxin.[18] Lastly, based on the previous two characteristics, the protein vaccine candidate must be immunogenic, meaning that the components must elicit a protective immune response. This requires the protein to also be surface exposed and able to interact with human cells without bacterial lysis. Proteins must be able to induce the generation of antibodies in the host, and those antibodies must be functional, that is bactericidal, opsonic, or confer blocking of adherence/infection.[18] Based on the criteria above, the research groups under the supervision of Dr. Lea Vacca Michel (RIT) and Dr. Michael Pichichero (Rochester General Hospital Research Institute, RGHRI) have selected several **protein vaccine candidates** for protection against NTHi AOM: (i) **Protein D** (PD); (ii) lipoprotein **P6**; (iii) **Outer Membrane Protein 26** (OMP26); (iv) **Protein F** (PF).[18, 22]

## *PROTEIN D*

PD is a highly conserved 42-kDa outer membrane associated immunoglobulin D (IgD) binding lipoprotein.[18] PD is encoded by gene *hpd Hi*, and it was the first cloned, sequenced, and expressed in *Escherichia coli* (*E. coli*) in 1991 by Janson and colleagues.[23] The *hpd* gene encodes for 364 amino acid residues. The N-terminus has characteristics of a bacterial signal peptide with the sequence Leu-Ala-Gly-Cys that is common for bacterial lipoproteins. PD is typically expressed at high levels (estimated to be 2800 molecules per cell). PD is also very conserved and present in most (if not all) *Hi* strains, making it an attractive vaccine candidate. [24]

It was mentioned previously that NTHi has a strong affinity for NP epithelial cells. Specifically, NTHi was found to be located intracellularly in epithelial and macrophage cells, and adhesion to those cells was shown to be moderated by PD.[24] In the study, 5-fold higher amounts of wild-type (WT) NTHi was detected intracellularly compared with the PD-deficient strain of NTHi.[24] When PD was expressed in the PD-deficient cells, the number of bacteria found intracellularly increased by 50%.[24]

PD also possesses glycerophosphodiesterase activity, which causes the release of glycerophosphorylcholine from host epithelial cells. This glycerophosphorylecholine serves as a ligand for the platelet-activating factor receptor of epithelial cells.[25] Through PD, NTHi binds to the platelet-receptor, which results in a multifactorial host cell signal cascade and bacterial invasion. Based on these findings, PD is thought to be a ***virulence factor***.[25] As such, NTHi strains lacking PD are 100-fold less infectious than WT NTHi.[25] Lastly, WT NTHi caused a significant decrease in the frequency of ciliary beating and an increased loss of cilia from epithelial cells in NP tissue compared to the PD mutant of NTHi. [18, 23-26]

Based on these results, PD has become a leading vaccine candidate for NTHi AOM. Interestingly, PD is contained as a conjugated component in the *Spn* Prevnar vaccine. In a 2006 study, a large group of infants received the *Spn* vaccine, which included 11 *Spn* strains conjugated to NTHi PD. Results showed 33.6% reduction in overall incidence of AOM and a 35.3% reduction in NTHi AOM in vaccinated children (compared to placebo).[27] Results from this study suggest that PD is immunogenic and that it likely elicits some protection against NTHi AOM *in vivo*.

## *Outer Membrane Protein 26*

OMP26 was successfully characterized in 1996 by Kyd and coworkers.[22] OMP26 has a molecular weight of 26 kDa. OMP26 is highly conserved and present in all known *Hi* strains.[22] The 174 amino acids of OMP26 exhibit between 96.5 and 99.5% similarity between different strains of *NTHi*, however the function of OMP26 is unknown.[20, 22] OMP26, though, has structural similarities to Skp proteins from two other GN bacteria, *P. multocida* and *Y. pseudotuberculosis. Skp* proteins are believed to play a role as a chaperone in extracytoplasmic compartments or as a folding catalyst.[22]

Kyd and coworkers used a rat model to determine the immune response to OMP26 *in vivo*. Rats were immunized with 10 or 40 mg of purified OMP26 from NTHi and boosted one week later with the same dose of OMP26. One week after the last booster, rats were pulmonary challenged with NTHi. The results showed significant bacterial lung clearance in OMP26-vaccinated rats (compared to sham mice).[22] After immunization of rats with OMP26, authors detected significant titers of IgG, IgA, and IgM to OMP26 in serum, and these levels increased

with increased levels of OMP26 in each vaccine. Low levels of OMP26-specific IgG, IgA, and IgM were also found in serum from whole bacterium-immunized rats.[22]

To determine OMP26's role as a mucosal immunogen, Kyd and coworkers intranasally (IN) challenged chinchillas with *NTHi* and directly injected the bacteria into the chinchilla's middle ear.[28] Pre-challenge immunization of the chinchillas with OMP26 caused rapid clearance of NTHi in the NP and reduced bacterial loads in the middle ear.[28] In 2014, Pichichero and coworkers detected antibodies against OMP26 in children with NTHi colonized in the NP and children with AOM.[29] The OMP26 was shown to be immunogenic in infants 6 to 30 months of age. These studies are supportive of the inclusion of OMP26 in a vaccine against NTHi.[29]

## *P6*

When NTHi proteins were first characterized, there were five major outer membrane proteins expressed by NTHi: P1, P2, P4, P5, and P6. Of these proteins, P6 demonstrated the highest conservation among NTHi strains, present in all 136 tested NTHi strains with nucleotide homology between 97-99%. P6 is highly abundant in NTHi, accounting for ~5% of the total surface expressed proteins produced by NTHi.[30, 31]

Dr. Lea Vacca Michel and her research group at Rochester Institute of Technology (RIT) have been studying P6 for the past nine years, for its role as a leading vaccine candidate against NTHi and its unique dual orientation, which will be described later. Although its exact function in NTHi is unclear, P6 is thought to play a structural role in helping to maintain the integrity of the outer membrane. The outer membrane of GN bacteria contains lipopolysaccharides in its outer leaflet, phospholipids in its inner leaflet, and numerous lipoproteins, which are integrated into the membrane via N-terminally attached lipid moieties.[32, 33] The homologue to P6 from *E.*

*coli*, Pal, has been shown to bind peptidoglycan via non-covalent interactions, as well as the outer membrane proteins Lpp, OmpA, TolB, and TolA. Together, these proteins participate in a complex web that anchors together the outer membrane, peptidoglycan, and inner membrane, to enhance the stability of the cell. [32, 33]

To further elucidate the function of P6, Murphy and coworkers prepared a P6 mutant of NTHi. The P6 deletion mutant grew at a slower rate compared to WT cells and demonstrated increased variability in size, decreased cell wall integrity, and increased vesicle formation and fragmentation of cells. Further the P6 mutant had increased susceptibility to selected antibiotics and increased susceptibility to complement-mediated killing by human serum.[34] These results all pointed to P6's role in the maintenance of structural integrity of the NTHi cell.

DeMaria and coworkers immunized 21 chinchillas with 50 mg of P6 once a week for three weeks. Shortly after immunization, but before bacterial challenge with NTHi, blood samples were collected for antibody analysis. Bactericidal antibodies against NTHi were detected in all 21 samples.[35] Vaccinated chinchillas showed significant protection from NTHi AOM, suggesting that P6 was a viable vaccine candidate.[20, 35]

The Pichichero group at RGH-RI has performed an impressive long-term prospective study, collecting NP, throat, sera, and sometimes middle ear samples from healthy kids and kids with AOM.[29] An analysis of some of the samples showed that P6 was immunogenic in infants 6 to 30 months of age, and they also showed that anti-P6 antibodies in 75% of the sera samples were bactericidal against NTHi.[29, 36] Taken together, these studies suggest that P6 is a promising candidate for an AOM vaccine.

Both P6 from NTHi and Pal from *E. coli* have been shown to exhibit dual orientation.[32] In other words, a subpopulation of P6/Pal faces out toward the extracellular space and is surface

exposed, while a second subpopulation of P6/Pal faces into the cell, allowing it to interact with peptidoglycan (**Fig. 2**).[32, 33] Further studies showed that, under experimental conditions, only a small percentage of cells contained surface exposed P6. However, P6 antibodies have been shown to be ***bactericidal*** against NTHi, suggesting that P6 is localized to the surface of NTHi in suitable amounts to be an effective target for bactericidal antibodies.[32]

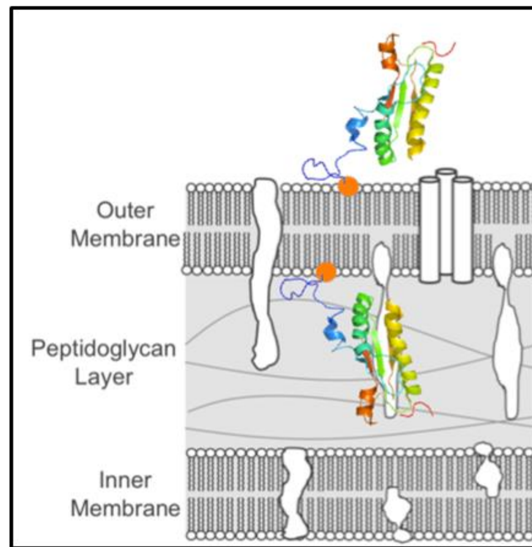



**Figure 2.** Representation of dual orientation of P6 in NTHi (PDB ID 2AIZ). P6 exhibits dual orientation with one population on the surface and another population interacting with peptidoglycan.

## *Protein F*

Among the four proteins described here, PF has been the least studied. PF is 30 kDa surface exposed protein that is encoded by the *hfp* gene. The gene is one of the four structural genes in the adenosine triphosphate–binding cassette (ABC) transporter operon and is present in all 20 NTHi strains that were tested with >98% amino acid identity.[18, 37]

Until 2013, there was no information on the function of PF; however, Jalavand and coworkers performed a structural analysis on PF and determined that it had close homology to

the Laminin-binding proteins of *S. pyrogenes* and *S. agalactiae*. [38] Laminin is a large 900 kDa, multidomain protein found in all human tissues. Laminin's main functions are to attach epithelial cells to other cell membranes and aid the development and migration of specific cell types in growth and maturation. As a result, many pathogenic bacteria use laminin as an adhesion during tissue invasion. Therefore, it is proposed that PF helps NTHi bind to laminin and human epithelial cells. Production of PF is stimulated by low concentrations of iron. NP tissues have low free iron concentrations, which should upregulate transcription of *hpf* gene up to 12-fold and enhance the binding of bacteria to epithelial cells.[37, 38]

Even though PF has not been assessed as a protein vaccine candidate for AOM, there was one study, which demonstrated its role in the pulmonary clearance of NTHi in a mouse lung infection model. Mice were immunized with 50 µg of PF four times, after which the animals were challenged IN with $10^6$ colony forming units (CFU) of NTHi. Lungs and blood were collected to quantify bacterial loads and PF antibodies.[39] Results suggested that mice immunized with PF exhibited significantly better NTHi clearance at both 3 hour and 5 hour time points, as compared to the control group.[39] Group also determined that immunized rabbits showed production of specific anti-PF directed toward specific amino acid sequences of PF. Blood serum showed high amount of $PF^{44-68}$, $PF^{84-108}$ , $PF^{104-128}$ and $PF^{225-255}$.[39]

In another study, healthy adults who previously had AOM in childhood were tested for the presence of PF antibodies. Approximately 26% of the 60 healthy blood donors were positive for PF IgG.[23-48] Based on these studies, PF may also be a strong protein vaccine candidate for protection against NTHi AOM.[39]

## *Current AOM Models*

Over the past 100 years, animal models have played a crucial role in the development of human vaccines. Animal models have been used for decades to assess vaccine safety and to determine the vaccine effectiveness of vaccines at providing protection against infection. Animal models are also used to help determine optimal vaccine dosage, formulation, and delivery modality. The goal of this work is to assess several protein vaccine candidates for their abilities to stimulate a protective immune response and memory to NTHi. In order to test these candidates, we first had to develop a reliable animal AOM model. Based on the currently employed AOM animal models and the progression of AOM in humans, we hypothesized that the model might require a viral coinfection with NTHi. Choosing the right animal model can be critical for the success of the vaccine and can also reduce the number of animals required for the study. Currently, the two most widely used AOM models are in chinchillas and mice. [35, 40-42]

The anatomical structures of chinchillas' inner and middle ears make them ideal models for human otological diseases. Since 1975, chinchillas have been used to study hearing loss due to *Spn* AOM.[35] Importantly, the chinchillas undergo similar AOM disease progression as humans. In one chinchilla model, the animals were infected with a respiratory virus (IN) prior to IN instillation of *Spn*; this coinfection strategy allowed for the "sporadic" development of a middle ear infection.[35, 40] The major setback for this model was its high cost. In contrast, mouse models are more widely used due to their relatively lower costs and shorter timelines. Mice are easier to handle and can be housed in larger groups; they also have a relatively short gestation period and are amenable to genetic manipulation.

The most often used animal AOM model employs a direct injection method of infection, also known as direct transtympanic inoculation of bacteria into the middle ear. The advantage of

this method is the ability to precisely and reproducibly inoculate an exact number of microorganisms into the middle ear, thereby guaranteeing induction of disease with low variation between individual animals.[43] Transtympanic inoculation is a straight forward procedure, during which a small needle is inserted through the tympanic membrane and bacteria are directly inoculated into the middle ear. On the negative side, direct ME inoculation is an artificial route of infection, and the disease does not progress in the same manner as in humans. Also, injected fluid can drain from the ME via the hole in the tympanic membrane, and if the insertion is not done properly, bacteria can get into the blood stream and cause sepsis.[43]

The IN infection model is less commonly used, but more closely mimics the human development and pathogenesis of AOM. There have been less studies where the IN model was employed in mice, most likely due to poor reproducibility. Several IN models were developed in JUNBO mice, which are genetically modified to express specific genotypes. For example, for one AOM study with JUNBO mice, the mice were modified to exhibit an immune deficiency.[42] To date, many more published studies have focused on *Spn* AOM in mice, most likely because *Spn* is more virulent in mice and more reliably leads to sporadic infection.

To our knowledge, there has only been one published study that employed non-JUNBO mice to study NTHi AOM. The study developed a virus-NTHi coinfection model to investigate complement-mediated killing of NTHi.[2] The study concluded that priming the NP with virus was necessary for the mice to develop robust colonization of bacteria in in middle ear.[2] However, the authors did not determine whether or not their model would be appropriate for the assessment of vaccines. Here, we describe our efforts to develop a viral/bacterial coinfection mouse model to study NTHi AOM, specifically with the goal of assessing the effectiveness of several protein vaccine formulations.

# MATERIALS AND METHODS

## *Bacterial strains and preparation*

All *NTHi* cultures were grown on brain heart infusion (BHI) medium supplemented with 20 µg/ml NAD (Sigma) and 10 µg/ml hemin (Sigma). WT *NTHi* (86-028NP) was a pediatric isolate (gift from Lauren Bakaletz, The Research Institute at Nationwide Children's Hospital).[44] WT *NTHi* was cultured on supplemented BHI medium under aerobic conditions at 37°C until the optical density at 490 nm (OD490) reached 0.6 (log phase). One mL glycerol stocks were prepared from the *NTHi* culture and frozen at -80°C. A series of $10^x$ dilutions of the thawed culture were grown on chocolate agar to quantify the bacteria stock.

## *Virus preparation*

For all coinfection studies, the mouse-adapted H1N1 influenza virus strain PR/8/43 (PR8) was prepared from one of two sources. The first source was obtained from the ATCC and expanded in embryonated chicken eggs.[45] A $TCID_{50}$ assay was performed to determine the influenza virus infection inoculum and titers, as described in the literature.[46] C57BL/6J mice (Jackson Laboratories) were administered IN inoculation (both nares) of PR8 at 50 times the $TCID_{50}$ (in a volume of 10 µl). PR8 was also purchased from Charles River Laboratories as a sterile, clarified allantoic fluid, purified to 2 mg/ml, and further diluted 1:125 in sterile phosphate buffered saline (PBS); this PR8 was also administered via IN inoculation in a 10 µl volume.

## *IN coinfection model*

C57BL/6J adult mice (approximately 6 weeks old) were administered an IN inoculation (both nares) of PR8 (prepared as described above) in a 10 µl volume. Either 3 days or 7 days later, *NTHi* stocks were prepared, as followed. *NTHi* glycerol stocks (described above) were gently thawed and centrifuged at 12,000 rpm, 4˚C for 5 minutes; cell pellets were resuspended in sterile PBS and washed two additional times in PBS before diluting to final concentration of 5.0 x $10^7$ CFU/mL or $10^8$ CFU/mL (IN inoculation) or $10^5$ CFU/mL or $10^6$ CFU/mL (direct injections). Adult (6-week-old) C57BL/6J mice were anesthetized with isoflurane (1%) in 100% oxygen with a delivery rate of 4 L/min, and *NTHi* was delivered via IN inoculation (both nares) in a volume of 10µl. Mice were housed in a sterile environment and monitored for any symptoms of infection. Mice were sacrificed three days post-*NTHi* challenge. Upper respiratory tract lavage fluid was collected from the nostrils using 200 µL of PBS as described in the literature. [46, 47] The middle ear lavage fluid was collected with 50-100 µL of PBS. Middle ear bullae were collected and homogenized in sterile PBS. Blood (50-100 µL) was also collected and plated onto chocolate agar to assess for sepsis. The nasal and middle ear lavage fluids and homogenized ear bullae were diluted on chocolate agar and incubated overnight (37°C), and colonies were enumerated the next day. Total CFU were calculated based on the volume of lavage fluid recovered.

## *Direct injection model*

Six-week-old mice were administered PBS or PR8 via IN inoculation (as described above). One week later, mice were anesthetized with ketamine/xylazine (20 µL at 80mg/kg). One of two NTHi stock solutions ($10^5$ or $10^6$ CFU/mL) was administered through transtympanic injection under an operating microscope into left and right ears (tympanic cavity) in a volume of

25 µl. Mice were placed into a sterile environment and monitored for symptoms of pain or distress. Mice were sacrificed two days post-NTHi injection. The middle ear lavage fluid was collected with 100 µL of PBS; middle ear bullae were collected and homogenized; blood (100 µL) was collected and plated onto chocolate agar to assess for sepsis. The middle ear lavage fluids and homogenized ear bullae were diluted onto chocolate agar and incubated overnight (37°C), and colonies were enumerated the next day. Total CFU were calculated based on the volume of lavage fluid recovered.

## *Protein vaccine study #1*

In the first pilot study, six-week-old mice were vaccinated intramuscularly (IM) with 50 µl (25 µl per hind leg) of a protein vaccine formulation. Vaccine formulations were prepared using purified recombinant proteins, as followed: A) a trivalent protein formulation (P6, OMP26, and PD, each at 10 µg per vaccine dose) with aluminum hydroxide as adjuvant (alum) (at 25 µg per vaccine dose); B) PF (5 µg per vaccine dose with alum (at 25 µg per vaccine dose); or C) an alum (25 µg per vaccine dose).

Mice (7 mice- trivalent group, 7 mice- PF group, and 7 mice- alum group) were administered three doses of the appropriate vaccine, with boosters 1 and 3 weeks after the initial vaccine injection. All female mice were primed with PR8 one week after the final vaccine booster, and then challenged with NTHi ($10^8$ CFU/mL) seven days later. Blood was collected from all mice a few days prior to the NTHi infection to determine antibody titers to the vaccine antigens. Female mice were sacrificed three days post-NTHi IN infection, and bacterial counts were determined from nasal lavages, ear washes, and homogenized ear bullae.

## *Protein vaccine study #2*

In the second pilot study, six week old female mice were vaccinated with A) the same trivalent protein formulation as described previously (7 mice), B) PD (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (6 mice); C) OMP26 (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (6 mice); D) P6 (10 µg per vaccine dose) with alum adjuvant (at 25 µg per vaccine dose) (5 mice); or E) an alum control group (25 µg per vaccine dose) (6 mice). Mice in each group were administered three doses of the appropriate vaccine, with boosters 1 and 3 weeks after the initial vaccine injection. All mice were primed with PR8 one week after the final vaccine booster, and then challenged with NTHi (IN: $10^8$ CFU/mL NTHi) seven days later. Data collection was performed as described above.

## *Protein vaccine study #3*

In the third pilot study, six week old female mice were vaccinated ) original trivalent vaccine formulation as described previously (5 mice), B) PD (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (4 mice); C) OMP26+PD (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (5 mice); D) P6+PD (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (4 mice); E) P6+OMP26 (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (4 mice); F) P6 (10 µg per vaccine dose) with alum (at 25 µg per vaccine dose) (4 mice); G) new trivalent vaccine (Tri*) (1µg- 10µg-1µg per vaccine dose respectively with alum adjuvant (at 25 µg per vaccine dose) (4 mice); or H) an alum control (25 µg per vaccine dose) (4 mice). Mice in each group were administered three doses of the appropriate vaccine, with boosters 1 and 3 weeks after the initial vaccine injection. All mice were primed with PR8 one

week after the final vaccine booster, and then challenged with NTHi (IN: $10^8$ CFU/mL NTHi) seven days later. Data collection was performed as described above.

## *Blood Serum Preparation*

Few days post last booster vaccine, 100µL of blood samples obtained by retro orbital bleed from mice before NTHi challenge. Blood samples were spun down at 2,000g for 20 min at room temperature. Pelleted red blood cells were carefully discarded, supernatant blood serum was saved and stored at -20˚C for further use.

## *Antibody titers*

Protein specific antibody titers were determined by enzyme linked immunosorbent assay (ELISA) using recombinant proteins. Purified recombinant proteins (P6, PD, PF, and OMP26) were diluted to 0.5 µg/ml in bicarbonate coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6). Proteins were added (100 µl/well) to a medium-binding 96-well plate (Greiner Bio-One) and incubated overnight at 4˚C. The wells were washed three times (200 µL/well) with wash buffer (PBS/0.1% Tween 20) and then incubated with blocking buffer (200 µL/well) (3% non-fat milk in wash buffer) for 1 hour at 37˚C. The wells were washed three times (200 µL/well) with wash buffer and then incubated with sera samples serially diluted in blocking buffer (collected from blood, centrifuged at 2,000 rpm for 20 minutes at room temperature) for 1 hour at room temperature. Initial sera samples were diluted 1:100 in blocking buffer. An in-house positive control serum (mixture of human sera) was run on each plate. After three additional washes, wells were incubated with goat anti-mouse antibody conjugated to horse radish peroxidase enzyme (HRP) (Bethyl Laboratories), diluted 1: 5,000 in blocking buffer (100 µL/well) for 1

hour at room temperature. After three additional washes, HRP substrate (Bethyl Laboratories) was incubated in each well (100 µL/well) for approximately 15 minutes. The reaction was stopped using 100µL/well of 0.1M phosphoric acid, and the plates were analyzed at 450 nm using a Spectra Max plate reader (Molecular Devices) and the Softmax end point titer dilution protocol.

## *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

The SDS-PAGE gels were prepared using a standard 10% recipe [resolving gel: 3.27 mL of 30% acrylamide/bis-acrylamide, 3.33 mL Tris/SDS (Tris/SDS solution: 182 g Tris base, 1.5 g SDS, pH 8.0), 1.38 mL nanopure water, 2.12 mL 50% glycerol, 10% ammonium persulfate (APS), 10 µL tetramethylethylenediamine (TEMED); stacking: 405 µL 30% acrylamide/bisacrylamide, 775 µL Tris/SDS, 1.95 mL nanopure water, 20 µL 10% APS, and 5 µL TEMED]. A BioRad gel system was used to cast the gels, and the gel was run in standard buffers (10X Cathode buffer: 60.6 g Tris base, 89.6 g Tricine, 5 g SDS, 500 mL water; 10X Anode buffer: 121.1g of Tris base, 500 mL water, pH adjusted to 8.9 using 12 M hydrochloric acid). The protein samples were prepared by mixing 1:1 with either 2X-sample buffer: 4 mL of 10% SDS, 2 mL Glycerol, 1.2 mL of 1 M Tris pH adjusted to 6.8, 2.8 mL of H2O, 0.001-0.002 g. bromophenol blue and boiled for 10 minutes.

All protein samples were loaded onto the gel 14 µL) alongside a Kaleidoscope protein ladder (BioRad and ThermoScientific) (5µL). Proteins were separated for between 30-45 minutes (120-150 V). Gels that were not transferred for Western blot analysis were incubated for 15 minutes in 15 mL of InstaBlue gel dye (Fisher).

## *Semi-Dry Transfer*

Protein bands were transferred to a nitrocellulose membrane using a semi-dry transfer technique. 2 filter papers (8 cm x 10.5 cm), a nitrocellulose membrane (0.45 micron), and SDS-PAGE gel equilibrated in transfer buffer (2.91 g Tris base, 1.47 g glycine, 100 mL methanol, 400 mL of nanopure water) for 10 minutes. A semi-transfer was prepared on the bottom electrode plate of the BioRad Trans-Blot SD semi-dry transfer system: filter paper, nitrocellulose membrane, gel, filter-paper (layered bottom-up). Air bubbles were removed, and the stainless-steel cathode was placed over the sandwich. The proteins were transferred at 15 V for 20 minutes.

## *Western Blot Protocol*

Transferred nitrocellulose membranes were blocked in 10 mL 5% (m/v) evaporated milk in 1x Tris-buffered saline (TBS) (50 mL of 10X TBS: 80g NaCl, 2g KCl, 30g Tris Base dissolved in 950 mL H2O pH adjusted to 7.4 with HCl, and sterile filtered; diluted to 500 mL with H2O) for 30 minutes, followed by a one hour room temperature incubation with primary antibody [1.5 µL of polyclonal anti-PD or in 8 mL of 1% (m/v) evaporated milk in TBST (100 mL 1x TBS, 50 µL TWEEN-20, 1 g powdered milk)] or incubated overnight at 12°C. After two 10min washes (1x TBST), the membranes were incubated in secondary antibody [0.5 µL of Goat anti-mouse IgG-H+L HRP conjugate (BETHYL laboratories) in 8 ml of 1% (m/v) evaporated milk in TBST] for 30 minutes. After three 10 min washes (1x TBST) and an additional two 5min washes (1x TBS), Pal was detected using a LumiGlo reserve HRP chemiluminescent substrate and a BioRad BioDoc system (Quantity One software).

## *Statistics*

The statistical tests were performed using Prism software (Graph Pad, La Jolla CA). Differences between data sets were analyzed by unpaired parametric or t-test for antibody levels, and bacterial loads CFU. For the purpose of statistical analysis, undetectable samples were arbitrarily assigned a value equivalent to one half the lower limit of detection. $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.0001$ were considered significantly different (*, **, ***, and **** respectively).

# RESULTS

## *Developing a PR8-NTHi Coinfection Mouse Model*

We quickly determined that coinfection with virus was required to establish robust NTHi colonization in the NP. Adult male C57-BL/6J mice infected with $5x10^7$ CFU/mouse NTHi (strain 86-028NP) cleared the bacteria from their NP within a few days, as determined by quantifying the bacteria in the nasal wash samples (**Table 1.1**). For all remaining studies, adult male C57-BL/6J mice will be infected with $5x10^7$ CFU/mouse NTHi (strain 86-028NP), unless noted otherwise.

Next, we compared NTHi colonization in NP in mice primed with Influenza A/Puerto Rico/8/1943 (PR8) virus either three or seven days prior to NTHi challenge. Adult male mice primed with PR8 seven days prior to NTHi infection had significantly ($p<0.0001$) higher intranasal bacterial counts compared to unprimed mice (**Table 1.1 and Fig. 1.1**). Mice primed with PR8 seven days prior to NTHi infection also had significantly ($p=0.0104$) higher NP bacterial loads compared to mice primed three days before challenge. For all remaining studies, mice were primed with PR8 seven days prior to NTHi infection.

| Nasal Lavages | Bacterial Count (CFU/mL | | |
|---|---|---|---|
| | Day -3 | Day -7 | NO- PR8 |
| 1 | $1.9 \times 10^3$ | $7.0 \times 10^3$ | 0 |
| 2 | 0 | $4.1 \times 10^4$ | 0 |
| 3 | $6.0 \times 10^3$ | $1.0 \times 10^4$ | 0 |
| 4 | $7.0 \times 10^3$ | $1.8 \times 10^5$ | 0 |
| 5 | $1.0 \times 10^4$ | $1.2 \times 10^4$ | |

**Table 1.1** *NTHi* (86-028NP) count in the NP area in mice that were primed intranasally with PR8 3, 7 days (6-10) or no priming before *NTHi* challenge.

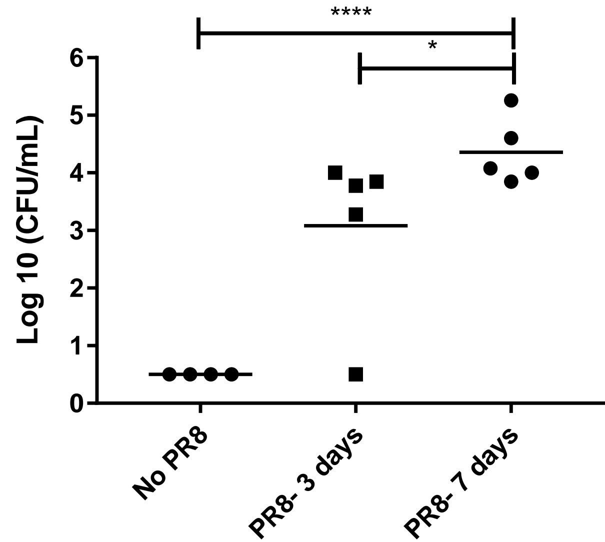



**Figure 1.1** Intranasal bacterial loads in unprimed (no PR8) mice or mice primed three or seven days prior to NTHi infection.

Children will typically present with AOM a week after a viral respiratory infection. To determine whether or not the mice sporadically developed AOM in our coinfection model, we performed ear washes to quantify the bacteria present in the middle ear three and seven days after NTHi challenge.

Mice had significantly (p=0.015) higher bacterial loads in their nasal wash three days post challenge compared seven days post challenge (**Table 1.2** and **Fig 1.2**). Three out of four mice contained quantifiable bacterial loads three days post-challenge, while all mice cleared NTHi from their middle ear seven days post-challenge.

| Nasal Lavages | Bacterial Count (CFU/mL) | | Ear Lavages | Bacterial Count (CFU/mL) | |
|---|---|---|---|---|---|
| | Day 3 | Day 7 | | Day 3 | Day 7 |
| 1 | $3.2 \times 10^4$ | $1 \times 10^2$ | 1 | $2.0 \times 10^1$ | 0 |
| 2 | $1.2 \times 10^5$ | $1.2 \times 10^3$ | 2 | $4.0 \times 10^1$ | 0 |
| 3 | $1.1 \times 10^3$ | 0 | 3 | 0 | 0 |
| 4 | $6.0 \times 10^3$ | 0 | 4 | $8.0 \times 10^1$ | 0 |

**Table 1.2** Bacterial count of *NTHi* (86-028NP) from nasal lavages and middle ear in mice that were treated with PR8 virus 7 days prior to bacterial challenge with *NTHi* ($5.0 \times 10^7$ CFU/mouse). Nasal lavages and ear washes were performed 3 and 7 days after the bacterial challenge.

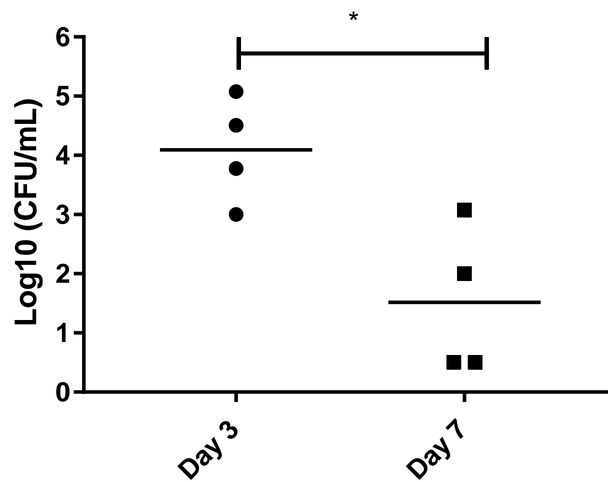



**Figure 1.2** Comparison of bacterial loads in the nasal lavage 3 and 7 days after bacterial challenge, priming with PR8 7 days prior to *NTHi* ($5.0 \times 10^7$ CFU/mouse) challenge.

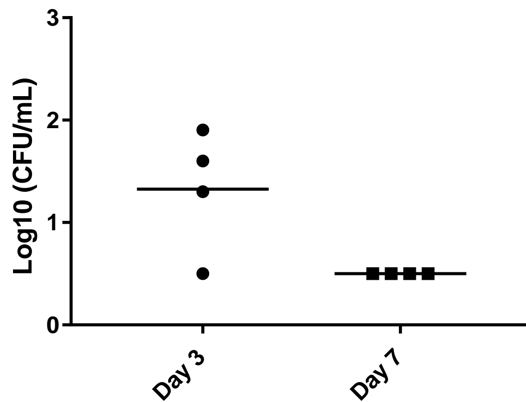



**Figure 1.3** Comparison of bacterial loads in the ear lavage 3 and 7 days after bacterial challenge, priming with PR8 7 days prior to *NTHi* ($5.0x10^7$ CFU/mouse) challenge.

To establish a more robust bacterial presence in the ME, we increased the NP inoculation level from $5.0x10^7$ to $10^8$ CFU/mouse NTHi. Most mice showed NP and ME colonization of NTHi at both 3 and 7 days post-challenge (**Table 1.3, Figs. 1.4 and 1.5**). Although the differences were not significant, mice inoculated with $10^8$ CFU/mouse NTHi showed a higher trend in NP colonization compared to mice inoculated with $5x10^7$ CFU/mouse NTHi (**Fig. 1.4b**).

| Nasal Lavages | Bacterial Count (CFU/mL) | | Ear Lavages | Bacterial Count (CFU/mL) | |
|---|---|---|---|---|---|
| | Day 3 | Day 7 | | Day 3 | Day 7 |
| 1 | $6.9 \times 10^4$ | $9 \times 10^2$ | 1 | 0 | 0 |
| 2 | $9 \times 10^4$ | $3.9 \times 10^4$ | 2 | 0 | $1.3 \times 10^3$ |
| 3 | $4 \times 10^4$ | $2.6 \times 10^3$ | 3 | TNTC | $6.4 \times 10^3$ |
| 4 | $1.2 \times 10^5$ | $8 \times 10^3$ | 4 | $2 \times 10^1$ | $3 \times 10^1$ |
| 5 | $1.5 \times 10^5$ | 0 | 5 | $1.7 \times 10^2$ | $8.4 \times 10^3$ |

**Table 1.3** Bacterial count of *NTHi* in nasal lavages and middle ear in mice that were treated with PR8 virus prior to bacterial challenge with $10^8$ CFU/mouse *NTHi.*
*Too numerous to count (TNTC)- for the purpose of the representation TNTC=$10^6$ CFU/mL.

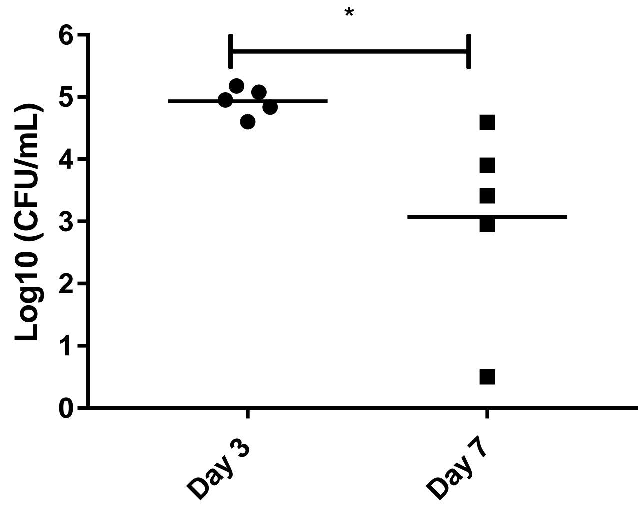



**Figure 1.4** Intranasal challenge with $10^8$ CFU/ml *NTHi* leads to significantly (p=0.03) higher bacterial colonization in the NP at three days after challenge.

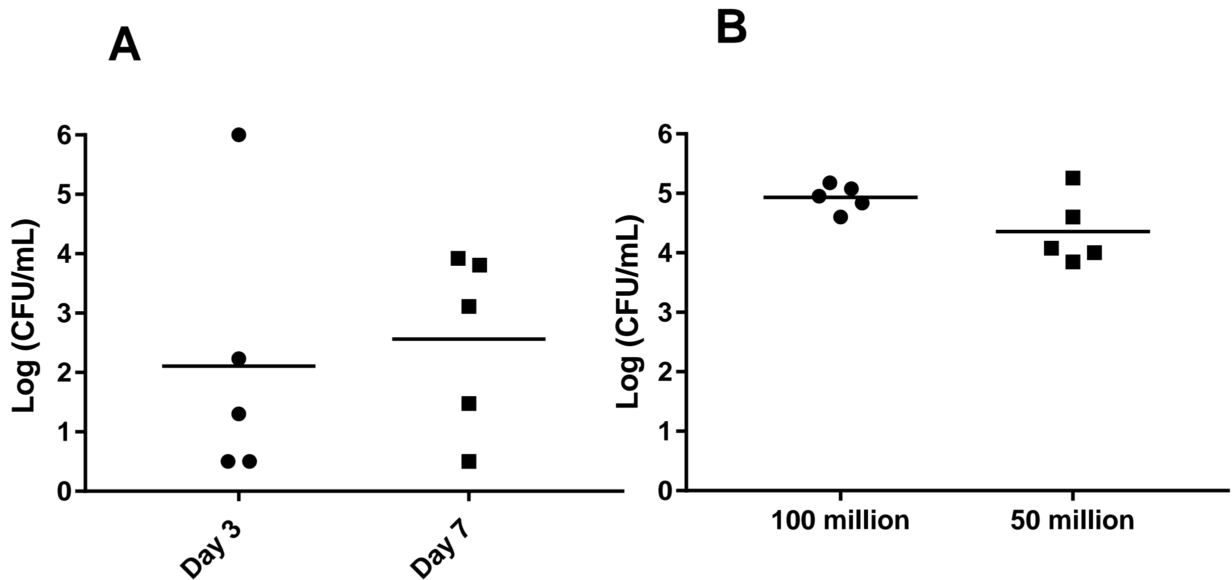



**Figure 1.5 a)** Bacterial colonization in the middle ear three and seven days post $10^8$ CFU/mouse *NTHi* challenge. **b)** Comparison of bacterial load in the NP, after the IN challenge with 100 million (1.0 x $10^7$ CFU/mouse) or 50 million (5 x $10^7$ CFU/mouse) NTHi.

Most of the previously described studies used adult male mice, but we considered the possibility that female mice might give significantly different results. As shown in **Fig 1.7**, bacterial loads in the ear washes of male mice were not significantly different than bacterial loads in the ear washes of female mice, although one female mouse appeared to clear NTHi from its ME. When possible, we used all male or all female mice in a single study to limit any potential variability due to differences between the sexes.

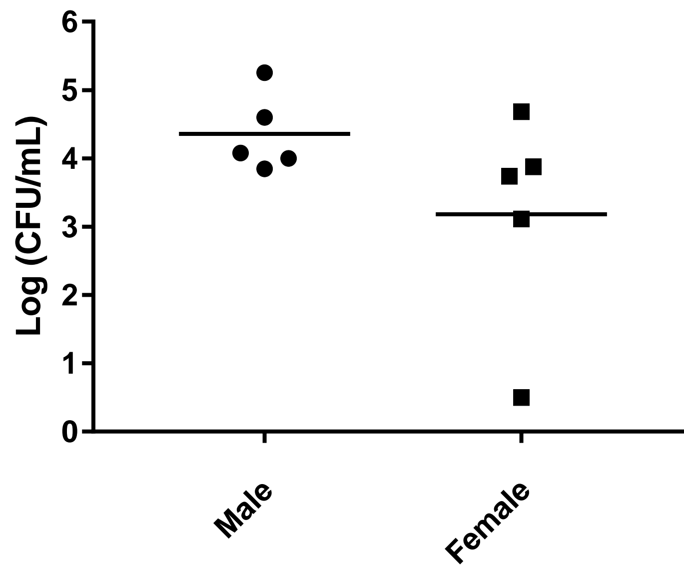



**Figure 1.7** Bacterial count in the middle ear of female and male mice 3 days after intranasal bacterial challenge. No significant difference in male or female AOM mouse model.

Personal communication with some of the world's leading OM scientists suggested that NTHi AOM mouse models were not preferred due to challenges in reproducibility. We observed similar issues, as a few mice per experiment appeared to clear NTHi from their ME, thus increasing the variability of the data. We considered the possibility that some of the NTHi bacteria were more tightly associated with the ME epithelia. We hypothesized that extracting the ear bullae (tissue) would allow for a more reliable quantification of ME bacteria. To test this

hypothesis, we employed the coinfection experiment with $5x10^7$ CFU/mouse NTHi and performed both ear washes and ear bullae extractions three days post-challenge. We quantified bacteria in both sets of samples (**Fig. 1.6**). Overall, bacterial counts were similar in the ear wash and ear bullae samples, although one mouse with no detectable bacteria in its ear wash had 2.6 x $10^3$ CFU/mL in its ear bullae sample. For all future studies, when possible, ear washes and ear bullae were collected to quantify bacterial loads.

As a side note, blood was collected and plated on chocolate agar for most of the previously described studies; no NTHi was ever detected in the blood, suggesting that the NTHi inoculation levels used in our study did not result in sepsis.

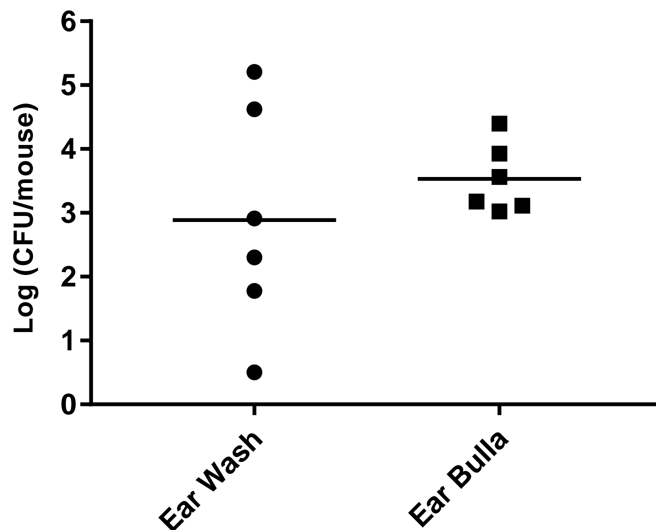



**Figure 1.6** NTHi bacteria were detected in the ear wash and ear bulla of mice coinfected with PR8 and NTHi (5 x $10^7$ CFU/mouse) via intranasal inoculation.

As described above, many groups have had success in modeling AOM in mice by directly injecting bacteria into the ME.[43] We wanted to determine whether priming the mouse with PR8 (IN) prior to direct ear injection of NTHi had any effect on NTHi colonization in the ear. PBS or

PR8 was used to prime the mice seven days prior to direct injection of $10^6$ or $10^5$ CFU/mL NTHi in each ear. Bacterial loads were quantified from ear wash samples two days post-infection; left and right ear samples were not combined in this study. Mice primed with PR8 had, overall, lower bacterial counts compared to mice primed with PBS (p=0.0196 in $10^5$ NTHi samples). The two inoculums ($10^6$ and $10^5$) of NTHi yielded similar bacterial loads, as detected in the ear washes.

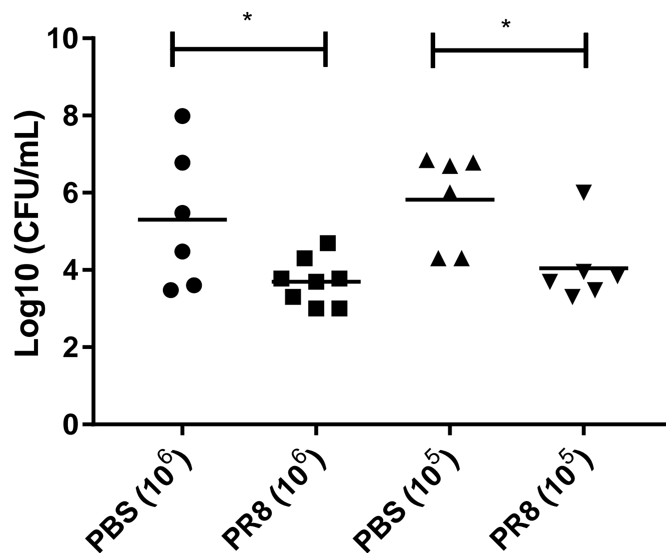



**Figure 1.8** Mice were primed with PR8 seven days prior to direct injection of either $10^5$ or $10^6$ CFU/mL NTHi in each ear and sacrificed two days after the NTHi challenge.

AOM in the ME requires both the presence of bacteria *and* inflammation. To demonstrate inflammation in the ME, we detected IFNγ in the ear wash samples of coinfected and naïve mice. IFNγ is one of the major pro-inflammatory cytokines that is released by adaptive and innate immune systems and triggers release of macrophages. Naïve mice had no detectable levels of IFNγ, while coinfected mice had an average of 41.13 pg/mL IFNγ in their ear wash samples

(**Fig. 1.9**). This final study confirms that, using our coinfection mouse model, NTHi travels to middle ear *and* causes inflammation.

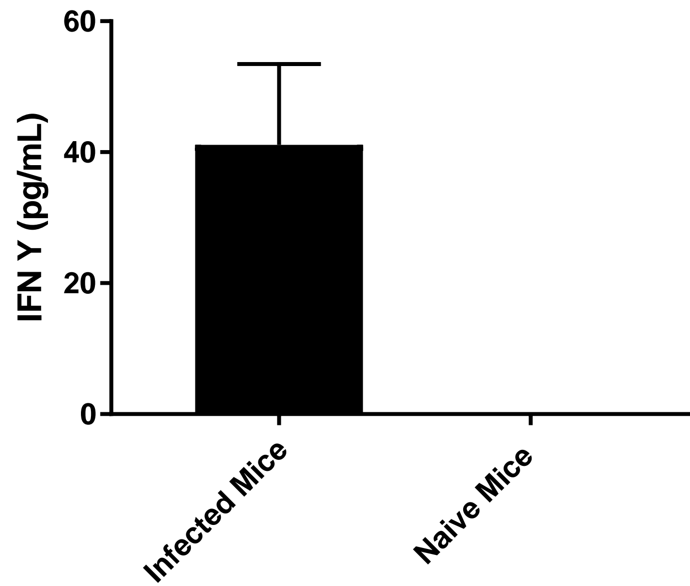
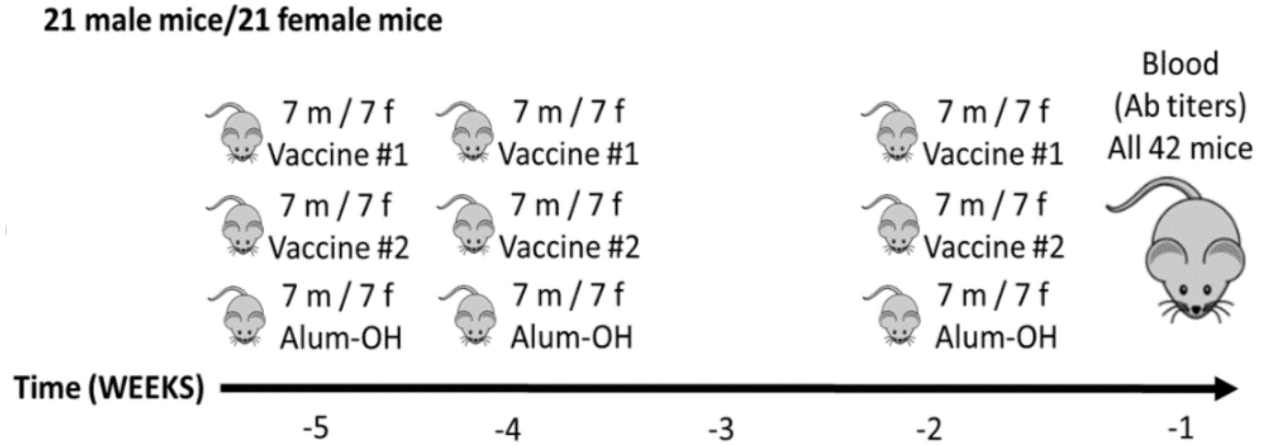



**Figure 1.9** Presence of proinflammatory cytokine interferon gamma (IFNγ) in mice coinfected with PR8 and NTHi and compared to naïve mice.

## *Testing Vaccine Formulations (Trial #1)*

Several protein vaccine formulations were prepared and assessed for protection using our newly developed direct ear injection and IN coinfection models. Mice were vaccinated with A) a trivalent protein formulation (P6, Omp26, and PD) mixed with an alum; B) PF with alum; or C) alum control. Mice in each group (14 mice per group) were administered three doses of vaccine, with boosters 1 and 3 weeks after the initial vaccine dose (**Fig. 2.1**) Blood samples were collected one week after the final booster to measure antibody titers to the vaccine antigens. A cohort of female mice (7 per group) was primed with PR8 one week after the final booster and then IN challenged with NTHi ($10^8$ CFU/mouse) one week after the PR8 infection (**Fig. 2.1**); nasal lavage, ear wash, and ear bullae samples were collected three days post-NTHi infection. A cohort of male mice (7 per group) was challenged by direct injection of NTHi ($10^5$ CFU/mL) into each ME; ear wash and ear bullae samples were collected two days post-NTHi injections (**Fig. 2.1**).



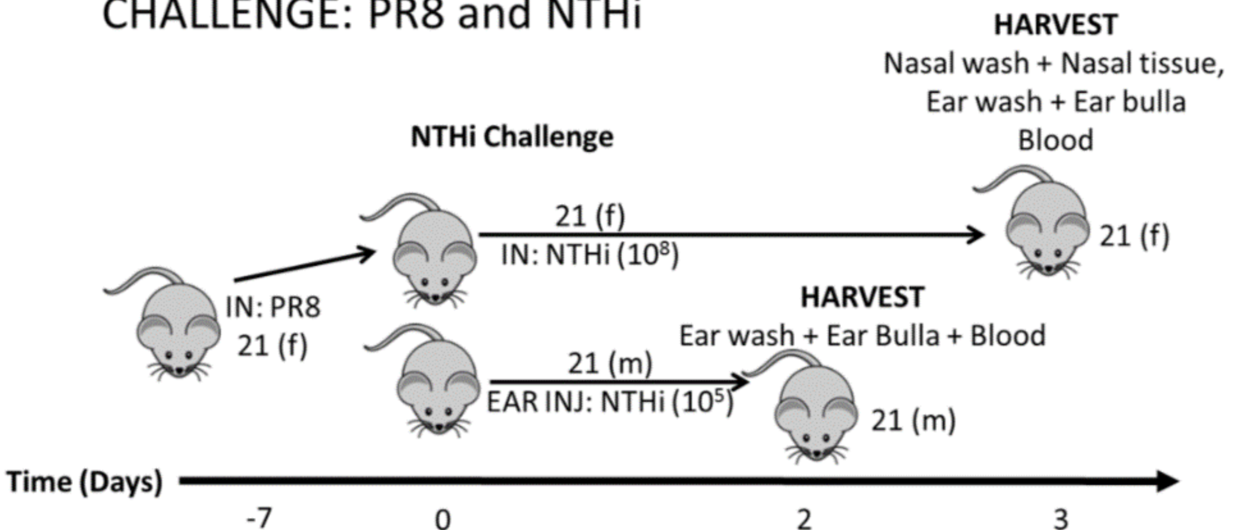




**Figure 2.1** Timeline for immunization of mice with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge. Established AOM mice model applied to determine the vaccine protection from NTHi.

*IN Coinfection mice*

Trivalent vaccinated mice had significantly ($p \leq 0.05$) lower (2.5 logs) NP bacterial loads compared to control mice. Protein F vaccinated mice had significantly lower NP bacterial loads (~1 log) compared to control mice (**Table 2.1**. and **Figure 2.2**). Four out of seven trivalent immunized mice had no detectable NTHi in their nasal lavages, while alum-control mice had an

average of $4.5x10^4$ CFU/mL NTHi per nasal lavage sample (**Table 2.1**. and **Fig. 2.2).** Three out of seven PF immunized mice had no detectable NTHi in their nasal lavages samples.

| Nasal Lavage | Bacterial Count (CFU/mL) | | |
|---|---|---|---|
| | Alum | Trivalent | PF |
| 1 | 4.0 x $10^2$ | 5.4 x $10^3$ | 0 |
| 2 | 3.0 x $10^5$ | 0 | 6.0 x $10^2$ |
| 3 | 1.0 x $10^3$ | 6 x $10^2$ | 6.0 x $10^3$ |
| 4 | 6.0 x $10^2$ | 0 | 0 |
| 5 | 6.0 x $10^3$ | 2.9 x $10^3$ | 5.0 x $10^2$ |
| 6 | 7.0 x $10^2$ | 0 | 1.0 x $10^2$ |
| 7 | 1.2 x $10^4$ | 0 | 0 |

**Table 2.1** Bacterial counts in the nasal lavage samples of the mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge.

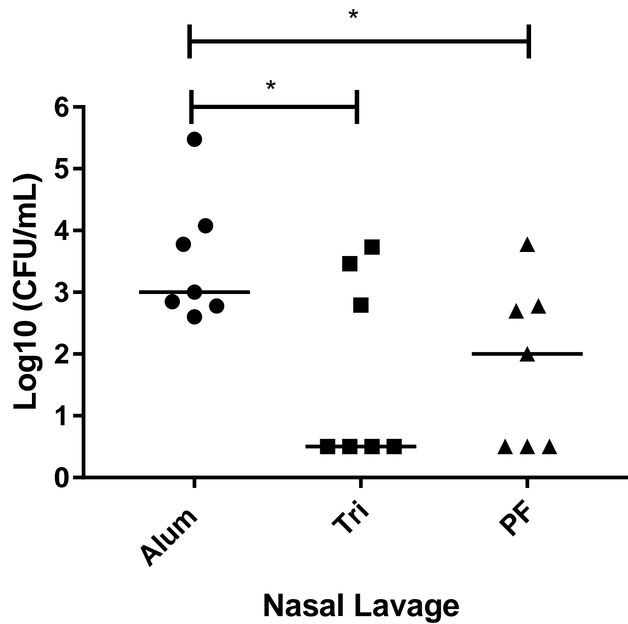



**Figure 2.2 a)** Bacterial count in the nasal lavage samples of mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge.

Trivalent vaccinated mice had significantly lower bacterial loads in ear washes (p=0.0041) and ear bullae (p=0.048) compared to control mice. Protein F vaccinated mice had a trend lower bacterial loads in ear washes/bullae compared to control mice, but these differences were not statistically significant (p>0.05) (**Table 2.2**. and **Figure 2.3)**.

ELISA's were used to quantify antibody titers to each vaccine antigen in the sera of vaccinated mice. In general, the protein vaccines elicited a strong antibody response to each antigen in the formulations; however there were no detectable PD antibodies in the trivalent vaccinated mice (**Fig. 2.4**).

| Ear Wash | Bacterial Count (CFU/mL) | | |
|---|---|---|---|
| | **Alum** | **Trivalent** | **PF** |
| 1 | $2.4 \times 10^3$ | $2 \times 10^2$ | $8 \times 10^2$ |
| 2 | $4.0 \times 10^5$ | $1.5 \times 10^3$ | $7 \times 10^2$ |
| 3 | $7.9 \times 10^4$ | $8 \times 10^2$ | $8.4 \times 10^3$ |
| 4 | $4.1 \times 10^7$ | $1.1 \times 10^3$ | $4 \times 10^3$ |
| 5 | $4.1 \times 10^7$ | $2.1 \times 10^4$ | $1.6 \times 10^5$ |
| 6 | $1.5 \times 10^3$ | $9 \times 10^2$ | $1.2 \times 10^5$ |
| 7 | $4.0 \times 10^5$ | $1.5 \times 10^3$ | $1.1 \times 10^3$ |

**Table 2.2** Bacterial count in the ear wash samples from the middle ear in the mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge.

| Ear Bullae | Bacterial Count (CFU/mouse) | | |
|---|---|---|---|
| | **Alum** | **Trivalent** | **PF** |
| 1 | $1 \times 10^2$ | $0.5 \times 10^2$ | $1.5 \times 10^2$ |
| 2 | $1.2 \times 10^4$ | $0.5 \times 10^2$ | $0.5 \times 10^2$ |
| 3 | $1.1 \times 10^3$ | $0.5 \times 10^2$ | $4 \times 10^2$ |
| 4 | $1.0 \times 10^2$ | 0 | $2 \times 10^2$ |
| 5 | $1.5 \times 10^5$ | 0 | 0 |
| 6 | $2.5 \times 10^2$ | $1.0 \times 10^2$ | $2.2 \times 10^3$ |
| 7 | $3.4 \times 10^3$ | $1.5 \times 10^4$ | $3.4 \times 10^3$ |

**Table 2.3** Detected bacterial count in the middle ear tissue samples in the mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge.

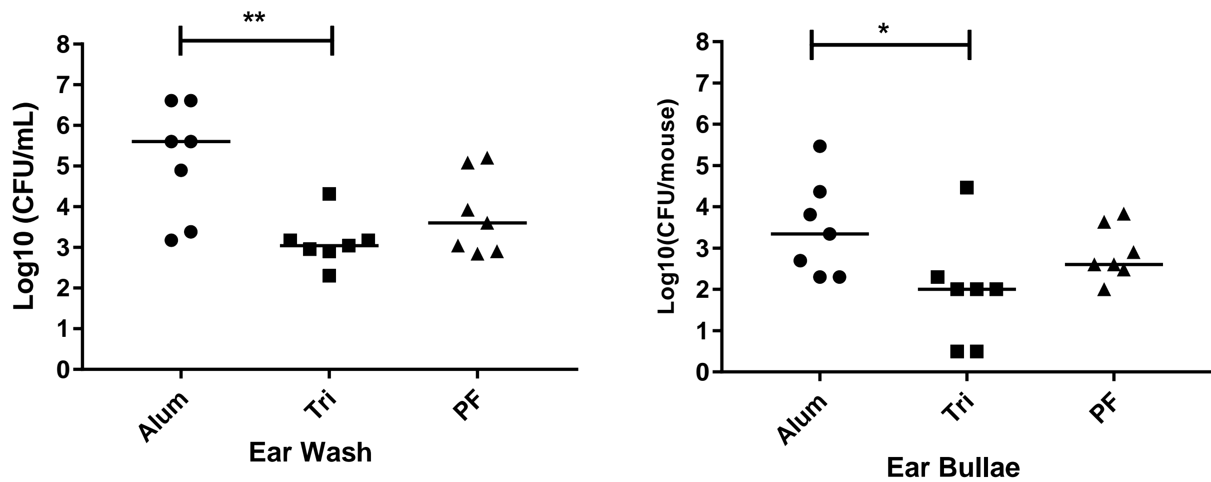



**Figure 2.3 a)** Bacterial count in the ear wash samples from the middle ear and **b)** middle ear bulla tissue samples of mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to viral and bacterial challenge.

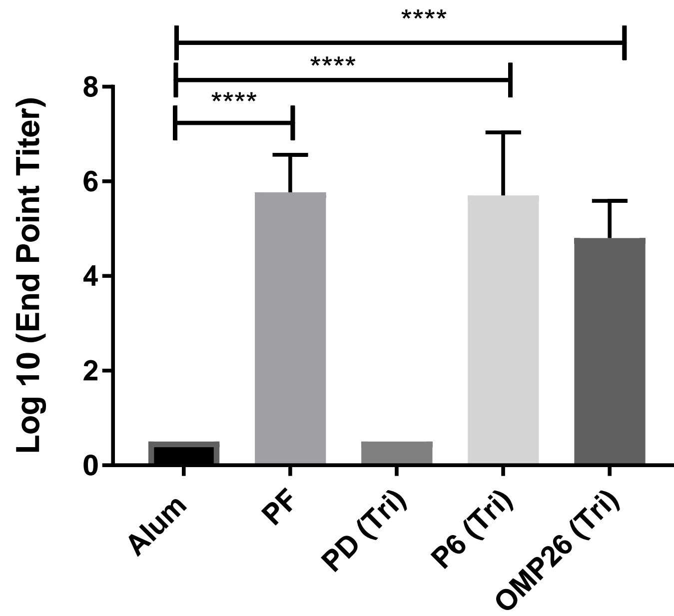



**Figure 2.4** End point titers for antibodies against proteins PF, PD, P6 and OMP26 in blood serum of mice immunized with the vaccines (trivalent, and single protein vaccines compared to alum).

*Direct Injection mice*

There were no significant differences (p>0.05) in ear wash/bullae bacterial loads between vaccinated and control mice (**Fig. 2.5**). ELISA's were used to quantify antibody titers to each vaccine antigen in the sera of vaccinated mice. In general, the protein vaccines elicited a strong antibody response to each antigen in the formulation; however, there were no detectable PD antibodies in the trivalent vaccinated mice (**Fig. 2.6**). The male mice produced similar levels of antibody titers to female mice; however, male mice had, on average, higher titers to PF compared to female mice (3 log difference). The exact same vaccine formulations were used on male and female mice, and the mice were vaccinated at the same time and in the same manner.

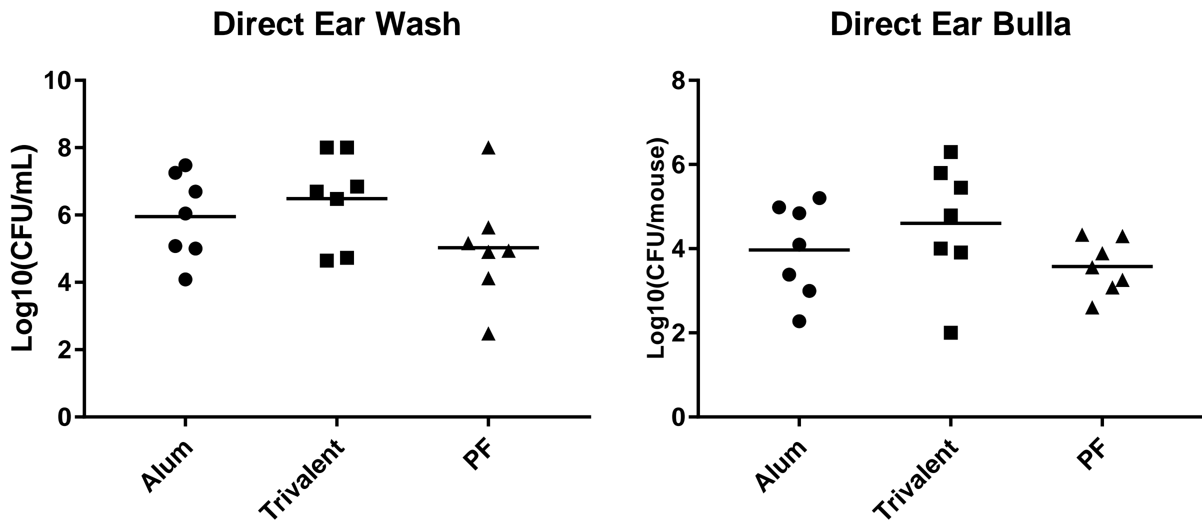



**Figure 2.5** Bacterial count in the ear wash samples from the middle ear and middle ear bulla tissue samples of mice that were immunized with trivalent (PD, P6, OMP26) vaccine, PF vaccine or alum control prior to direct bacterial challenge.

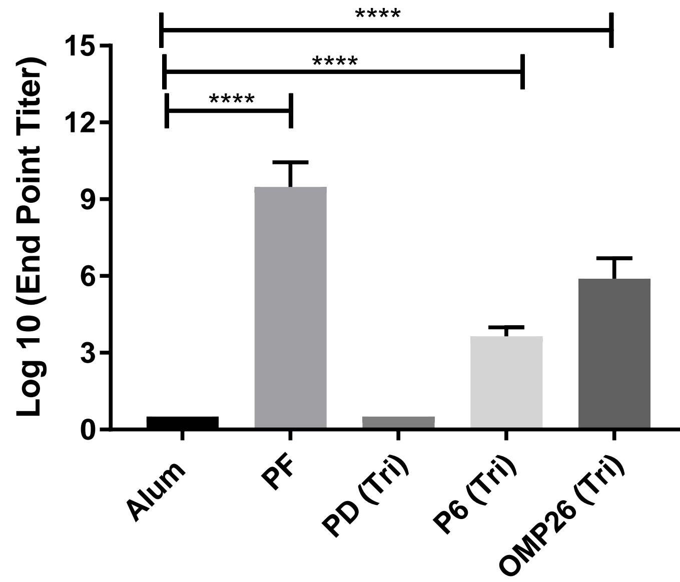



**Figure 2.6** End point titers for antibodies against proteins PF, PD, P6 and OMP26 in blood serum of mice immunized with the vaccines (trivalent, and single protein vaccines compared to alum) prior direct challenge.

## *Testing Vaccine Formulations (Trial #2)*

In the second vaccine trial, female mice were vaccinated with A) the same trivalent protein formulation as described previously (7 mice), B) PD (6 mice); C) OMP26 (6 mice); D) P6 (5 mice); or E) alum control (6 mice). Mice in each group were administered three doses of the vaccines, as described above, primed with PR8 one week after the final vaccine booster, and then challenged with NTHi (IN challenge, $10^8$ CFU/mL NTHi) seven days later. Blood was collected from each mouse a few days prior to the NTHi challenge to determine antibody titers to the vaccine antigens. NTHi bacteria counts were quantified in nasal lavages, ear washes, and homogenized ear bullae. In addition, nasal tissue was collected from each mouse and homogenized to quantify bacterial loads.

Median NTHi concentrations in nasal lavages/tissue of vaccinated mice were all lower than the median NTHi concentration of alum mice, with the exception of P6-vaccinated mice (**Table 3.1** and **Fig. 3.1a**). However, none of these differences were statistically significant ($p>0.05$).

| Nasal Lavage | Bacterial Count (CFU/mL) | | | | |
|---|---|---|---|---|---|
| | **ALUM** | **OMP26** | **PD** | **TRIVALENT** | **P6** |
| 1 | $4.1 \times 10^3$ | $3.0 \times 10^2$ | $1.6 \times 10^3$ | $1.3 \times 10^4$ | $4.0 \times 10^4$ |
| 2 | $7.0 \times 10^3$ | $2.0 \times 10^3$ | $2.0 \times 10^2$ | $4.4 \times 10^3$ | $3.6 \times 10^5$ |
| 3 | 0 | $2.0 \times 10^3$ | $1.0 \times 10^2$ | $4.0 \times 10^2$ | $2.0 \times 10^2$ |
| 4 | $5.3 \times 10^3$ | $6.0 \times 10^2$ | $3.0 \times 10^2$ | $4.0 \times 10^2$ | $1.0 \times 10^3$ |
| 5 | $3.0 \times 10^2$ | 0 | $1.0 \times 10^2$ | $5.3 \times 10^3$ | $6.0 \times 10^4$ |
| 6 | $2.2 \times 10^5$ | 0 | $4.0 \times 10^2$ | 0 | |
| 7 | | | | 0 | |

**Table 3.1** Bacterial count in the nasal lavage samples of the mice that were immunized with trivalent, PD, OMP26 vaccines or alum control prior to viral priming and bacterial challenge.

| Nasal Tissue | Bacterial Count (CFU/mouse) | | | | |
|---|---|---|---|---|---|
| | ALUM | OMP26 | PD | TRIVALENT | P6 |
| 1 | $7.5 \times 10^3$ | $2.5 \times 10^2$ | $1.15 \times 10^3$ | $1.8 \times 10^3$ | $3.0 \times 10^4$ |
| 2 | $7.0 \times 10^3$ | $2.65 \times 10^3$ | $0.5 \times 10^2$ | $1.0 \times 10^3$ | $1.0 \times 10^5$ |
| 3 | $3.5 \times 10^2$ | $1.2 \times 10^3$ | $0.5 \times 10^2$ | $5.0 \times 10^2$ | $1.1 \times 10^4$ |
| 4 | $2.2 \times 10^4$ | $3.5 \times 10^2$ | $0.9 \times 10^2$ | $2.5 \times 10^3$ | $1.0 \times 10^3$ |
| 5 | $2.5 \times 10^2$ | $9.0 \times 10^2$ | 0 | $4.5 \times 10^3$ | $3.0 \times 10^3$ |
| 6 | $3.0 \times 10^4$ | $2.2 \times 10^3$ | $1.1 \times 10^3$ | 0 | |
| 7 | | | | 0 | |

**Table 3.2** Bacterial count in the nasal tissue samples of the mice that were immunized with trivalent, PD, P6, OMP26 vaccines or alum control prior to viral priming and bacterial challenge.

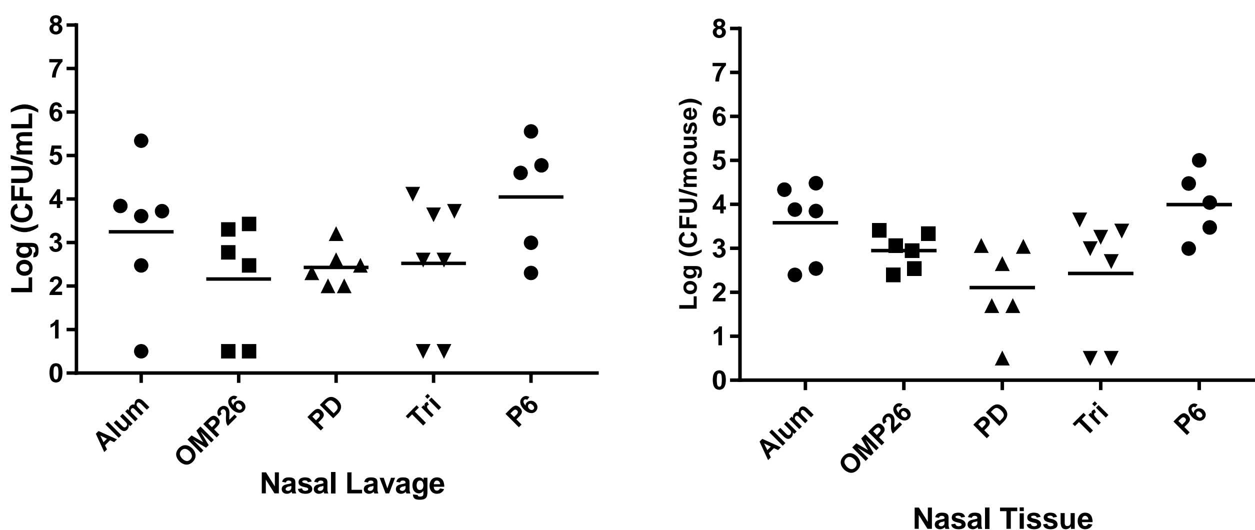



**Figure 3.1 a)** Bacterial count in the nasal lavage samples and **b)** in the nasal tissue samples of mice that were immunized with trivalent, PD, P6, OMP26 vaccine or alum control prior to viral priming and bacterial challenge.

Mice immunized with PD showed significantly lower concentrations of NTHi in ear wash samples (p=0.0051) and ear bullae (p=0.0022) compared to control mice **(Table 3.3 and Fig. 3.2a).** Three out of six PD immunized mice had no detectable NTHi in their ear wash and three mice had no detectable NTHi in their ear bullae samples (**Fig. 3.2b).**

The median ear wash NTHi concentration of trivalent immunized mice was lower than that of alum control mice, but the difference was not significant, likely due to the variability of the alum samples. However, the median ear bullae NTHi concentration was more than 3 logs lower in trivalent vaccinated mice compared to alum control mice, and this difference was significant ($p=0.0002$) (**Fig. 3.2b**). P6 vaccination provided no protection against NTHi colonization in the ear wash/bullae, and Omp26 vaccination led to lower NTHi concentrations in the ear bullae only ($p=0.005$).

| **Ear Wash** | **Bacterial Count (CFU/mL)** | | | | |
|---|---|---|---|---|---|
| | **ALUM** | **OMP26** | **PD** | **TRIVALENT** | **P6** |
| 1 | $1.8 \times 10^7$ | TNTC | 0 | $4.0 \times 10^4$ | $2.2 \times 10^7$ |
| 2 | $2.0 \times 10^2$ | TNTC | 0 | $2.6 \times 10^3$ | $1.4 \times 10^7$ |
| 3 | 0 | TNTC | $1.0 \times 10^2$ | $1.8 \times 10^3$ | $2.0 \times 10^3$ |
| 4 | $1.8 \times 10^5$ | TNTC | 0 | $4.7 \times 10^3$ | $2.0 \times 10^6$ |
| 5 | $8.0 \times 10^4$ | TNTC | $1.0 \times 10^2$ | $1.6 \times 10^3$ | $2.0 \times 10^7$ |
| 6 | $2.8 \times 10^7$ | $3.3 \times 10^5$ | $1.0 \times 10^2$ | $2.6 \times 10^3$ | |
| 7 | | | | $1.9 \times 10^3$ | |

**Table 3.3** Bacterial count in the ear wash samples of the mice that were immunized with trivalent, PD, OMP26 vaccines or alum control prior to viral priming and bacterial challenge.

| **Ear Bullae** | **Bacterial Count (CFU/mouse)** | | | | |
|---|---|---|---|---|---|
| | **ALUM** | **OMP26** | **PD** | **TRIVALENT** | **P6** |
| 1 | $4.5 \times 10^5$ | 0 | 0 | $1.0 \times 10^2$ | $2.2 \times 10^4$ |
| 2 | $0.5 \times 10^2$ | $1.0 \times 10^2$ | 0 | 0 | $2.7 \times 10^4$ |
| 3 | $3.0 \times 10^2$ | 0 | $0.5 \times 10^2$ | 0 | $2.3 \times 10^3$ |
| 4 | $1.3 \times 10^3$ | 0 | 0 | 0 | $2.0 \times 10^3$ |
| 5 | $4.0 \times 10^4$ | 0 | $0.5 \times 10^2$ | 0 | $1.2 \times 10^5$ |
| 6 | $0.9 \times 10^5$ | $1.05 \times 10^3$ | $0.5 \times 10^2$ | 0 | |
| 7 | | | | 0 | |

**Table 3.4** Bacterial count in the middle ear bulla samples of the mice that were immunized with trivalent, PD, P6, OMP26 vaccines or alum control prior to viral priming and bacterial challenge.

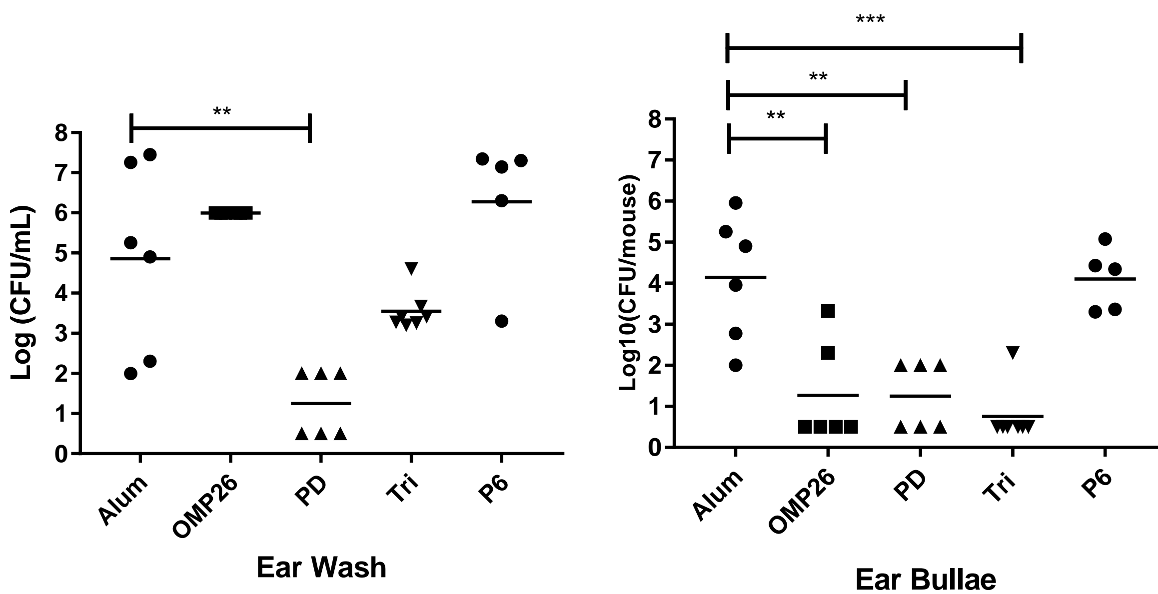



**Figure 3.2 a)** Bacterial count in the ear wash samples and **b)** in the middle ear bulla samples of mice that were immunized with trivalent, PD, P6, OMP26 vaccine or alum control prior to viral priming and bacterial challenge.

Sera from trivalent immunized mice contained OMP26 and P6 antibodies with average endpoint titers of $2.9 \times 10^4$ and $1.0 \times 10^{12}$, respectively (**Fig. 3.3**). P6 alone elicited significantly ($p \leq 0.01$) less P6 antibodies than P6 in the trivalent vaccine. Mice vaccinated with PD alone elicited a robust antibody response, compared to trivalent vaccinated mice, whose sera contained almost no PD antibodies ($p < 0.0001$) (**Fig 3.3**).

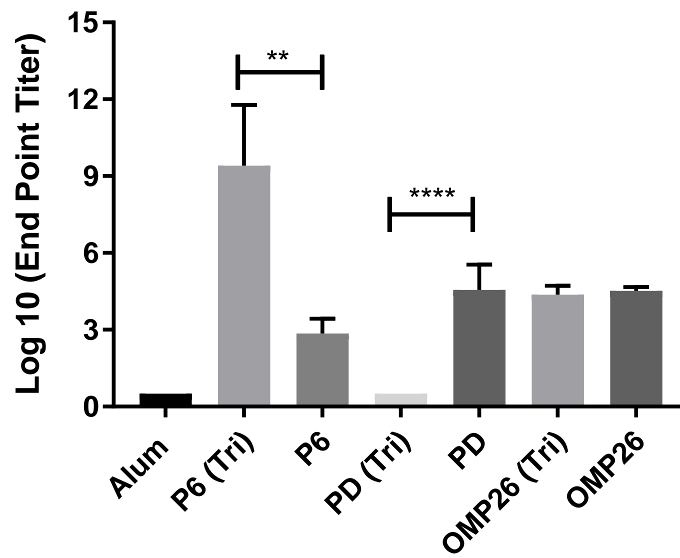



**Figure 3.3** Endpoint titers for antibodies against proteins PD, P6 and OMP26 in blood serum of mice immunized with the vaccines (trivalent, and monovalent protein vaccines compared to alum). (Performed by Jareth Wischmeyer)

## *Testing Vaccine Formulations (Trial #3)*

In the previous two vaccine trials, the trivalent formulation of PD, P6, and OMP26 resulted in (in most cases) significant reduction of NTHi in the ME. However, in both trials, PD failed to elicit an antibody response in trivalent vaccinated mice. In this trial, divalent vaccine formulations of each protein combination were prepared and assessed for antibody production and protection against NTHi colonization.

Adult female mice were vaccinated with: A) original trivalent vaccine (10µg of each antigen/vaccine dose) (5 mice), B) PD (4 mice); C) OMP26+PD (5 mice); D) P6+PD (4 mice); E) P6+OMP26 (4 mice); F) P6 (4 mice); G) new trivalent vaccine (Tri*) (OMP26: 1µg; PD: 10µg; P6: 1µg per vaccine dose) (4 mice); or H) an alum control (4 mice). Mice in each group were administered three doses of the vaccines, as described above, primed with PR8 one week after the final vaccine booster, and then challenged with NTHi (IN challenge, $10^8$ CFU/mL NTHi) seven days later. Blood was collected from each mouse a few days prior to the NTHi challenge to determine antibody titers to the vaccine antigens. NTHi bacteria counts were quantified in nasal lavages, ear washes, and homogenized ear bullae.

NTHi bacterial loads in the nasal lavage samples were highly variable, yielding no statistically significant protection in any of the protein vaccine groups. However, there were significantly lower NTHi concentrations in the ear washes of trivalent vaccinated mice, P6+OMP26 vaccinated mice, and PD vaccinated mice compared to alum mice. Further, there were significantly lower NTHi concentrations in the ear bullae of trivalent vaccinated mice, P6+OMP26 vaccinated mice, and P6+PD vaccinated mice compared to alum mice.

| Nasal Lavages | Bacterial Count (CFU/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Alum | Tri | OMP+PD | P6+PD | PD | P6+OMP | P6 | Tri* |
| 1 | $2.0x10^2$ | $1.0x10^2$ | $9.0x10^3$ | 0 | $3.1x10^3$ | 0 | $6.9x10^4$ | 0 |
| 2 | $1.4x10^3$ | $2.4x10^3$ | $4.3x10^4$ | 0 | $4.7x10^3$ | 0 | $7.3\ x10^4$ | $1.0x10^2$ |
| 3 | 0 | $1.0x10^2$ | $5.4x10^4$ | $5.0x10^2$ | $8.0x10^2$ | $1.4x10^3$ | $4.7\ x10^4$ | $3.0x10^2$ |
| 4 | 0 | 0 | $3.0x10^2$ | 0 | 0 | 0 | $2.0\ x10^3$ | $4.0x10^2$ |
| 5 | | $4.0x10^2$ | $1.3x10^4$ | 0 | | | | |

**Table 4.1** Bacterial count in the nasal lavage samples of the mice that were immunized with trivalent, divalent or single protein vaccines prior to viral priming and bacterial challenge

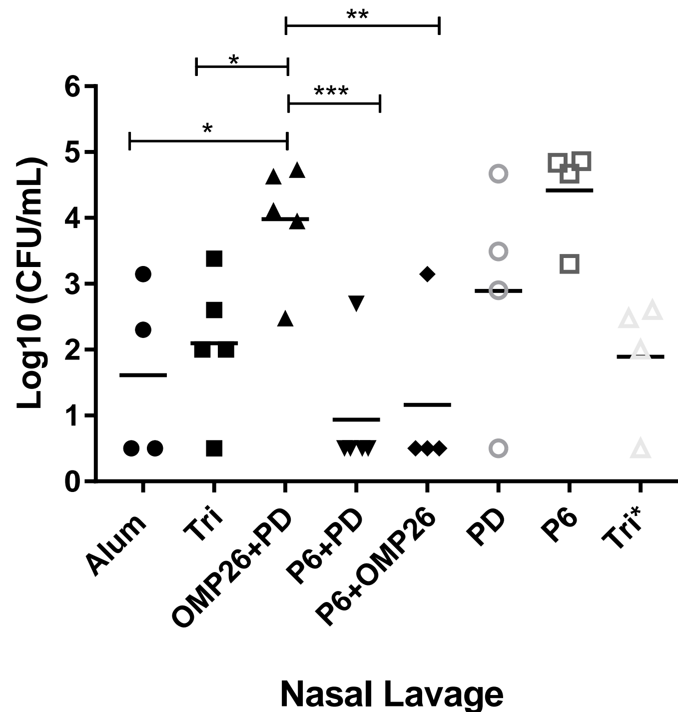



**Figure 4.2** Comparison of the bacterial count in the nasal lavage samples of the mice that were immunized with trivalent, divalent or monovalent protein vaccines prior to viral priming and bacterial challenge. (Tri* - new trivalent formulation).

P6 elicited P6 antibody production in mice when formulated as a single antigen vaccine, a trivalent protein vaccine with PD and OMP26, and as a divalent vaccine with OMP26 or PD. The trivalent* formulation, which contains 10fold less P6 compared to the original trivalent formulation, resulted in significantly less P6 antibodies, as expected.

OMP26 elicits a very strong antibody response in every vaccine formulation that includes the OMP26 antigen. OMP26 also appears to cross-react with sera from P6 vaccinated mice, perhaps due to the N-terminal Histidine tag, which is present in both recombinant proteins. PD only elicits PD antibody production in mice when formulated as a single antigen vaccine or as a divalent vaccine with P6.

| Ear Wash | Bacterial Count (CFU/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Alum | Tri | OMP+PD | P6+PD | PD | P6+OMP | P6 | Tri* |
| 1 | $6.4x10^7$ | $1.5x10^4$ | 0 | $6.1x10^4$ | $3.7x10^3$ | $1.8 x10^3$ | 1.5x10 | $4.0x10^4$ |
| 2 | $8.6x10^4$ | $2.0x10^2$ | $7.5x10^6$ | $3.8x10^3$ | $1.7 x10^3$ | $1.8 x10^3$ | $2.8x10^5$ | $3.7x10^4$ |
| 3 | $2.4x10^4$ | $1.0x10^2$ | $2.6x10^4$ | $3.9x10^3$ | $1.7x10^3$ | $1.6 x10^3$ | $4.2x10^7$ | $3.3x10^4$ |
| 4 | $1.7x10^4$ | 0 | $3.4x10^3$ | $4.9x10^3$ | $1.8 x10^3$ | $2.2 x10^3$ | $3.5x10^5$ | $5.1x10^4$ |
| 5 | | $5.0x10^2$ | $1.7x10^3$ | $5.9x10^4$ | | | | |

**Table 4.2** Bacterial count in the ear wash samples of the mice that were immunized with trivalent, divalent or monovalent protein vaccines prior to viral priming and bacterial challenge.

| Ear Bullae | Bacterial Count (CFU/mouse) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Alum | Tri | OMP+PD | P6+PD | PD | P6+OMP | P6 | Tri* |
| 1 | $2.8x10^4$ | 0 | $7.0x10^3$ | $3.0x10^3$ | $9.9x10^4$ | 0 | $3.0x10^6$ | $1.0x10^3$ |
| 2 | $1.0x10^4$ | $2.0x10^3$ | $7.0x10^6$ | 0 | $1.9x10^6$ | $5.0x10^2$ | $7.1x10^5$ | $1.5x10^3$ |
| 3 | $1.0x10^4$ | 0 | $5.2x10^4$ | 0 | $5.5x10^3$ | 0 | $2.0x10^6$ | $3.0x10^3$ |
| 4 | $1.5x10^4$ | 0 | $8.5x10^6$ | 0 | $3.4x10^5$ | $4.0x10^3$ | $8.0x10^5$ | $5.0x10^2$ |
| 5 | | $1.0x10^3$ | $2.1x10^6$ | $5.0x10^2$ | | | | |

**Table 4.3** Bacterial count in the middle ear bullae samples of the mice that were immunized with trivalent, divalent or sing protein vaccines prior to viral priming and bacterial challenge.

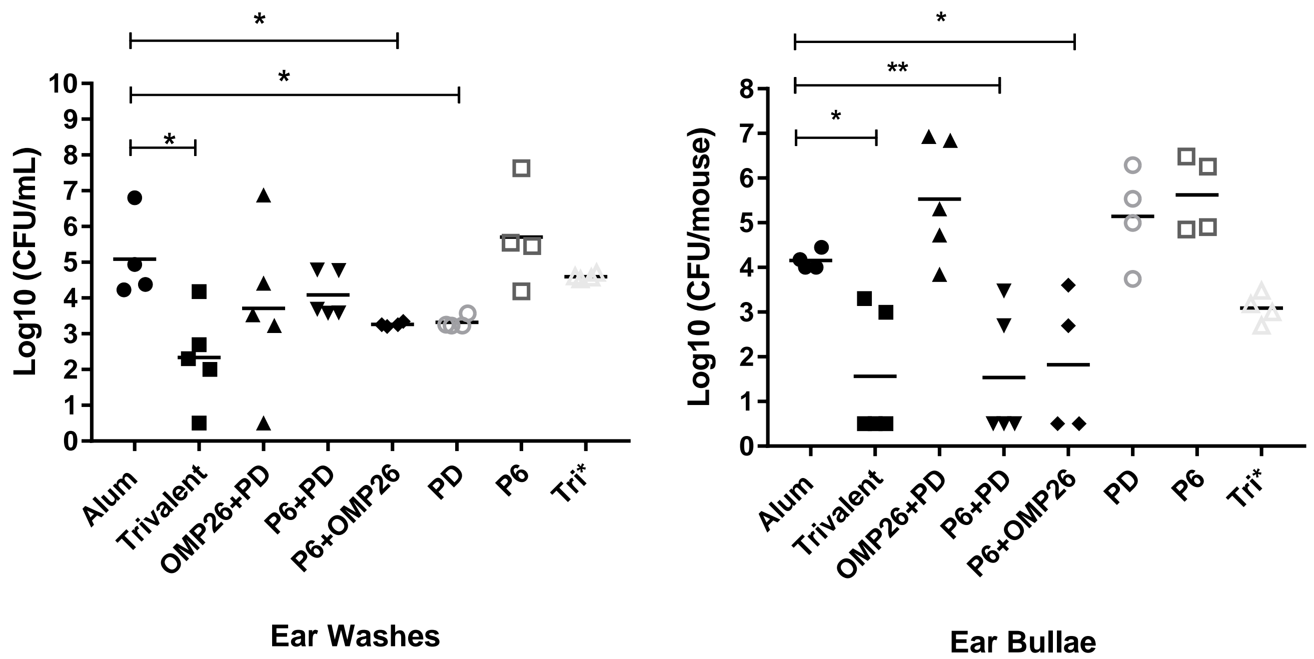



**Figure 4.3 a)** Comparison of the bacterial count in the ear wash samples and **b)** in the middle ear bulla samples of mice that were immunized with trivalent, divalent or monovalent protein vaccines prior to viral priming and bacterial challenge.

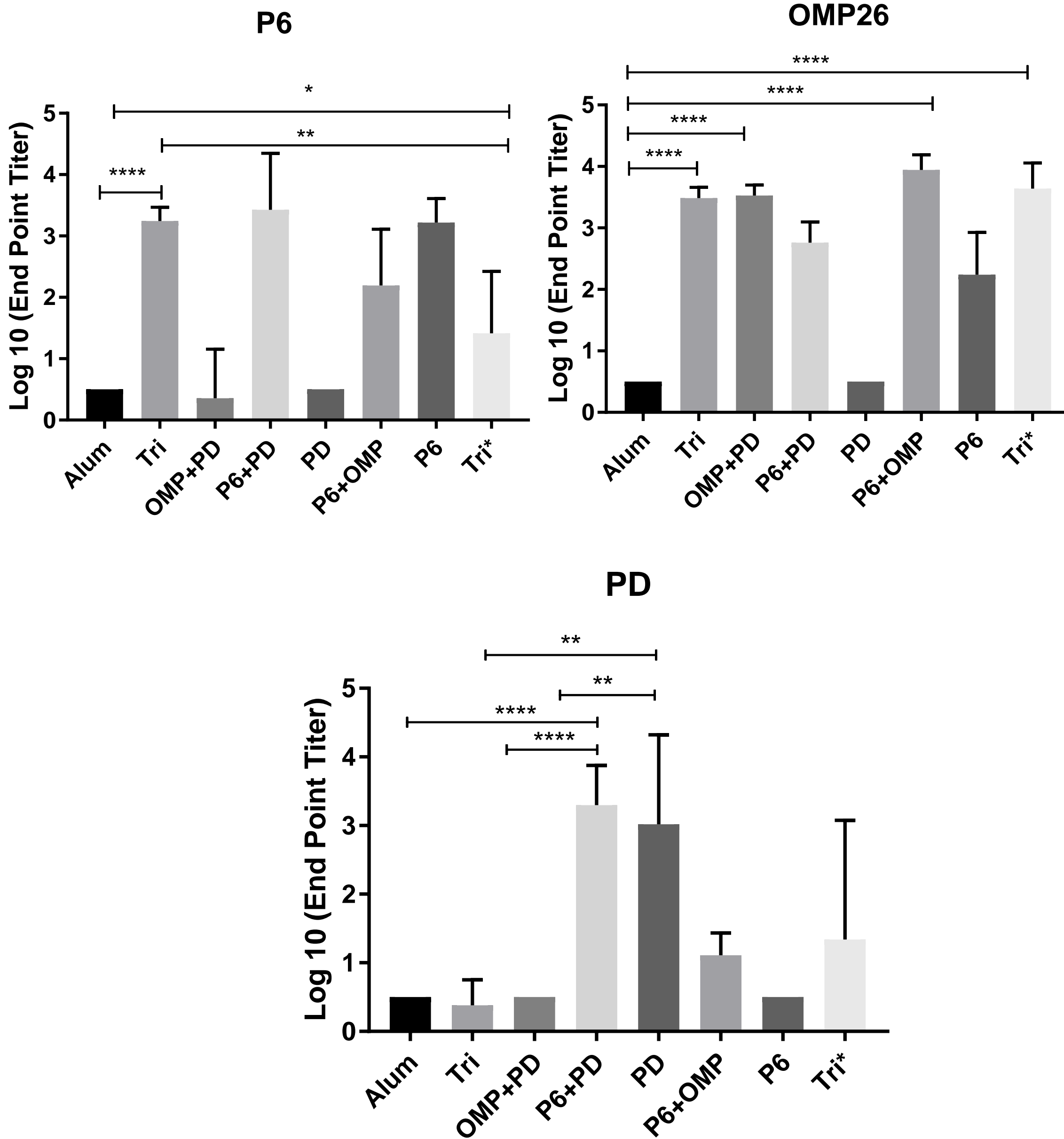



**Figure 4.4** Endpoint titers for antibodies against proteins PD, P6 and OMP26 in blood serum of mice immunized with the following vaccines (trivalent, divalent and single protein vaccines compared to alum). (Performed by Jareth Wischmeyer)

To confirm that PD was not degraded by proteases in the trivalent (OMP26+PD+P6) and divalent (OMP26+PD) formulations, the protein samples were analyzed using SDS-PAGE and Western blotting. PD was detected in all three vaccine formulations (**Fig. 4.1**).

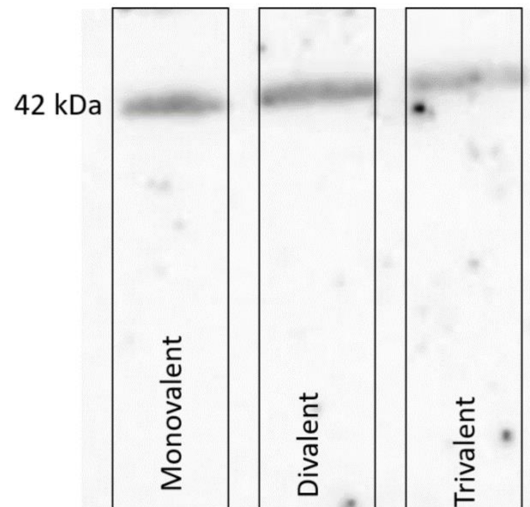



**Figure 4.1** Western blot analysis of vaccines for presence of PD in monovalent, divalent, and trivalent vaccines.

# DISCUSSION

The recent emergence of antibiotic-resistant bacterial strains has reinvigorated the search for new antibiotics and broad-coverage vaccines. AOM continues to be the leading illness for which antibiotics are prescribed to children, pointing to the urgent need for vaccines against the organisms that cause AOM. While there is a widely used vaccine for *Spn* AOM, there is currently no vaccine in use to protect against NTHi AOM in the US. A protein based vaccine would be highly attractive, as it would likely offer broad protection against the many (>100) NTHi strains.

The US Food and Drug Administration (FDA) requires all vaccines to go through rigorous animal testing before they can be tested in humans. To that end, animal models of disease must first be developed to accurately mimic the human diseased state and then to demonstrate the safety, feasibility, and effectiveness of potential vaccines. While an effective mouse model has already been established and implemented for *Spn* AOM, most NTHi AOM models have employed chinchillas or JUNBO mice. In an effort to reduce costs and increase the flexibility and efficiency of our model, we modified an existing NTHi mouse model to optimize it for the assessment of several protein vaccine formulations.

Results from our initial NTHi AOM mouse studies helped us to optimize our model. First, we determined that mice needed to be primed (IN) with virus one week prior to IN NTHi challenge. During a viral upper respiratory infection, mucous can be altered in the NP.[9, 49] Changes to mucous and/or the NP epithelial lining may lead to increased bacterial pathogenicity and enhanced NTHi colonization. An upper respiratory tract infection may lead to an inflammatory response obstruction of ET and resulting in negative middle ear pressure, allowing for efficient efflux of NTHi into the ME and consequential AOM. [50]

Next, we determined that A) NTHi can colonize in the NP up to 7 days post-NTHi challenge (during viral coinfection); B) NTHi can colonize in the ME up to 7 days post-NTHi; C) optimal dosage for IN NTHi infection is $10^8$ CFU/mouse; and D) there is no significant difference between female and male mice in our coinfection model.

However, as expected, NTHi colonization in the ME appeared to be somewhat variable. We noticed that, by day 3, some mice cleared NTHi from their ME, as assessed by ear washes, or those mice never developed ear infections at all. To address this variability, we began to remove the ear bullae, in addition to performing ear washes (which were always done prior to bullae removal). We were able to detect quantifiable NTHi in the ear bullae samples when we could not detect ME count in the middle ear. We proposed that at least some of the bacteria were strongly attached to the epithelial cells, and therefore were only detectable in homogenized bulla samples. This change in protocol allowed us to decrease variability among groups and to capture NTHi colonization in mice that previously showed no sign of NTHi in their ear washes. Results from our preliminary experiments using this new protocol suggested that our PR8-NTHi coinfection model resulted in the progression of NTHi to the ME.

We also confirmed that NTHi were not just travelling to the ME, but were also was causing inflammation, as demonstrated by IFNγ cytokine production in the ear washes of coinfected mice. Together, inflammation and NTHi colonization in the ME point to successful induction of AOM in coinfected mice.

With our NTHi intranasal mouse colonization model established, we wanted to further test the model for its usefulness in assessing the effectiveness of protein vaccines. The proteins we used had already been shown to be promising vaccine candidates for protection against NTHi. Immunogenicity is one of the critical factors for selection of desirable vaccine candidate

antigens. Previous studies have shown that proteins PD, P6, OMP26, and PF can stimulate an immune response.[22, 29, 36] Additionally, several of these proteins were shown to be bactericidal.[35, 36, 39]

In our first vaccine trial, several protein vaccine formulations were prepared and tested. Female mice were vaccinated with A) a trivalent protein formulation (P6, OMP26, and PD) with aluminum hydroxide adjuvant; B) PF with alum; or C) an aluminum hydroxide control. Interestingly, NP bacterial loads were only moderately reduced in immunized mice. We suggest that we saw only a modest reduction in NP bacterial loads, because we infect the mice with such high concentrations of NTHi during challenge. These high NTHi titers are necessary to "force" the bacteria to travel to the ME, but unfortunately result in non-physiologically high NP colonization levels that are less affected by vaccine protection. This effect is more pronounced in the later studies.

We were able to observe significant reduction (on average 1 log decrease) in bacterial load in the ear washes and bullae of trivalent vaccinated mice, suggesting that both our mouse NTHi AOM model and the trivalent vaccine were "working." We did not observe significant differences in ME bacterial loads in mice immunized with PF (compared to alum control mice), suggesting that PF by itself did not provide observable protection.

On opposite, direct ear injections showed no differences between vaccinated and control mice. We suspected that the NTHi levels injected into the ME are too high to demonstrate reductions in bacterial loads in vaccinated mice- basically, we overwhelmed the ME with too much NTHi so vaccination effectiveness cannot be easily evaluated. To implement this AOM model, we would need to optimize the parameters (injected bacterial levels, timing of harvest, etc.); however, the protocol itself is challenging and time consuming, so we have chosen to

postpone optimization of this protocol so that we can focus on the more physiologically relevant IN coinfection model.

In the second vaccine trial, we compared the trivalent protein vaccine to single protein vaccines: A) the same trivalent protein formulation, as described above; B) PD with aluminum hydroxide adjuvant; C) OMP26 with alum; D) P6 with alum; and E) an aluminum hydroxide control. In this trial, there were no significant differences in nasal lavage bacterial loads between vaccinated and alum control mice; as described above, we propose that the excessively high NTHi titers required for our model prevent observable differences in NP colonization levels. Alternatively, our protein vaccines may only be affecting NTHi movement to the ME and not NP colonization.

The P6 vaccine performed the worst, with no significant reductions in either the ear wash or ear bulla samples. However, we did see significant reductions in ear wash bacteria levels in PD immunized mice compared to control mice and in ear bulla bacteria levels in OMP26, PD, and trivalent protein immunized mice compared to control mice. Although P6 is a leading NTHi vaccine candidate, and P6 antibodies have been also shown to exhibit bactericidal activity, we propose that the version of purified recombinant P6 that we used in this study has decreased immunigenicity.[22, 29, 36] In this study (and during most of this project), we used nonlipidated-P6 (NL-P6) in our formulations. NL-P6 is expressed without its N-terminal lipid, since the lipid can cause the protein to aggregate, which makes purification more challenging. However, it has been suggested in the literature that a lipoprotein received much of its immunogenicity from the lipid itself. To address these concerns, we have begun studies using lipidated P6 (L-P6) and have observed greater protection in L-P6 vaccinated mice.

Trivalent immunized mice had lower levels of NTHi colonization, on average, in their ME. Six mice out of seven trivalent immunized mice had no detectable NTHi in their ear bullae. These results were similar to those from our first vaccine trial. We propose that antibodies produced in trivalent vaccinated mice reduce bacterial concentration in the middle ear by killing NTHi and/or disrupting the bacterial adhesion to epithelial cells, thus preventing strong adhesion to the ear bulla tissue.

In the third vaccine trial, we assessed two trivalent vaccine formulations as well as several different divalent formulations: A) original trivalent vaccine (10µg of each antigen/vaccine dose) and alum; B) PD and alum; C) OMP26+PD and alum; D) P6+PD and alum; E) P6+OMP26 and alum; F) P6 and alum; G) new trivalent vaccine (Tri*) (OMP26: 1µg; PD: 10µg; P6: 1µg per vaccine dose) and alum; and H) an aluminum hydroxide control.

Again, bacterial loads in the nasal lavage samples were highly variable, and many of the vaccinated mice exhibited higher bacterial loads than the alum control mice. For the third time, we were able to observe significant reductions in NTHi in the ear wash and ear bulla samples of trivalent vaccinated mice compared to control mice. However, decreasing the amount of P6 and OMP26 in the new Tri* vaccine formulation resulted in similar bacterial loads in the ME of those mice and alum control mice, suggesting that P6 and/or OMP26 contributed to protection in the original trivalent vaccine. OMP26+P6 vaccinated mice showed reductions in bacterial loads in both ear wash and ear bulla samples. Median NTHi levels in the ear bullae of OMP26+PD, PD, and P6 mice were all higher than the median NTHi level of control mice.

In summary, we observed that several protein vaccine formulations were successful at decreasing NTHi levels in the ME, as determined by the ear wash and/or ear bulla samples. In general, the trivalent vaccine formulation containing equal amounts of PD, OMP26, and P6

showed the greatest and most significant reductions in ME bacteria levels. The bacterial loads in nasal lavage samples were more variable and did not always correlate with changes in the ME.

To confirm that our vaccines are immunogenic, we performed immunoassays to determine the presence of antibodies. These studies showed some unexpected results. In the first trial, trivalent immunized mice did not produce PD antibodies. We suspected an error in the vaccine formulation and performed second study with a trivalent vaccine. Mice vaccinated with the trivalent protein formulation did not produce PD antibodies however mice vaccinated only with PD protein had a robust antibody response. We hypothesized that one of the proteins or alum in the trivalent formulation is preventing/impeding PD antibody production. In the third vaccine trial we had similar picture as before. Mice vaccinated with the trivalent protein formulation did not produce PD antibodies (PD antibody levels similar to Alum mice); Mice vaccinated with PD alone or P6+PD induced a robust PD antibody response; Mice vaccinated with PD+OMP26 produced LOW levels of PD antibody, similar to alum/trivalent mice; Mice vaccinated with Tri* formulation lower levels of P6/OMP26 had in between levels of PD antibodies. Our final hypothesis was that presence of OMP26 with PD inhibits production of PD antibodies. The western blot analysis showed that PD is present in all three formulations (PD, OMP26+PD, OMP26+PD+P6). We believe, OMP26 may bind to PD and prevent its interaction with mouse immune cells, thus inhibiting PD antibody production. Currently, this interaction is poorly understood, and we are looking to expand our knowledge through next several studies.

# FUTURE STUDIES

To further explore the OMP26 and PD interaction, we performed a study with divalent (OMP26+PD) and trivalent (OMP26+PD+P6) vaccines, where the OMP26 and PD antigens

were delivered as separate vaccine formulations and injected into separate legs or delivered as a single formulation. Although not a part of this thesis, these studies confirm our hypothesis that OMP26 and PD interact in vivo in mice and depress PD antibody production. When the two antigens are delivered via separate vaccines (injected at the same time, in separate legs), PD is able to elicit antibody production. Antibody titers for the divalent/separate vaccines are about half that for the monovalent PD vaccine. In light of these results, a new trivalent vaccine formulation containing PD, P6, and Protein F will be prepared and tested in mice in the near future.

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