Safety and Immunological Outcomes Following Human Inoculation With Nontypeable Haemophilus influenzae

Patricia L. Winokur,1,5 Kathryn Chaloner,2 Gary V. Doern,3 Jennifer Ferreira,6 and Michael A. Apicella4

1Department of Internal Medicine, 2Department of Biostatistics, 3Department of Pathology, and 4Department of Microbiology, University of Iowa, and 5Iowa City VA Health Care System, Iowa City; and 6EMMES Corporation, Rockville, Maryland

(See the editorial commentary by Barenkamp on pages 717–9.)

Background. Nontypeable Haemophilus influenzae (NTHi) exclusively infects humans, causing significant numbers of upper respiratory tract infections. The goal of this study was to develop a safe experimental human model of NTHi nasopharyngeal colonization.

Methods. A novel streptomycin-resistant strain of NTHi was developed, and 15 subjects were inoculated in an adaptive-design phase I trial to rapidly identify colonizing doses of NTHi. Bayesian analysis was used to estimate the human colonizing dose 50 and 90 (HCD50 and HCD90, respectively). Side effects and immunological responses to whole-cell sialylated NTHi were measured.

Results. Nine subjects were colonized and tolerated colonization well. Immunological analyses demonstrated that 7 colonized subjects and 0 noncolonized subjects had a 4-fold rise in serum levels of immunoglobulin A, immunoglobulin M, or immunoglobulin G. Preexisting immunity to whole-cell NTHi did not predict success or failure of colonization.

Conclusions. The statistical design incorporated a slow escalation to higher dose levels. HCD50 and HCD90 Bayesian estimates were identified as approximately 2000 and 150 000 colony-forming units, respectively; credible interval estimates were broad. This study provides a potential platform for early proof of concept studies for NTHi vaccines, as well as a way to evaluate bacterial factors associated with colonization.

Keywords. Haemophilus influenzae; nasopharyngeal colonization; adaptive design; Bayesian analysis; respiratory pathogens; upper respiratory tract.

Haemophilus influenzae is gram-negative bacterium that exclusively colonizes and infects the human host. Nontypeable H. influenzae (NTHi) strains lack an outer capsule and are commensals of the human upper respiratory tract. Between 20% and 80% of healthy children are colonized with NTHi at any one time [1–4], and colonization is a dynamic process, with new strains being acquired and replacing old strains periodically [5].

Although less virulent than encapsulated strains, NTHi can cause upper respiratory tract infection and, less commonly, distal invasive infection [6]. NTHi is the responsible agent in approximately 30%–40% of cases of otitis media in children [7–9] and a frequent cause of sinusitis in healthy adults [10]. Smokers and those with chronic lung disease are at risk for lower respiratory tract infection, including acute exacerbation of bronchitis and pneumonia [11]. Colonization is considered the first step in invasive infection, although specific mechanisms that lead from colonization to invasive disease are incompletely understood. In some cases, intercurrent viral infection may promote invasion [12, 13].

NTHi has become a desirable vaccine target. The pneumococcal vaccine Synflorix uses protein D of H. influenzae conjugated to pneumococcal polysaccharides. This vaccine has been shown to reduce the
incidence of *H. influenzae*–associated otitis media by 31% [14]. NTHi has other outer membrane structure, such as fimbrae, outer membrane proteins (OMPs), and lipoooligosaccharide, that play a role in virulence and could be potential vaccine antigens [15–17].

NTHi infections can be induced in animal models but do not occur naturally and do not fully reproduce human colonization and infection [18]. Previous human experimental models of *Streptococcus pneumoniae,* *Neisseria gonorrhoeae,* and *Haemophilus ducreyi* infections have provided useful information regarding pathogenesis and protective immune responses [19–24]. The goal of this study was to develop a safe experimental human model of NTHi nasopharyngeal colonization, using a novel strain of NTHi. Colonization results, safety, reactogenicity, and immunological responses are presented. This model could provide a way to rapidly evaluate the early efficacy of candidate vaccines. Because other bacterial pathogens, such as *S. pneumoniae,* *Neisseria meningitidis,* and *Bordetella pertussis,* initiate similar interactions with their obligate human host by colonization of the upper respiratory tract, this model could also be a mechanism for studying the pathogenesis of respiratory colonization.

**MATERIALS AND METHODS**

**Human Volunteers**

Healthy men and women aged 18–40 years were recruited, and written informed consent was received from enrolled participants. Specific exclusion criteria included allergic rhinitis requiring therapy; tobacco use within the past year; a history of sinusitis, otitis, chronic bronchitis, pneumonia, bronchospasm, or asthma in the past 5 years; use of any antibiotic within the past month; allergy to penicillin, macrolide, cephalexin, or fluoroquinolone antibiotics; American Indian, Native Alaskan, or Native Australian heritage (these populations have increased risk for invasive bacterial respiratory diseases [25]); close contact with persons <5 or >55 years of age, with chronic smokers, or with persons with human immunodeficiency virus infection, cancer, or chronic immunosuppressive diseases; and asthma or chronic lung disease.

All study activities were approved by the University of Iowa institutional review board; the Office of Clinical Regulatory Affairs, National Institute of Allergy and Infectious Diseases, National Institutes of Health; and the Food and Drug Administration.

**Bacterial Strain**

NTHi 2019 is a clinical isolate obtained from a patient with chronic obstructive pulmonary disease (COPD) and chronic bronchitis in 1985. Strain 2019 has been used to study colonization in vitro and in animal models [26–30]. NTHi 2019StrR1 was created using a specific chromosomal point mutation in the ribosomal RNA gene *rpsL,* which encodes the S12 polypeptide to encode streptomycin resistance. The *rpsL* 12 DNA sequence of *H. influenzae* Rd KW20 (an *H. influenzae* strain known to contain the appropriate resistance marker, kindly provided by Arnold Smith, MD) was used to construct 2 primers, 5′-GCTTCAAACACTACGACGAC-3′ and 5′-GACTTCTTCTTA CGCCATATAAAC-3′, for polymerase chain reaction (PCR) analysis. A 1060-bp fragment of DNA that included *Hi* 0581 (the *rpsL* 12 gene) from strain NTHi strain 2019 was amplified and cloned into Zero-Blunt TOPO (Invitrogen, Carlsbad, CA). PCR was performed by means of the Clontech Diversify PCR Random Mutagenesis Kit, using conditions to insert 2 nucleotide switches per 1000 bp. These amplified fragments were used to transform NTHi strain 2019, as previously described [31]. Transformed bacteria were plated onto brain heart infusion agar supplemented with hemin and nicotinamide adenine dinucleotide (NAD) (Sigma, St. Louis, MO) and streptomycin (50 μg/mL).

Genomic preps were made using the PureGene DNA Isolation Kit (Genta, Minneapolis, MN). Sequencing verified that nucleotide 128 was altered, resulting in a change in amino acid 43 from lysine to arginine. To ensure safety from zoonotically transmissible microbes and retained streptomycin, NTHi 2019StrR1 was passaged 5 times on defined, antibiotic-free Roswell Park Memorial Institute (RPMI) 1640 medium. NTHi 2019StrR1 was tested using NCCLS broth dilution standards and was susceptible to amoxicillin, doxycycline, trimethoprim-sulfamethoxazole, cefuroxime-axetil, ceftriaxone, and ciprofloxacin. This organism was resistant to streptomycin (minimum inhibitory concentration, ≥256 μg/mL). Aliquots of NTHi 2019StrR1 were tested for purity at the University of Iowa Clinical Microbiology laboratory. The final aliquots were stored frozen at −80°C.

**Inoculum Preparation**

A single frozen vial for each study subject was thawed the night prior to inoculation, and an aliquot was plated onto RPMI 1640 agar supplemented with hypoxanthine/uracil, sodium pyruvate, NAD, protoporphyrin IX, and sialic acid as previously described [32]. Plates were grown overnight in 5% CO2 at 35°C. Four-five colonies were selected to make a 0.5 McFarland cell suspension (OD, 0.1–0.12, equivalent to 3–5 × 10^8 colony-forming units [CFU]/mL) in 5 mL of sterile saline, using a turbidity meter. A Gram stain was performed to confirm purity, and the preparation was diluted to the appropriate concentration for administration. Titers were performed on the final diluted inoculum to determine the exact inoculum delivered to the subject. Additionally, overnight cultures from the inocula were performed to ensure purity.

**Human Inoculation and Assessment**

Volunteers who qualified for enrollment were admitted to the inpatient Clinical Research Unit at the University of Iowa. Serum, saliva, nasal wash, and nasopharyngeal samples were taken prior to inoculation. A total of 0.2 mL of diluted NTHi 2019StrR1 was administered once by nose drops (0.1 mL per nostril).
Volunteers were observed for 15 minutes after inoculation and remained hospitalized for the first 3 days after inoculation. Clinical assessments were performed daily for 6 days. Solicited reactogenicity events included fevershiness, headache, myalgias, tachycardia, nasal congestion, nasal drainage, sneezing, sore throat, hoarseness, ear pain, cough, shortness of breath, and chest pain. Nasal wash specimens (obtained using 5 mL of sterile saline) and nasopharyngeal swab specimens (Diagnostic Hybrids, UT M with Nasopharyngeal FLOQSwab) were collected on days 3, 4, 5, and 6 after inoculation. By use of previous decolonization studies of N. meningitidis as a model, all subjects received levofloxacin 500 mg daily for 3 days (days 6–9) to eradicate carriage of the study organism [33, 34]. Repeat serum, saliva, nasal wash, and nasopharyngeal swab specimens were obtained on days 14 and 28 after inoculation. Adverse events were recorded throughout 28 days after inoculation, and serious adverse events were recorded for 6 months. Inoculations were performed April through September to minimize the risk of concomitant respiratory virus infection.

Detection of Colonization in Volunteers
A total of 100 μL of nasal wash or the nasopharyngeal swabs were placed on brain heart infusion agar containing protoporphyrin IX, NAD, and 200 μg/mL streptomycin. Potential H. influenzae colonies were further analyzed by Gram stain and porphyrin testing [35].

Enzyme-Linked Immunosorbent Assays (ELISAs)
Serum samples were analyzed for immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) responses to whole-cell sialylated NTHi 2019StrR1, using an ELISA previously described elsewhere [36]. All samples were run in triplicate, and all samples from a single patient were run on the same day. Each microtiter plate had positive and negative controls run daily. Positive titers exhibited a fluorescence intensity that was 2 times the background level of fluorescence for wells without added test serum.

Statistical Design of the Colonization Model
This was an unblinded, adaptive-design, phase I study conducted in 2 stages. The first 6 volunteers (stage I) were assigned to receive doses one at a time, to gain experience with safety and reactogenicity and to rapidly establish successful colonization doses. Stage I was a modification of the up-and-down and continuous-reassessment designs [37, 38]. The first volunteer was inoculated with 1000 CFU of NTHi 2019StrR1 and was assessed for colonization on days 3–6. The second subject was to be inoculated with either a half log lower (10^{2.5}) or higher (10^{3.5}) dose, based on the success or failure of colonization with 1000 CFU in the first volunteer. Similarly, the third through sixth subjects were inoculated following a prespecified algorithm depending on whether colonization was successful at previous doses. Information on the human colonizing dose 50 and 90 (HCD_{50} and HCD_{90}, respectively) was maximized, a range of doses was explored, and simultaneously the probability of well-defined maximum likelihood estimates (MLEs) was increased by limiting replication at any 1 dose. All 64 potential inoculation dose and outcome sequences of the first 6 subjects (stage I) were specified along with a stage II design for each stage I outcome. Doses in both stages were limited to 8 choices between 32 CFU and 100 000 CFU equally spaced on the logarithmic scale. The stage II design involved 3 subjects at each of 3 doses, for a total of 9 subjects. If the MLE from stage I was well defined, a stage II design minimizing the posterior variances of the HCD_{50} and HCD_{90} in the log_{10}(CFU) scale was specified, and if the MLE was not defined, the design incorporated a maximization of the probability of MLEs being defined. The small sample properties of the designs were evaluated by simulation. Details of the design are available by request.

The data from stages I and II were combined and analyzed, using logistic regression on log_{10}(CFU) with a Bayesian analysis, to provide posterior means and 95% credible intervals of HCD_{50} and HCD_{90}. The prior distribution was prespecified, and WinBUGS was used for analysis [39].

The prior distribution assumed that the HCD_{50} was uniformly distributed on the log_{10} scale between 1 and 5. The log_{10} (HCD_{90}) was constrained to be larger than the log_{10}(HCD_{50}) by a fixed value of 0.1 log_{10}(CFU) plus a random quantity corresponding to a β(1, 4) distribution, scaled by a factor of 5 to be in the interval [0, 5.0] log_{10}(CFU); that is, the random quantity is 5 times a β distribution with a mean of 0.2 and an SD of √{2}/ √{3}. The prior mean (±SD) of the log_{10}(HCD_{50}) was therefore 3.0 ± 2/√{3}, and the increase in log_{10}(CFU) from the log_{10}(HCD_{50}) to the log_{10}(HCD_{90}) was independent of the log_{10} (HCD_{50}) with a mean (±SD) of 1.1 ± √{2}/√{3}.

RESULTS

Human Subjects
Fifteen adults were enrolled in the study; 9 were men, and 6 were women. Three participants were Asian, and 12 were white. The mean age was 27 years (range, 21–34 years). All volunteers completed the study.

Colonization Versus Dose
Colonization was defined as a single NTHi 2019StrR1-positive culture during days 3–6 after inoculation. The first 6 subjects were inoculated with live NTHi 2019StrR1, with each subject followed for safety and colonization response for at least 6 days before the next subject was inoculated. The first subject received 1000 CFU and did not demonstrate colonization, so the second subject received a half-log-higher dose of 3200 CFU. The sequence of inoculations seen in Table 1 demonstrates the up-down logic of the design. Two of the first 6 subjects were
colonized, with intended doses of 3200 CFU and 32 000 CFU. The estimated (MLE) HCD50 and HCD90 based on the intended dose were $10^{3.79}$ and $10^{4.75}$, whereas values based on the confirmed doses were $10^{3.71}$ and $10^{5.33}$. Because the proposed maximum dose was $10^5$ CFU, these values gave similar stage II designs. In stage II, 9 subjects received a dose, 3 each at doses of 10 000, 32 000, and 100 000 CFU. In 7 of the 9 second-stage subjects, colonization was achieved. Overall, we were able to culture NTHi 2019StrR1 from 9 of 15 subjects. We did not detect obvious preexisting wild-type NTHi in any samples prior to inoculation. However, both the nasal wash and nasopharyngeal samples showed mixed flora, and extensive subculturing to identify NTHi was not performed.

There was disparity between the confirmed doses and the intended doses (Table 1): a single dose error occurred (for subject 18), while the remaining differences were due to experimental variability. When colonization was evaluated by either intended or confirmed inoculation dose, higher doses were more likely to be associated with colonization. Confirmed dose results of the Bayesian analysis indicate that the estimated HCD50 is $1991$ CFU (95% credible interval, $102^{19}453$ CFU) and that a dose of 10 000 CFU has 95% posterior probability of colonizing at least 47% of the population; similarly, a dose of 100 000 CFU has 95% probability of colonizing at least 70%. The estimated HCD90 is $150^{314}$ CFU (95% credible interval, $8375^{11114}{813}536$ CFU), with the length of the credible interval indicating considerable uncertainty in the estimate.

Analysis of nasal wash samples was more likely to detect NTHi 2019StrR1 than was analysis of nasopharyngeal swab

### Table 1. Outcome of the 2-Stage Bayesian Statistical Design and Inoculation Doses

<table>
<thead>
<tr>
<th>Stage, Dosing Sequence (Subject ID)</th>
<th>Total Inoculation Concentration, CFU/0.2 mL</th>
<th>Intended</th>
<th>Confirmed* and Delivered</th>
<th>Colonized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (06)</td>
<td>1000</td>
<td>112</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>2 (09)</td>
<td>3200</td>
<td>940</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>3 (04)</td>
<td>320</td>
<td>206</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>4 (11)</td>
<td>10 000</td>
<td>10 100</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>5 (13)</td>
<td>32 000</td>
<td>53 000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6 (14)</td>
<td>100</td>
<td>126</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 (16)</td>
<td>10 000</td>
<td>1660</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>8 (15)</td>
<td>10 000</td>
<td>5400</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>9 (19)</td>
<td>10 000</td>
<td>12 200</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>10 (18)</td>
<td>32 000</td>
<td>1600</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>11 (21)</td>
<td>32 000</td>
<td>22 000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>12 (22)</td>
<td>32 000</td>
<td>10 000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>13 (32)</td>
<td>100 000</td>
<td>82 000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>14 (31)</td>
<td>100 000</td>
<td>152 000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>15 (27)</td>
<td>100 000</td>
<td>76 000</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

The first 6 subjects were inoculated one at a time, and the success of colonization and doses for all prior subjects were used to determine the dose for the next subject. The results for the first 6 subjects determined the dosing for stage 2. For this outcome of stage 1, three subjects each were inoculated with the 3 highest doses for this study. The progression of dosing was based on the total intended concentration of inoculation.

### Table 2. Nasal Wash Versus Nasopharyngeal Swab Cultures and Pattern of Detection of Nontypeable Haemophilus influenzae (NTHi) 2019StrR1

<table>
<thead>
<tr>
<th>Total Inoculation Concentration, CFU/0.2 mL</th>
<th>Nasal Wash</th>
<th>Nasopharyngeal Swab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonization Duration, Days, No.</td>
<td>NTHi Culture Positivity, Study Day(s)</td>
<td>Colonization Duration, Days, No.</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Subject ID</td>
<td>Intended</td>
<td>Confirmed* and Delivered</td>
</tr>
<tr>
<td>015</td>
<td>10 000</td>
<td>5400</td>
</tr>
<tr>
<td>019</td>
<td>10 000</td>
<td>12 200</td>
</tr>
<tr>
<td>018</td>
<td>32 000</td>
<td>1600</td>
</tr>
<tr>
<td>022</td>
<td>32 000</td>
<td>10 000</td>
</tr>
<tr>
<td>021</td>
<td>32 000</td>
<td>22 000</td>
</tr>
<tr>
<td>013</td>
<td>32 000</td>
<td>53 000</td>
</tr>
<tr>
<td>032</td>
<td>100 000</td>
<td>82 000</td>
</tr>
<tr>
<td>031</td>
<td>100 000</td>
<td>152 000</td>
</tr>
</tbody>
</table>

Colonization was observed in nasal wash specimens from 9 subjects (60%) and nasopharyngeal swab specimens from 6 (40%).

### Abbreviations
- CFU, colony-forming units;
- ID, identification number.

### Notes
- Colony counts were determined after the doses were prepared.

### Table 2. Nasal Wash Versus Nasopharyngeal Swab Cultures and Pattern of Detection of Nontypeable Haemophilus influenzae (NTHi) 2019StrR1

- Colony counts were determined after the doses were prepared.

- Defined as the no. of days between the first and last occurrence of shedding. Mean values (±SD) of $3.1 ± 1.2$ days and $2.2 ± 1.5$ days were observed for nasal wash specimens and nasopharyngeal swab specimens, respectively.
specimens (Table 2). Of the 9 subjects with evidence of coloni-
zation, all had nasal wash samples positive on days 3, 4, 5, or 6,
and all except 1 subject had nasal wash cultures positive on >1
day. In general, higher inoculums were associated with earlier
detection of colonization. Six subjects were positive by day 3, 2
were positive by day 4, and 2 were positive by day 5. Once the
nasal wash sample was culture positive, almost all cultures were
positive on subsequent days. Nasopharyngeal swab specimens
were positive in 6 of the 9 colonized subjects, and 3 had only a
single day of in which results of culture were positive; these 3
subjects had NTHi 2019StrR1 detected on day 6. No subjects
had NTHi 2019StrR1 detected following the administration of
levo-
lo-
ffoxacin. Growth was quantitated as 1+ through 4+ on the
basis of the last quadrant positive for growth on the agar streak
plate. There was no trend in maximum quantitation of growth,
based on intended or confirmed dose (data not shown). Five of
9 subjects had 1+ growth on the first day of colonization, and
the others started with 2+ growth. During the short duration of
colonization, quantitation frequently increased but did not cor-
relate with clinical symptoms. By day 6, the last day prior to an-
tibiotic treatment, 2 volunteers had no positive culture results,
4 had 1+ growth, and 3 had 2+ or 3+ growth.

Clinical Response to Experimental Colonization
Volunteers were assessed specifically for fever, systemic reacto-
genicity, and upper respiratory tract signs and symptoms, as
well as for unanticipated adverse events. None of the subjects
experienced fever, and there were no events that were graded as
severe. Ten volunteers experienced mild symptoms, and 4 had

Figure 1. Maximum severity of solicited reactogenicity events, by day.

Figure 2. Correlation of sore throat symptoms to time after inoculation, culture results, and confirmed inoculation dose. Stars represent the days each subject had a positive culture. Black lines represent the days each subject reported sore throat. The maximum severity of sore throat reported by each subject is designated by symbols on the right of the figure; circles denote mild severity, and triangles denote moderate severity.
moderate symptoms (Figure 1). There was one instance each of mild feverishness, fatigue, general malaise, or myalgias. Five subjects had headache, although only 1 rated the headache as moderate in severity. Six subjects had mild runny nose or nasal congestion, 1 subject had transient ear congestion, and 1 had ear fullness with otherwise normal examination findings. Four subjects had mild cough. Eleven subjects had a sore throat, with 7 rating the severity as mild and 4 as moderate (Figure 2). Of the 8 colonized individuals with sore throat, 6 had sore throat develop within 24 hours before or after detection of NTHi 2019StrR1; resolution of symptoms occurred spontaneously in 2 and within 24 hours after receiving the first dose of levofloxacin in 6 (data not shown). Sore throat symptoms in noncolonized individuals tended to occur more sporadically, although 2 developed sore throat on day 6, raising the possibility that colonization might have been detected within the next 24 hours if antibiotic therapy had been withheld.

Two subjects had mild cervical lymphadenopathy within the 8 days after inoculation, and 2 subjects had mild pharyngeal erythema but no exudates.

Immunological Responses
IgA, IgM, and IgG serum immune responses to whole-cell sialylated NTHi 2019StrR1 were analyzed using ELISA. Seven of 9

Figure 3. Scatterplot of fold rise in immunoglobulin A (IgA; A), immunoglobulin G (IgG; B), and immunoglobulin M (IgM; C) titers, using the preinoculation titer on day 0 as baseline. Maximum titers were not always sustained over the 28-day period.
individuals who became colonized demonstrated a $\geq 4$-fold rise in IgA, IgM, and/or IgG titer (Figure 3). None of the noncolonized individuals showed a $\geq 4$-fold rise in titer for any of the 3 immunoglobulin classes. IgG responses were the most likely to show a $\geq 4$-fold rise in titer, with 5 of the 9 individuals showing an appropriate response. Four individuals showed a rise in IgA titers, and 2 showed an IgM response. Subject 31 is the only individual who showed a rise in titers of all 3 immunoglobulin classes. This individual delayed taking levofoxacin dosing for 3 additional days despite study instructions. All individuals had detectable titers for all immunoglobulin classes prior to receipt of the nasal inoculation, with IgG titers demonstrating substantially higher preinoculum levels than IgA or IgM titers (Figure 4). There was no clear relationship between preexisting titer and success or failure of colonization.

**DISCUSSION**

Studies involving patients with COPD have shown that acquisition of a new strain of NTHi can lead to an inflammatory state that is associated with acute exacerbation of chronic bronchitis [40]. In this study, 8 of 9 subjects who became colonized developed mild-to-moderate sore throat, suggesting that a local
inflammatory process may be seen in some people who acquire a new strain of NTHi in the upper respiratory tract. The statistical significance of this finding is obscured by the fact that 3 of 6 noncolonized subjects also developed sore throat. This could suggest that an intercurrent respiratory illness occurred in the population during the study. Three colonized individuals who developed sore throat had extensive viral and bacterial testing performed, and no common respiratory pathogens were identified (unpublished data). It is also possible that noncolonized individuals had bacterial invasion or colonization that was undetectable by means of traditional culture methods. Microbiome 16S DNA sequence and other molecular studies are underway to determine whether more-sensitive molecular analyses can detect NTHi 2019StrR1 in negative cultures. Nasal wash samples have also been tested for various inflammatory cytokines, using Luminex assays. Small subsets of colonized subjects had elevated levels of some of the inflammatory cytokines, such as interleukin 1β, interleukin 1Ra, interleukin 8, but there were no obvious trends (data not shown). However, the nasal wash process substantially dilutes the nasal secretions and could undermine interpretation.

Overall, we were able to culture NTHi 2019StrR1 from 9 of 15 subjects. Higher doses were more likely to be associated with colonization. However, even a dose as high as 76,000 CFU was not always successful, suggesting that there is individual susceptibility to colonization. Susceptibility could be related to preexisting...
immunity. Human antibody responses to NTHi in patients with COPD are strain specific, and antibodies typically bind surface epitopes on the intact bacterium [11, 41]. In these patients, acute exacerbation of bronchitis was often associated with acquisition of a new strain of NTHi, and 61% demonstrated an immune response to the homologous new strain, whereas only 21% had a response to the preexisting strain of NTHi [41]. In this study design, antibody responses could easily be interrogated using the homologous, inoculated NTHi 2019StrR1 strain. All subjects had detectable levels of IgA, IgM, and IgG to NTHi 2019StrR1 prior to inoculation. IgG responses were the most likely to show a ≥4-fold rise in titer, with 5 of the 9 individuals showing an appropriate response. All individuals had been immunologically primed by H. influenzae exposure before the age of 18 years, so an IgG

![Figure 4. Box plot analysis of immunoglobulin A (IgA; top 2 plots), immunoglobulin G (IgG; middle 2 plots), and immunoglobulin M (IgM; bottom 2 plots) titers, by study day. The center horizontal line is drawn at the 50th percentile (median), and the bottom and top edges of the box denote the sample 25th and 75th percentiles, respectively. Diamonds represent the mean, and the whiskers extend from the box to a distance of the 5th and 95th percentiles. Enzyme-linked immunosorbent assays were performed using whole-cell sialylated nontypeable Haemophilus influenzae 2019StrR1 as the target antigen. A modest rise in IgG and IgM titers is seen in subjects colonized on days 14 and 28. However, there is no correlation between starting titer and success or failure of colonization.](image-url)
response would be expected. However, one might anticipate that lower titers could be associated with successful colonization. Box plots do not demonstrate a clear relationship between the starting titers for IgM, IgA, or IgG in those who became colonized, and some individuals with IgG titers greater than 1:16,000 became colonized, while others did not. It is likely that individuals have a broad range of antibodies to *H. influenzae* and that only antibodies to specific antigens may be protective or functional. These specific antibodies are likely overshadowed by the broad range of nonprotective antibodies. In other studies, antibody responses to protein D, P6, and OMP26 have been seen in children with nasopharyngeal colonization and acute otitis media [42]. Additional studies evaluating immune responses to specific surface proteins and bactericidal activity are needed to further understand antibody responses to colonization.

Further studies are also needed to refine the HCD$_{50}$ and HCD$_{90}$ doses and provide smaller SDs and shorter credible interval, especially for the HCD$_{90}$. Use of a broth log-phase culture as opposed to isolation from an agar plate might provide a more standardized inoculum for future studies, although this introduces risk of contamination and would introduce more opportunity for phase variation of genes important in colonization. Identification of specific protective antibodies could improve the model since individuals could be screened for protective titers prior to inoculation. This would enhance the ability to achieve an HCD$_{90}$, making the model more effective as a proof of principle method to analyze vaccine candidates.

Overall, the study product was well tolerated and safe. All subjects were successfully decolonized with levofloxacin. The first stage in this Bayesian statistical model allowed a step-wise exploration of a wide range of doses, and the second stage improved the precision of the Bayesian estimates of the HCD$_{50}$ and HCD$_{90}$ for use in future studies. Nasal colonization was associated with modest increases in immunoglobulin titers to whole-cell NTHI 2019Str$^{R1}$, although further studies are needed to correlate specific antibodies to susceptibility or resistance to NTHI colonization. This model provides a potential platform for early proof of concept studies of NTHI vaccines, as well as a way to study bacterial factors associated with colonization and pathogenesis.

**Notes**

**Acknowledgments.** We thank Nancy Wagner, Geri Dull, Kris Heilmann, and Meg Ketterer, who provided clinical and laboratory support for this project; and Yu-Hui Huang Chang, Eric Foster, and Wei Zhang, for help with the statistical design and analyses. The Division of Microbiology and Infectious Diseases and Respiratory Diseases branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, provided invaluable guidance for this study.

**Financial support.** This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (contract NIH-N01AI-30040-07 to M. A. A. and P. L. W.), Y.-H. H. C. and E.F. were supported by NIGMS training grant T32 GM077973 (to K. C.). The Clinical Research Unit is supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through grant 2 U1 TR000442-06.

**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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