Use of the Chinchilla Model to Evaluate the Vaccinogenic Potential of the *Moraxella catarrhalis* Filamentous Hemagglutinin-like Proteins MhaB1 and MhaB2

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Abstract

*Moraxella catarrhalis* causes significant health problems, including 15–20% of otitis media cases in children and ~10% of respiratory infections in adults with chronic obstructive pulmonary disease. The lack of an efficacious vaccine, the rapid emergence of antibiotic resistance in clinical isolates, and high carriage rates reported in children are cause for concern. In addition, the effectiveness of conjugate vaccines at reducing the incidence of otitis media caused by *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* suggest that *M. catarrhalis* infections may become even more prevalent. Hence, *M. catarrhalis* is an important and emerging cause of infectious disease for which the development of a vaccine is highly desirable. Studying the pathogenesis of *M. catarrhalis* and the testing of vaccine candidates have both been hindered by the lack of an animal model that mimics human colonization and infection. To address this, we intranasally infected chinchilla with *M. catarrhalis* to investigate colonization and examine the efficacy of a protein-based vaccine. The data reveal that infected chinchillas produce antibodies against antigens known to be major targets of the immune response in humans, thus establishing immune parallels between chinchillas and humans during *M. catarrhalis* infection. Our data also demonstrate that a mutant lacking expression of the adherence proteins MhaB1 and MhaB2 is impaired in its ability to colonize the chinchilla nasopharynx, and that immunization with a polyepitope shared by MhaB1 and MhaB2 elicits antibodies interfering with colonization. These findings underscore the importance of adherence proteins in colonization and emphasize the relevance of the chinchilla model to study *M. catarrhalis*-host interactions.

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Introduction

*Moraxella catarrhalis* is a leading cause of otitis media worldwide along with *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi) [1,2,3,4,5,6,7,8]. More than 80% of infants experience at least one episode of this disease by the age of three, and *M. catarrhalis* is the causative agent in ~20% of these cases. Likewise, otitis media is the number one reason for which children are prescribed antibiotics [9,10]. In the U.S., ~25 million visits are made annually to pediatrician offices for treatment of this painful infection and of these, 3–5 million are precipitated by *M. catarrhalis* [1,2,3,4,5,6,7,8,11,12,13,14,15]. The annual costs associated with management of otitis media are upwards of $5 billion, and direct medical care expenditures alone account for $2–3 billion [1,2,5,15,16,17,18,19]. The disease is a significant source of distress, as it produces a rapidly expanding middle ear abscess that exerts pressure on the tympanic membrane and causes acute stabbing pain. After the onset of otitis media, fluid persists in the middle ear for weeks to months and interferes with hearing. Recurring ear infections are prevalent and occur during the crucial period when a child is developing speech and language skills. Hence, many children spend most of the first 2–3 years of life with some hearing impairment because of multiple episodes of otitis media, which can delay the development of communication and learning. The WHO has estimated that chronic/recurrent otitis media occurs in 65–330 million people and is the major cause of hearing loss in developing countries [20,21]. Clearly, otitis media is a significant health and economic problem, and *M. catarrhalis* contributes substantially to this burden.

*Moraxella catarrhalis* is also the second most common cause of respiratory infections in adults with chronic obstructive pulmonary disease (COPD) [19,22,23,24]. This disease is the fourth leading cause of death in the U.S., surpassed only by heart attack, cancer and stroke [25]. Each year ~10 million visits to physicians are related to COPD, and the costs associated with treatment are enormous – direct medical care costs alone are greater than $14 billion [26,27,28,29]. Worldwide, COPD ranks as the fourth leading cause of death, killing more people than TB or HIV/AIDS, and is predicted to be third by 2030 [30,31]. The course of this debilitating disease is characterized by intermittent exacerbations, half of which caused by bacterial infections. These infections, of which *M. catarrhalis* causes ~10% of cases, contribute prominently to the progression of COPD by augmenting inflammation, oxidative stress, and tissue damage in the airways.
M. catarrhalis infections are a matter of concern due to the rapid emergence of antibiotic resistance in clinical isolates, high carriage rates in children, and the current lack of a vaccine. More than 90% of M. catarrhalis strains are now resistant to β-lactams [47, 48, 49, 50, 51, 52, 53, 54], which are generally the first antibiotics prescribed to treat otitis media. The genes specifying this resistance appear to be of Gram-positive origin [55, 56], suggesting that M. catarrhalis can readily acquire genes conferring resistance to additional antibiotics via horizontal transfer. Carriage rates as high as 81% have been reported in children [6, 57]. In one study, Faden and colleagues analyzed the nasopharynx of 120 children over a two-year period and showed that 77% of patients became colonized with M. catarrhalis [58]. These investigators also observed a direct relationship between colonization with M. catarrhalis and development of otitis media. This high carriage rate, coupled with the emergence of antibiotic resistance, suggests that M. catarrhalis infections may become more prevalent and difficult to treat, emphasizing the need to improve our understanding of pathogenesis by this understudied bacterium in order to identify targets for intervention and prevention.

To cause disease, M. catarrhalis must first colonize the nasopharynx and then spread to distant sites such as the middle ear and the lower respiratory tract. Hence, one key event that occurs early in pathogenesis by the organism is adherence to the mucosal surface of the nasopharynx because it leads to colonization. Crucial to this process are afimbrial adherence proteins (adhesins), which mediate binding of bacteria to host cells (adhesins), which mediate binding of bacteria to host cells. In vitro, mutant strains to human airway cells characterized by demonstrating a decrease in the adherence of a 1-kb amphlicon encompassing amino acids 72–399 of the M. catarrhalis strain O35E MhaB1 protein was generated with primers P1 (5′-CGG GAT CCC TTA TTT CTG ACA GTG AAG CAA- 3′; BamHI site underlined) and P2 (5′-CGG GAT CCC AGT ATT ACC TTG CAA GTT GGC AGT- 3′; XhoI site underlined). This DNA fragment was excised from an agarose gel, purified with the High Pure PCR Product Purification Kit (Roche Applied Science), restricted with the endonucleases BamHI and XhoI (New England Biolabs), and ligated into the BamHI and XhoI sites of the vector pGEX4T-2 (GE Healthcare Life Sciences), yielding plasmid pGEX-MhaB. This plasmid was sequenced to verify that no mutations were introduced during PCR and to confirm that the protein expressed from pGEX-MhaB corresponds to residue 72–399 of M. catarrhalis O35E MhaB1 fused to an N-terminal Glutathione-S-transferase (GST) tag. Plasmid DNA used as template in sequencing reactions was obtained with the QIAprep Spin Miniprep Kit (Qiagen). A similar approach was used to obtain the plasmid pGEX-McaP, which expresses residues 51–338 of M. catarrhalis O35E McaP joined to GST. The PCR product cloned into pGEX-McaP was amplified with primers P3 (5′-CGG GAT CCC AAG AAT TTA GGC AAA CCG TA- 3′; BamHI site underlined) and P4 (5′-CGG GAT CCC AGT ATT ACC TTG CAA GTT GGC AGT- 3′; XhoI site underlined). M. catarrhalis O35E genomic DNA was used as the template in all PCR-based cloning experiments.

Materials and Methods

Plasmids, Bacterial Strains, Growth Conditions, and Culture of Human Epithelial Cells in vitro

Strains and plasmids are described in Table 1. Wild-type (WT) M. catarrhalis isolates were routinely cultured using Todd-Hewitt agar plates (THA, BD Diagnostic Systems). The M. catarrhalis isogenic mutant strain O35E.B1B2 was propagated on THA supplemented with 15 μg/mL spectinomycin and 5 μg/mL zeocin. The hag transposon mutant O35E.TN2, the ompCD mutant strain O35E.CD1, and the uspA2 serum-sensitive mutant O35E.2 were cultured using THA containing 20 μg/mL kanamycin. For colonization experiments, tissues and nasopharyngeal lavages collected from infected animals were plated onto THA supplemented with 5 μg/mL Vancomycin and 2.5 μg/mL Tri-methoprim to suppress the growth of the chinchilla flora. Escherichia coli was grown using Luria-Bertani (LB) medium (Fisher BioReagents) containing 15 μg/mL chloramphenicol or 100 μg/mL ampicillin. All strains were cultured at 37°C in the presence of 7.5% CO₂. The human cell line HEp-2 (laryngeal epithelium; ATCC CCL-2) was cultured as previously reported [74].

Recombinant DNA Methods, PCR, and Cloning

Standard molecular biology techniques were performed as described elsewhere [70,72,74,75]. Genomic DNA was obtained using the Easy-DNA™ kit (Invitrogen™ Life Technologies™). Platinum® Pfu DNA Polymerase was used in cloning experiments per the manufacturer’s recommendations (Invitrogen™ Life Technologies™). A 1-kb amphlicon encompassing amino acids (aa) 72–399 of the M. catarrhalis strain O35E MhaB1 protein was generated with primers P1 (5′-CGG GAT CCC TTA TTT CTG ACA GTG AAG CAA- 3′; BamHI site underlined) and P2 (5′-CGG GAT CCC AGT ATT ACC TTG CAA GTT GGC AGT- 3′; XhoI site underlined). This DNA fragment was excised from an agarose gel, purified with the High Pure PCR Product Purification Kit (Roche Applied Science), restricted with the endonucleases BamHI and XhoI (New England Biolabs), and ligated into the BamHI and XhoI sites of the vector pGEX4T-2 (GE Healthcare Life Sciences), yielding plasmid pGEX-MhaB. This plasmid was sequenced to verify that no mutations were introduced during PCR and to confirm that the protein expressed from pGEX-MhaB corresponds to residue 72–399 of M. catarrhalis O35E MhaB1 fused to an N-terminal Glutathione-S-transferase (GST) tag. Plasmid DNA used as template in sequencing reactions was obtained with the QIAprep Spin Miniprep Kit (Qiagen). A similar approach was used to obtain the plasmid pGEX-McaP, which expresses residues 51–338 of M. catarrhalis O35E McaP joined to GST. The PCR product cloned into pGEX-McaP was amplified with primers P3 (5′-CGG GAT CCC AAG AAT TTA GGC AAA CCG TA- 3′; BamHI site underlined) and P4 (5′-CGG GAT CCC AGT ATT ACC TTG CAA GTT GGC AGT- 3′; XhoI site underlined). M. catarrhalis O35E genomic DNA was used as the template in all PCR-based cloning experiments.

Nucleotide Sequence Analysis

Plasmids were sequenced at the University of Michigan sequencing core (http://sequore.brcf.med.unichicago.edu/). Chromatograms were analyzed and assembled with the Sequencer software (Gene Codes Corporation). Sequence analysis was performed using Vector NTI (Invitrogen™ Life Technologies™).

Protein Preparation

Outer membrane proteins were obtained from M. catarrhalis strains using the EDTA procedure of Murphy and Loeb [76]. The method used to prepare whole-cell lysates is described elsewhere [77,78]. The His-tagged recombinant protein His-MhaB was obtained as previously outlined by Balder et al [67]. The plasmids pGEX-MhaB and pGEX-McaP were introduced in the E. coli strain TUNER™ (EMD Millipore) for the purpose of overexpressing and purifying the recombinant proteins GST-MhaB and GST-McaP, respectively. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration of 1 mM) to broth cultures and incubating for 5 hours at 37°C with agitation (200-rpm). Bacteria were pelleted, followed by treatment with the BugBuster® HT protein extraction reagent (EMD Millipore) supplemented with lysisozyme™ (EMD Millipore) under the recommended conditions. Recombinant proteins were then purified using a GST Spin Purification Kit (Thermo Scientific).
Table 1. Strains and plasmids used in this study.

| Strain         | Description                                      | Source                                      |
|----------------|--------------------------------------------------|---------------------------------------------|
| **M. catarrhalis** |                                                 |                                             |
| O35E           | WT isolate from middle ear effusion (Dallas, TX) | [82]                                        |
| O35E.B1B2      | mhaB1mhaB2 double isogenic mutant of strain O35E, spectinomycin and azitoin resistant | [67]                                        |
| O35E.TN2       | hag transposon mutant of strain O35E, kanamycin resistant | [132]                                       |
| O35E.2         | uspA2 isogenic mutant of strain O35E, kanamycin resistant | [133]                                       |
| O35E.CD1       | ompCD isogenic mutant of strain O35E, kanamycin resistant | [72]                                        |
| O12E           | WT isolate from middle ear effusion (Dallas, TX) | [66]                                        |
| McGHS1         | WT isolate from patient with respiratory infection (Toledo, OH) | [70]                                        |
| **E. coli**    |                                                 |                                             |
| EP300TM        | Cloning strain for recombinant DNA methods       | Epicentre® (Illumina®)                      |
| TUNERTM        | Expression strain for protein purification purposes | EMD Millipore                                |
| **Plasmids**   |                                                 |                                             |
| pGEX4T-2       | Protein expression vector, ampicillin resistant   | GE Healthcare Life Sciences                 |
| pGEX-MhaB      | pGEX4T-2 expressing O35E MhaB1 aa 72–399 joined to a GST N-terminal tag (GST-MhaB), ampicillin resistant | This study                                  |
| pGEX-McaP      | pGEX4T-2 expressing O35E McaP aa 51–333 joined to a GST N-terminal tag (GST-McaP), ampicillin resistant | This study                                  |
| pRBHis.MhaB.72.399 | pETcoco-1 expressing O12E MhaB1 aa 72–399 joined to 6 N-terminal histidine residues (His-MhaB), chloramphenicol resistant | [67]                                        |

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Scientific Pierce) per the manufacturer’s instructions. Protein concentrations were determined with a bicinchoninic acid (BCA) Protein assay kit (Thermo Scientific Pierce).

Analysis of Selected Antigens

Equivalent protein amounts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were visualized by staining gels with the ProtoBlue™ Safe reagent (National Diagnostics). Alternatively, the resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore) for western blot analysis. After transfer, the PVDF membranes were submersed in StartingBlock™ T20 (Thermo Scientific) and incubated for 1 hour at room temperature. The membranes were then probed overnight at 4°C with primary antibodies (Abs) diluted in StartingBlock™ T20. After this incubation, the membranes were washed with Phosphate-Buffered Saline (PBS) supplemented with 0.05% (vol/vol) Tween 20, followed by 1 hour incubation at room temperature with secondary Abs conjugated to Horse Radish Peroxidase (HRP) diluted in StartingBlock™ T20. After washing off the excess secondary Abs with PBS+0.05% Tween 20, protein bands were visualized by chemiluminescence using the Luminata™ Crescendo Western HRP substrate (EMD Millipore) and a Foto/Analyst Luminary/FX imaging system (Fotodyne Inc.).

For ELISA, duplicate wells of Immulon™ 2HB plates (Thermo Scientific Nunc) were coated overnight at 4°C with ~1 µg of purified GST-MhaB protein. Excess unbound protein was removed by washing the wells with PBS+0.05% Tween 20, and the wells were then filled with PBS+0.05% Tween 20 containing 3% (wt/vol) dry milk and incubated for 1 hour at room temperature. After washing with PBS+0.05% Tween 20, the wells were probed overnight at 4°C with primary Abs diluted in PBS+0.05% Tween 20+3% dry milk. After this incubation, the wells were washed with PBS+0.05% Tween 20, followed by overnight incubation at 4°C with secondary Abs conjugated to HRP and diluted in PBS+0.05% Tween 20+3% dry milk. After washing off the excess secondary Abs with PBS+0.05% Tween 20, 100 µL of the SureBlue™ TMB Microwell Peroxidase Substrate (KPL) was added to wells. Color development indicative of antibody binding was measured by determining the absorbance of well contents at a wavelength of 650 nm using a μQuant™ Microplate Spectrophotometer (BioTek®). End-point titers were determined as described by Song et al. [79] and correspond to the highest fold dilution giving an optical density at 630 nm greater than the mean value plus 3 standard deviations of pre-immune samples. Antibodies

The murine monoclonal Abs 10F3 [specific for the M. catarrhalis iron transport protein CopB [80]], 5D2 [specific for the M. catarrhalis adhesin Hag [81]], 17H4 [specific for the M. catarrhalis serum resistance protein UspA2 [82]], and 1D3 [specific for the M. catarrhalis adhesin OMPCD [83]], His-tag® (EMD Millipore) and GST-Tag™ were used as primary Abs in western blot experiments in combination with goat anti-mouse HRP (IgG+IgA+IgM) secondary Abs (SouthernBiotech). For experiments using chim-chilla samples as primary Abs (ELISA, Western blot), goat anti-rat Abs conjugated to HRP were utilized for detection. Goat anti-rat HRP (IgG) and HRP (IgG+IgA+IgM) were purchased from SouthernBiotech. Goat anti-rat HRP (IgA) Abs were obtained from Bethyl Laboratories, Inc.

Adherence Assays

The WT M. catarrhalis strains O35E, O12E and McGHS1 were preincubated for 30 min at 37°C with samples (serum, nasopharyngeal lavage fluids) collected from naive and vaccinated chinchillas. These bacteria were then used to perform adherence assays as previously described by Lipski and colleagues [73].

Table 1. Strains and plasmids used in this study.

| Strain         | Description                                      | Source                                      |
|----------------|--------------------------------------------------|---------------------------------------------|
| **M. catarrhalis** |                                                 |                                             |
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| O35E.2         | uspA2 isogenic mutant of strain O35E, kanamycin resistant | [133]                                       |
| O35E.CD1       | ompCD isogenic mutant of strain O35E, kanamycin resistant | [72]                                        |
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| McGHS1         | WT isolate from patient with respiratory infection (Toledo, OH) | [70]                                        |
| **E. coli**    |                                                 |                                             |
| EP300TM        | Cloning strain for recombinant DNA methods       | Epicentre® (Illumina®)                      |
| TUNERTM        | Expression strain for protein purification purposes | EMD Millipore                                |
| **Plasmids**   |                                                 |                                             |
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| pGEX-McaP      | pGEX4T-2 expressing O35E McaP aa 51–333 joined to a GST N-terminal tag (GST-McaP), ampicillin resistant | This study                                  |
| pRBHis.MhaB.72.399 | pETcoco-1 expressing O12E MhaB1 aa 72–399 joined to 6 N-terminal histidine residues (His-MhaB), chloramphenicol resistant | [67]                                        |
Briefly, bacteria were incubated for 30 min with HEp-2 human laryngeal cells seeded in 24-well tissue culture plates at a multiplicity of infection of 100 bacteria to 1 epithelial cell. The infected cells were then washed to remove unbound bacteria and treated with a solution containing saponin. Well contents were mixed, serially diluted, and spread onto agar plates to count colony-forming units (CFU). This value was used to calculate the number of inoculated bacteria that bound to HEp-2 cells. The adherence of *M. catarrhalis* preincubated with samples from control chinchillas (i.e. immunized with PBS) was set at 100%. The adherence of *M. catarrhalis* preincubated with samples from chinchillas vaccinated with the His-MhaB protein is presented as the percentage (± standard error) of that of *M. catarrhalis* preincubated with samples from control chinchillas. These assays were performed in triplicate in three or more separate experiments.

**Intranasal Inoculation of Chinchillas with *M. catarrhalis***

The method used to inoculate the nasopharynx of chinchillas was adapted from that described by Luke et al. [84], Bakaletz and colleagues [85], and more recently by Hoopman et al. [86]. Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher’s Chinchilla Ranch (LaRue, Ohio). Prior to inoculation, the animals were anesthetized with by injecting ketamine (10 mg/kg, Fort Dodge®) and xylazine (2 mg/kg, Lloyd Laboratories) intramuscularly (i.m.). Once anesthetized, the animals were placed on their stomach. Using a 26 ½ gauge needle attached to a 1 cc syringe, 0.2 mL of a *M. catarrhalis* suspension containing ~1×10^9 CFU was delivered intranasally (i.n.) by administering 5–10 µL droplets to alternating nasal openings and allowing droplets to be brought into the nasopharynx by the animal’s breathing. A total volume of 0.1 mL was administered per naris. *Mnnowella catarrhalis* strains used to inoculate chinchillas were cultured on THA for 16–20 hr at 37°C. These plate-grown bacteria were suspended to a concentration of ~5×10^9 CFU/mL in PBS supplemented with 0.15% gelatin (PBSG) to maintain the viability of the organism. The *M. catarrhalis* suspension was also diluted and 100 µL aliquots were immediately spread onto THA supplemented with vancomycin and trimethoprim to determine the number of CFU inoculated into the nasal passages of the chinchillas. Back titration of inoculum was performed for all challenge experiments.

Viable *M. catarrhalis* was recovered from the nasopharynx of infected animals by performing nasopharyngeal lavages or by collecting and homogenizing nasopharyngeal tissues. Lavages were performed under anesthesia. Using a 1 cc syringe and a 26 1/2 gauge needle, 0.5 mL of PBSG was delivered at the entrance of one naris (in the form of 5–10 µL droplets) by passive inhalation and collected from the other naris (as it is exhaled) utilizing a needle-free 1 cc syringe. Portions of these lavages were serially diluted and plated onto selective agar plates to suppress the growth of the chinchilla flora and accurately count viable *M. catarrhalis* bacteria present in the fluids.

To harvest nasopharyngeal tissues, chinchillas were first anesthetized as described above. While under anesthesia, the animals were euthanized by delivering 1 mL of Euthanasia®-D solution (Schering-Plough Animal Health) via cardiac injection. This was accomplished by inserting 21 gauge, 1 ½ inch needle into the chest cavity beneath the xyphoid process and injecting the euthanasia solution directly into the heart. After assurance of death, decapitation was performed. Standard dissection techniques were used to remove the eyes, mandibles, and soft tissues covering the skulls. Following this, the heads were bisected along the nasal septum to expose the interior structures of the nasopharynx. The mucosa of the nasopharynx and of the ethmoid and nasal turbinates were collected, weighed and placed in 2 mL of PBSG. The nasopharyngeal tissues were then shredded, homogenized using a sterile glass dounce and pestle (Kimble Chase Life Science and Research Products), serially diluted, and plated onto selective media to determine the number of viable *M. catarrhalis* organisms.

**Immunization of Chinchillas**

Serum and nasopharyngeal lavage fluids were collected from anesthetized chinchillas prior to immunization. Nasal fluids were collected as described above and stored at ~80°C for later use. Blood was drawn by cardiac puncture. This was accomplished by inserting 21 gauge, 1 ½ inch needle into the chest cavity beneath the xyphoid process and removing blood directly from the heart. The samples were allowed to clot, centrifuged to remove red blood cells, and the sera were stored at ~80°C. Blood samples and nasopharyngeal lavage fluids were also collected on days 19 and 44 post-immunization.

Vaccination was performed under anesthesia. Groups of chinchillas were immunized with PBS (control animals) or 80 µg of the His-MhaB protein. PBS and protein preparation were mixed with Complete Freund’s Adjuvant (CFA) in a 1:1 ratio (vol/vol) and administered subcutaneously (s.c.). Booster vaccinations were performed on days 23 and 72. Animals were boosted with PBS or 80 µg of His-tagged protein mixed with Incomplete Freund’s Adjuvant (IFA).

**Animal Research Ethic Statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Georgia. All efforts were made to minimize suffering.

**Statistical Analyses**

The paired *t* test was used to analyze data from adherence assays. *P* values <0.05 were found to be statistically significant. The results of nasopharyngeal colonization experiments were examined with the Wilcoxon signed rank test. All statistical analyses were performed using the Graph Pad Prism software.

**Results**

**Use of the Chinchilla Model to Examine Colonization of the Nasopharynx by *M. catarrhalis***

To study *M. catarrhalis* colonization and persistence *in vivo*, we developed the ability to utilize the chinchilla model of nasopharyngeal colonization. Figure 1 shows the results of calibration experiments in which chinchillas were inoculated intranasally (i.n.) with 10^7 colony-forming units (CFU) of the wild-type (WT) isolate O35E. At the indicated times post-infection, animals were anesthetized and nasopharyngeal lavage fluids were collected, diluted and spread onto selective agar plates to suppress the growth of the chinchilla flora and accurately count viable *M. catarrhalis* CFU. Following this, chinchillas were euthanized and nasopharyngeal tissues were harvested, weighed, homogenized, diluted and plated. After overnight incubation at 37°C, CFU were counted to determine the number of viable *M. catarrhalis* bacteria present in lavage fluids and tissues. The results shown in Figure 1 demonstrates that we obtain reproducible and consistent numbers, comparable to those reported by Luke et al. for the WT isolate 7169 [84] and Hoopman and colleagues for strain O35E [86].
M. catarrhalis Vaccine Studies in Chinchillas

Use of the Chinchilla Model to Perform Vaccine Studies

After establishing the model, we tested the hypothesis that mutants lacking expression of adherence proteins will not colonize as effectively as WT M. catarrhalis. To accomplish this, cohorts of chinchillas were challenged with WT M. catarrhalis O35E and the mutant strain O35E.B1B2, which is unable to express the filamentous hemagglutinin-like proteins MhaB1 and MhaB2 [67]. These molecules are associated with the outer membrane of M. catarrhalis and are secreted in a Two-Partner Secretion manner via the outer membrane protein MhaC. MhaB1 and MhaB2 are involved in adherence to several human epithelial cell types that are relevant to the pathogenesis of M. catarrhalis (lung, laryngeal, conjunctival). The adhesins also resemble the filamentous hemagglutinin FHA, which is the major colonization factor of Bordetella pertussis and a component of all vaccines that are currently licensed for use in children to protect against whooping cough (CDC website. Available: http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/pert.pdf. Accessed 2013 Jun 4). Figure 2 shows that lack of expression of MhaB1 and MhaB2 causes an 18.5-fold reduction in the number of viable M. catarrhalis bacteria recovered from nasopharyngeal tissues 72 hr post-infection. These results indicate that the filamentous hemagglutinin-like proteins are involved in M. catarrhalis ability to colonize and persist in the chinchilla nasopharynx. Lavages (prior to collecting tissues) were not performed in these experiments in order to generate a single value representing the total number of bacteria present in the nasal passageways at the experimental end-point.

Figure 1. Recovery of WT M. catarrhalis O35E from the nasopharynx of chinchillas. Animals were inoculated with ~1×10^9 CFU. Results are expressed as the mean (± standard error) CFU/mL (lavage fluids, black bars) or CFU/gr (nasopharyngeal tissues, open bars). Each column represents at least 4 animals, and each experimental condition was tested on at least two separate occasions. doi:10.1371/journal.pone.0067881.g001

MhaB2 in all M. catarrhalis isolates characterized to date, and murine Abs against this polypeptide were previously shown to react with both MhaB1 and MhaB2 [67]. This shared region of MhaB1 and MhaB2 also displays sequence similarity to the portion of B. pertussis FHA that is a component of all licensed vaccines for whooping cough (data not shown).

Serum and nasopharyngeal lavage fluids were collected from chinchillas and analyzed by western blot and ELISA. The results are shown in Figure 4 and demonstrate that the animals produced serum Abs reacting with the adhesins in the outer membrane of M. catarrhalis (Fig. 4A) and with a GST-tagged version of MhaB1/ MhaB2 (Fig. 4B and 4D). The data also indicate that chinchillas developed mucosal Abs binding to the shared region of MhaB1 and MhaB2 (Fig. 4C). Serum and lavage fluids from the control animals vaccinated with PBS did not contain Abs against the adhesins (data not shown). Following this, we performed in vitro adherence assays in which M. catarrhalis was incubated with serum or lavage fluids from immunized chinchillas prior to infecting HEp-2 laryngeal cells. These experiments revealed that chinchilla Abs against MhaB1 and MhaB2 significantly decrease the adherence of multiple WT M. catarrhalis isolates to epithelial cells (Fig. 5A and 5B). The data also indicate that this inhibitory effect is dependent on the concentration of Abs.

After confirming that chinchillas produced Abs against MhaB1 and MhaB2, and demonstrating that these Abs interfere with adherence to airway cells, we challenged the animals with ~10^9 CFU of the WT strain O35E and determined bacterial loads in nasopharyngeal tissues three days post-infection. Figure 6 shows that vaccination with the His-tagged MhaB protein causes a 9.3-fold reduction in the number of viable M. catarrhalis bacteria recovered from the nasopharynx of chinchillas compared to sham-immunized animals. These results substantiate the data obtained when comparing the ability of the mutant O35E.B1B2 to colonize the nasopharynx to that of its progenitor strain O35E (Fig. 2). The results also support the hypothesis that a vaccine containing M. catarrhalis adherence proteins will elicit the production of Abs blocking colonization and promoting clearance.

Figure 2. Recovery of M. catarrhalis from the nasopharynx of chinchillas three days post-infection. Animals were inoculated with ~1×10^9 CFU. Results are expressed as the mean (± standard error) CFU/gr of nasopharyngeal tissues. Strains were tested in parallel on two separate occasions. Each column represents 12 animals. The asterisk indicates that the reduction in the number of bacteria is statistically significant (Wilcoxon signed rank test). doi:10.1371/journal.pone.0067881.g002
**Moraxella catarrhalis** Proteins Targeted by the Chinchilla Immune Response During Colonization

To gain more insight into the immune response of the chinchilla to *M. catarrhalis*, we inoculated four animals i.n. with $10^9$ CFU of the WT strain O35E on three consecutive occasions (21 days apart). Seven days after the last challenge, serum samples were collected and analyzed by western blot. Figure 7 shows that chinchillas produced Abs against several *M. catarrhalis* antigens during colonization including the iron acquisition protein CopB, the serum-resistance factor UspA2, and the adhesins OMPCD and Hag. Of significance, these four molecules have been shown to be major targets of systemic and mucosal antibody responses in humans [83,87,88,89,90,91,92,93,94]. Infected chinchillas did not produce detectable levels of Abs against the shared region of MhaB1 and MhaB2 (data not shown).

**Discussion**

The success of the immunization program against *S. pneumoniae* has placed more emphasis on *M. catarrhalis* as a frequent cause of ear infection. Vaccination of children with Prevnar®, which contains capsular polysaccharides from seven different *S. pneumoniae* serotype strains, affords protection against otitis media caused by the organism (57% efficacy) [95]. Likewise, an investigational vaccine containing the capsule of 11 distinct *S. pneumoniae* serotype strains conjugated to protein D of *H. influenza* was shown to reduce the incidence of ear infection caused by *S. pneumoniae* (57% efficacy) and NTHi (33% efficacy) [96]. Significantly, Synflorix™, a capsule-protein D conjugate vaccine comprising capsular polysaccharides from 10 different *S. pneumoniae* serotype strains, was licensed in Europe in 2009. While these studies demonstrate that prevention of otitis media can be achieved, the widespread administration of capsule-protein D conjugate vaccines protecting against both *S. pneumoniae* and NTHi, along with the continued expansion of the *S. pneumoniae* vaccination program (a version of Prevnar® covering 13 capsule serotypes was licensed in 2010), will result in *M. catarrhalis* becoming an even more prevalent cause of infectious disease. Evidence of such a shift has been observed in children with otitis media as well as in children and adults with sinusitis [97,98,99]. Therefore, the prevention of *M. catarrhalis* infections would make a significant contribution to improving children's health. Though otitis media would be the primary target, a vaccine against the organism would also be of value to adults at high risk of infection, especially those with COPD.

*Moraxella catarrhalis* is an exclusively human organism and studying pathogenesis, as well as the stringent testing of vaccine candidates, has been hindered by the lack of an animal model that mimics human infection. To date, the most commonly used model...
has been a pulmonary clearance test in which bacteria are deposited in the lungs of mice [100,101,102,103,104,105,106]. Viable organisms are enumerated by aseptically removing the lungs, homogenizing the tissues, and spreading dilutions of the homogenates onto agar plates. While this model has provided important data, it is limited to measuring the rate at which bacteria are cleared over a very short period of time because \( M.\, catarrhalis \) persists for \(<24\text{-hr}\) in the murine lungs. Another drawback is that mice do not develop pneumonia. Hence, the rapid clearance and failure to cause disease limit the usefulness of this model.

Recent studies have demonstrated the value of the chinchilla to examine \( M.\, catarrhalis \) host-pathogen interactions in vivo [84,85,86,107,108]. Following intranasal inoculation, \( M.\, catarrhalis \) causes symptoms of disease (inflammation of the tympanic membrane, middle ear effusions) and colonizes the nasopharynx for \(~2\text{\ weeks} \) [85,86,108]. Therefore, chinchillas provide an advantage over the mouse pulmonary clearance test in that \( M.\, catarrhalis \) persists in their nasopharynx for several days. This imparts greater confidence in the data obtained by comparing the difference in colonization between two experimental conditions (vaccinated vs. sham-vaccinated animals, WT vs. mutant strains) as it provides a more physiologically relevant time frame to monitor bacterial clearance. The chinchilla model has been an invaluable tool to study the pathogenesis of NTHi and \( S.\, pneumoniae \) and to develop vaccines for these organisms [109,110,111,112]. The course of disease (nasopharyngeal colonization, ascension of the Eustachian tubes, development of middle ear effusions, clearance of fluids, return to homeostasis) is similar to that in children with otitis media [113,114,115,116,117,118]. Immunological parallels between chinchillas and humans have also been demonstrated. For example, middle ear fluids collected from chinchillas and children infected with NTHi contain Abs that bind to the same antigenic determinants of the adhesin OMP P5 [119]. Chinchillas also produce homologs of human antimicrobial peptides, and at least 2 of them (cBD-1 and cCRAMP) have been shown to have bactericidal activity against \( M.\, catarrhalis \) [120,121,122,123]. Kerschner and colleagues analyzed host cDNA libraries generated from the middle ear mucosa of chinchillas infected with NTHi, and discovered that the cDNA sequences displayed greater phylogenetic similarities to human genes than to other rodent species [124,125,126]. These investigators also noted similarities with human infection in the pattern of host defense genes expressed in chinchilla tissues. Our data showing that chinchillas infected with \( M.\, catarrhalis \) produce Abs against antigens known to be major targets of the immune response in humans further underscore the usefulness of the model (Fig. 7). To our knowledge, this is the first demonstration of immunological parallels between chinchillas and humans during \( M.\, catarrhalis \) infection.

We discovered that lack of expression of the filamentous hemagglutinin-like proteins MhaB1 and MhaB2 decreases recovery of viable \( M.\, catarrhalis \) cells from the chinchilla nasopharynx approximately \( 20\text{-fold} \) (Fig. 2). This reduction is most likely caused by a defect in adherence to the airway mucosa. MhaB1 and

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**Figure 5. Inhibition of adherence with samples from chinchillas immunized with His-tagged MhaB protein.** The WT \( M.\, catarrhalis \) strains O35E, O12E, and McGHS1 were preincubated for 30 min at 37°C with pooled samples from chinchillas sham-vaccinated with PBS (black bars) or with pooled samples from chinchillas immunized with His-tagged MhaB at dilutions of 1:50, 1:100, 1:200 and/or 1:2000. These bacteria were then used to perform adherence assays. The adherence of \( M.\, catarrhalis \) preincubated with samples from chinchillas vaccinated with PBS was set at 100%. The adherence of \( M.\, catarrhalis \) preincubated with samples from chinchillas immunized with His-tagged MhaB is expressed as the percentage (± standard error) of that of \( M.\, catarrhalis \) preincubated with samples from chinchillas vaccinated with PBS. Assays were performed in triplicate on three separate occasions. The asterisks indicate that the reduction in adherence is statistically significant (\( P \) values \(<0.05\), paired t test). Post-boost samples taken on Day 44 of vaccination experiments (see Fig. 3) were pooled and used in these assays.

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**Figure 6. Recovery of WT \( M.\, catarrhalis \) O35E from the nasopharynx of immunized chinchillas three days post infection.** Results are expressed as the mean (± std error) CFU/gr of nasopharyngeal tissues (note the log scale). The asterisk indicates that the reduction in the number of bacteria is statistically significant (Wilcoxon signed rank test, \( P \) value is shown in parentheses). Control and His-tagged MhaB groups were tested in parallel on three separate occasions. Each column represents 12 animals (groups of n = 4 animals/ experiment).

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MhaB2 mediate adherence to respiratory cells in vitro and resemble FHA, the major adhesin and colonization factor of *B. pertussis* [67]. Moreover, Abs against MhaB1 and MhaB2 reduce in vitro adherence of *M. catarrhalis* (Fig. 5) and decrease the number of viable organisms recovered from the nasopharynx of chinchillas infected with the WT strain O35E (Fig. 6). Taken together, our data suggest that MhaB1 and MhaB2 are critical factors for colonization. Hoopman and colleagues recently used the chinchilla and DNA microarray technology to determine global transcriptome expression by *M. catarrhalis in vivo* [86]. More than 100 ORFs of strain O35E, including mhaB1, were found to be upregulated 24-hr after introducing the organism in the nasopharynx. Another 200 genes were shown to be downregulated, and the ORF specifying MhaB2 (MchA1) exhibited some of the highest levels of repression. Therefore, it is tempting to speculate that lack of MhaB1 is responsible for the reduced number of viable O35E.B1B2 cells recovered from the chinchilla nasopharynx during our colonization experiments (Fig. 2). However, the contribution of MhaB2 cannot be ruled out. The transcriptome analysis showing decreased mhaB2 expression levels was performed with samples collected 24-hr post-inoculation, whereas we calculated bacterial loads in the nasopharynx 3 days after infection. It is possible that expression of mhaB2 (and mhaB1) changes during this 48-hr period. Interestingly, microarray data also indicate that expression of the uspA2 and hag genes is downregulated [86]. The western blot results of Fig. 7 show that infected chinchillas produce Abs against UspA2 and Hag, demonstrating their expression in vivo. Clearly, the disappearance of staining in the OMPCD region, and thus the absence of OMPCD, is consistent with the idea that infected chinchillas have reduced OMPCD. However, the presence of anti-OMPCD Abs suggests that the proteins will have broad-spectrum activity. Of note, this shared region of MhaB1 and MhaB2 (Fig. 5C) is also predicted to be present in the OMPCD region. This suggests that the proteins may have a role in colonization and persistence. The shared region of MhaB1 and MhaB2 blocks adherence of *M. catarrhalis* to respiratory cells in vitro and in vivo [67]. Moreover, Abs against MhaB1 and MhaB2 also indicate that expression of the shared region of MhaB1 and MhaB2 is critical for colonization. Subcutaneous immunization with a polypeptide common to both molecules elicits the production of serum Abs reacting with the proteins in the outer membrane of *M. catarrhalis* (Fig. 5A). Vaccinated animals also develop mucosal Abs binding to the shared region of MhaB1 and MhaB2 (Fig. 5C). These Abs not only block *M. catarrhalis* adherence in vitro, but also reduce nasopharyngeal colonization of the WT strain O35E by one order of magnitude (Fig. 6). The MhaB proteins function as adhesins and mediate a key step in pathogenesis by *M. catarrhalis*. To cause disease, the organism must first colonize the nasopharynx and then spread to distal sites such as the middle ear and the lower respiratory tract. Hence, adherence to the mucosal surface of the nasopharynx is critical. MhaB1 and MhaB2 are surface-located and thus are readily accessible to Abs and the host immune response. In addition, the proteins are well conserved among clinical isolates of diverse clinical and geographical origins [67,68]. Therefore, MhaB1 and MhaB2 possess many attributes of excellent vaccine candidates. Our results showing that Abs against the shared region of MhaB1 and MhaB2 blocks adherence of multiple WT *M. catarrhalis* isolates suggests that immunization with the proteins will have broad-spectrum activity. Of note, this shared region of MhaB1 and MhaB2 displays sequence similarity to the portion of *B. pertussis* FHA that is a component of all vaccines that are currently licensed for use in children to protect against *B. pertussis*. DOI:10.1371/journal.pone.0067881.g007

**Figure 7. Western blot analysis of serum from chinchillas inoculated with the WT *M. catarrhalis* strain O35E.** Equivalent amounts of whole cell lysates (WT *M. catarrhalis* O35E, uspA2 KO strain O35E2, hag transposon mutant strain O35E.TN2, and ompCD KO strain O35E.CD1) were resolved by SDS-PAGE, transferred to PVDF and probed with the indicated primary Abs. Panels A and B: Pre- and post-infection serum samples were pooled and used as primary Abs at a dilution of 1:250. Goat α-rat IgG conjugated to HRP were used as secondary Abs. Controls: The murine monoclonal Abs 10F3 (Panel C, α-CopB), SD2 (Panel D, α-Hag), 17H4 (Panel E, α-UspA2) and 1D3 (Panel F, α-OMP) were used as primary Abs in combination with goat α-mouse HRP-α-IgG-α-IgM secondary Abs. These controls were included to verify the identity of proteins recognized by post-infection chinchilla serum in panel B. MW markers are shown to the left of in kDa.
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