The Effects of Live Attenuated Influenza Vaccine on Nasopharyngeal Bacteria in Healthy 2 to 4 Year Olds
A Randomized Controlled Trial
Valtyr Thors¹*, Hannah Christensen², Begonia Morales-Aza¹, Ian Vipond³, Peter Muir³, and Adam Finn¹

¹School of Cellular and Molecular Medicine and ²School of Social and Community Medicine, University of Bristol, Bristol, United Kingdom; and ³Public Health Laboratory Bristol, Public Health England, Bristol, United Kingdom

Abstract

Rationale: Viral infections of the upper respiratory tract may influence the commensal nasopharyngeal bacteria. Changes in the bacterial niche could affect transmission dynamics. Attenuated vaccine viruses can be used to investigate this empirically in humans.

Objectives: To study the effects of mild viral upper respiratory infections on nasopharyngeal bacterial colonization using live attenuated influenza vaccine (LAIV) as a surrogate.

Methods: We used trivalent LAIV to evaluate the effects of viral infection on bacterial carriage and density of Streptococcus pneumoniae, Moraxella catarrhalis, Haemophilus influenzae, and Staphylococcus aureus. A total of 151 healthy children were randomized 1:1 to receive the vaccine starting either at recruitment (n = 74) or 28 days later (n = 77) in a stepped wedge fashion, allowing comparisons between recipients and nonrecipients as well as whole-group comparisons pre- and postvaccination. Bacterial carriage and density were determined using quantitative polymerase chain reaction assays.

Measurements and Main Results: A total of 151 children were recruited, 77 in the LAIV group and 74 in the control group. LAIV recipients (n = 63 analyzed) showed an apparent transient increase in H. influenzae carriage but no further significant differences in carriage prevalence of the four bacterial species compared with controls (n = 72 analyzed). S. pneumoniae density was substantially higher in vaccine recipients (16,687 vs. 1935 gene copies per milliliter) 28 days after the first dose (P < 0.001). Whole-group multivariable analysis (prevaccine, after one dose, and after two doses) also showed increases in density of other species and H. influenzae carriage prevalence.

Conclusions: In the absence of any safety signals despite widespread use of the vaccine, these findings suggest that bacterial density, and thus transmission rates among children and to people in other age groups, may rise following attenuated influenza infections without associated clinical disease. LAIV could therefore be used as an experimental tool to elucidate the dynamics of transmission of nasopharyngeal bacteria.

Keywords: bacterial colonization; bacterial density; children; live attenuated influenza vaccine

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*Present address: Pediatric Infectious Diseases & Immunology, Children’s Hospital Reykjavik, Reykjavik, Iceland.

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Author Contributions: A.F. and V.T. conceived of and designed the study; V.T. collected the samples; V.T., I.V., and B.M.-A. performed the laboratory analysis; V.T., H.C., P.M., and I.V. analyzed and interpreted the data; V.T. and A.F. drafted the manuscript; V.T., H.C., I.V., B.M.-A., P.M. and A.F. critically reviewed and edited the manuscript; and V.T. and H.C. did the statistical analysis. All authors had full access to all the data in the study, and the corresponding author had responsibility for submitting the manuscript for publication.

Correspondence and requests for reprints should be addressed to Valtyr Thors, M.D., School of Cellular and Molecular Medicine, University of Bristol, Education Centre, Upper Maudlin Street, Bristol BS2 8AE, UK. E-mail: valtyr@lsh.is

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Thors, Christensen, Morales-Aza, et al.: Effects of LAIV on Colonizing Bacteria
Pneumococcal conjugate vaccines are effective principally through population-wide effects on carriage and transmission (1). Despite this, the biology of colonization and determinants of transmission remain poorly understood. Evidence from both animals (2) and humans (3) suggests that respiratory viral infections can affect upper respiratory tract bacteria. However, the relevance of experiments in animal models to humans is uncertain, and deductions about causation based on observational studies are difficult. Studies by our group and others have shown that presence and colonization density of commensal bacteria of the nasopharynx (NP) are associated with symptoms of rhinitis and presence of respiratory viruses (4, 5).

Intranasal live attenuated influenza vaccine (LAIV) was licensed in Europe in 2012 for children aged 2–18 years, and until 2014 (when a second B strain was added) it consisted of three replication-competent viruses (two type A strains and one type B strain) attenuated by recombination of wild-type (WT) and attenuated donor viruses (6) and by cold adaptation through passages of progressively lower temperature in eggs (7). The vaccine viruses induce broad cell-mediated and local mucosal antibody responses (8). They replicate well at the lower temperatures of the upper respiratory mucosa but poorly in the lower airways or systemic circulation (9). LAIV is well tolerated, safe, and effective in preventing culture- and/or polymerase chain reaction (PCR)–proven influenza in children (10, 11), among whom influenza is a common and sometimes serious infection. However, when compared with trivalent inactivated influenza vaccine (TIV), higher rates of medically significant wheezing were observed during the second to fourth weeks postvaccination in children 6–23 months of age after the first dose of vaccine (5.9% in LAIV recipients vs. 3.8% in TIV recipients, as shown in Figure 3 in the online supplement in Belshé and colleagues [12]). In addition, increased all-cause hospitalization in children aged 6–11 months within 180 days of vaccination (6.1% in LAIV recipients vs. 2.6% in TIV recipients) (11) was observed, leading to restriction of licensure to children aged 2 years and older (13, 14). The pathophysiology of these adverse events is not understood.

Mina and colleagues (15) found a transient increase in density and delayed clearance of NP Streptococcus pneumoniae and Staphylococcus aureus in a mouse model after administration of either a single cold-adapted LAIV strain or WT influenza virus. No clinical disease was seen in animals given attenuated virus, although up to 5% weight loss was observed if S. pneumoniae was present before vaccination. Thus, both WT and attenuated influenza infection could influence bacterial load in the human NP, although extrapolation to humans and LAIV should be done with caution.

Demonstration of a similar effect in humans might help elucidate the relationships between respiratory viral infections and carriage prevalence and density dynamics of NP bacteria. The availability of LAIV that induces a mild upper respiratory tract infection without significant clinical illness offers an opportunity to undertake randomized interventional experimental studies in humans to explore this possibility.

Real-time quantitative PCR (qPCR) can enumerate bacterial species–specific gene copies and thus both viable and nonviable bacteria in biological samples over wide concentration ranges with less effort and expense than required with conventional quantitative plate cultures. Using qPCR, we recently described upper respiratory bacterial density ranges as wide as 6 orders of magnitude in healthy children (16). Such differences may result in wide variation in efficiency of onward transmission among carriers. Although alternatives exist, nasopharyngeal swabbing is the most widely used method to sample the nasal flora, is easy to perform, and is reasonably well accepted by children.

To explore the effect of LAIV on carriage rates and density of four bacterial species that commonly colonize the human NP, we recruited 151 children into a prospective randomized study to compare vaccinated and unvaccinated children at 1 and 4 weeks postvaccine and colonization before and after vaccination in the whole group. At the time of the study, no routine influenza immunization was being offered to healthy children in the United Kingdom. Some of the results of this study were reported previously in the form of an abstract (17).

**Methods**

**Study Design**

We conducted a cohort stepped wedge study of an LAIV containing three (two A and one B) influenza strains in children at 10 day care centers (DCCs) in Bristol, UK.

**Participants and Randomization**

Children aged 2–4 years attending the DCCs were eligible to participate. We attempted to provide written information sheets with an invitation to take part in the study to parents of all of the 659 eligible children in the 10 DCCs. We were able to make contact with 80% of the parents and invite them to take part in the study between September 3 and October 15, 2012. Only children for whom the vaccine was contraindicated were excluded (Figure 1). Informed consent was obtained for all enrolled subjects. At enrollment, children were individually randomized 1:1 within nurseries using the Bristol Randomized
Trials Collaboration electronic randomization system (Bristol Randomized Trials Collaboration, School of Social and Community Medicine, University of Bristol) by the study team to receive the vaccine at Day 0 (visit 1 [V1]) and on or around Day 28 (V3) (LAIV group) or on or around Day 28 (V3) and Day 56 (V5) (control late vaccine group) with additional swabbing visits (V2 and V4) on or around Days 7 and 35, respectively. A small number of children who had received inactivated influenza vaccine in either of the previous two seasons received only the first LAIV dose in the study (Figure 1). If the child refused vaccination, only NP swabbing was attempted. Laboratory staff were blinded to the intervention assignment during analysis.

**Intervention and Procedures**

Vaccine doses were given intranasally following monitored cold chain storage and after medical assessment of each subject. Following immunization, children were observed for 30 minutes. At all study visits, which were conducted at the DCCs between October 2012 and February 2013, a fine-tip pediatric NP swab (Peel Pouch Dryswab; Medical Wire and Equipment, Corsham, UK) was taken. For the swabbing procedure, the child was seated comfortably with the head slightly extended. The swab was inserted horizontally until resistance was met when the swab was retracted with a slight twisting action along the axis of the shaft and inserted into a 2-ml tube prefilled with 1.5 ml of skim milk, tryptone, glucose, and glycerol broth, which was labeled with a random number to blind laboratory analysis. The records linking the study participant and visit numbers with the blinding numbers were kept in a locked facility until after the completion of laboratory analysis. Tubes were kept at 0–4°C until transferred to storage at −80°C within 4 hours of collection. At the time of collection of each swab, the severity of any rhinitis symptoms was recorded (Symptoms of Nasal Outflow Tally score of 0–3 as previously reported [4] and as described in the online supplement).

**Laboratory Analysis**

Nucleic acid was extracted from all samples using the Virus/Pathogen Mini Kit from QIAGEN (Valencia, CA), yielding 110-μl extracts from 300 μl of skim milk, tryptone, glucose, and glycerol broth. These extracts were stored at −80°C. Monoplex qPCR assays were used to detect *S. pneumoniae, Moraxella catarrhalis, Haemophilus influenzae*, and *S. aureus* (16). The cycle threshold (Ct) value used to define detection was set at less than or equal to 35 cycles. For density values, Ct values were converted into gene copies (GC)/ml using

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**Figure 1.** Consolidated Standards of Reporting Trials flow diagram of the study. LAIV = live attenuated influenza vaccine.
standard curves (16). qPCR assays for adenovirus, bocavirus, influenza (A and B), respiratory syncytial virus, rhinovirus, human metapneumovirus, parainfluenza (types 1–3), parechovirus, coronavirus (229E, OC43, and NL63), and enterovirus were performed using methods described previously (4, 18). In addition, an assay for coronavirus HKU1 (see Table E1 in the online supplement) was used.

**Outcomes**
The primary endpoint was a comparison of the proportion of children positive for *S. pneumoniae* 1 week after a first dose of LAIV compared with the control group. Additional planned endpoints included similar comparisons for *M. catarrhalis*, *H. influenzae* or *S. aureus* at 1 and 4 weeks after LAIV; the density of all four species after the same intervals, both singly and combined; the proportions of positive subjects; and carriage density for the four species before and after one or two doses of LAIV over all study visits, both in all children studied and in those who were or were not carriers of each species at the first visit. Exploratory and descriptive analyses, respectively, were also done with respect to rhinitis symptom scores and presence of respiratory viral nucleic acid in samples.

**Statistical Analysis**
Statistical analyses were done using Stata version 12.0 software (StataCorp, College Station, TX). To assess balance at randomization, differences in characteristics were analyzed using Pearson’s χ² test and t tests when appropriate. We estimated the odds ratio of carriage for each bacterial species in the early vaccine group compared with the control (late vaccine) group at V2 and V3 postenrollment using logistic regression, including a fixed effect for the vaccine group and a random effect (intercept) for nurseries to allow for between-nursery variability. We also estimated the odds ratios of carriage after one and two doses of LAIV (i.e., children contributed measurements before and after their vaccination) using logistic regression and adjusting for confounders (detailed in footnotes to Tables 1 and 2), with random effects (intercept) for nurseries and children to allow for repeated measures in the same individuals and between-nursery variability. We estimated the difference in mean density (GC/ml) of bacteria in those children positive for carriage (at baseline and the visit of interest) using γ-regression (because GC/ml were positively skewed), including the same fixed and random effects as we used for the odds of carriage analysis. We modeled the effect of the vaccine from one visit after the vaccine was administered. Analyses were done on the modified intention-to-treat population, which included all enrolled participants who provided an assessable swab sample, regardless of whether they actually received the vaccine doses. Analyses were undertaken using the GLLAMM program in Stata (19–21).

**Trial Registration**
Trial registration was initiated (EudraCT identifier 2011-002964-25), but, owing to regulatory authority determination that this was not an investigational medicinal product trial because the vaccine was licensed for this age group, registration could not be completed.

**Ethical Approval**
The study was approved by the U.K. National Research Ethics Service – Central Bristol Committee (11/SW/0186), and responsibility for governance oversight was taken by the University of Bristol, UK.

**Results**
A Consolidated Standards of Reporting Trials (CONSORT) diagram detailing screening, enrollment, randomization, loss to follow-up, and completion is shown in Figure 1 (see details by nursery in Table E2). A total of 125 children completed the vaccination protocol, and 120 completed all visits (six withdrew before or at the time of the first visit, two departed from the area during the study, and the others repeatedly refused sampling). The number of subjects recruited in each DCC ranged from 4 to 29. The median number of days after first vaccine dose at V2 sampling was 7 days for both groups (range, 5–26 d) and, at V3, 28 days (range, 27–43 d). The median durations after the second vaccine dose were (V4) 7 days (range, 6–16 d) and (V5) 28 days (range, 21–48 d). The control (late vaccine) group had a greater mean number of siblings, but otherwise the groups were similar following randomization (Table 3). There was no evidence to suggest a difference in antibiotic use between the groups at baseline (3 of 70 in the vaccine group and 1 of 72 in the control group), V2 (1 of 66 and 2 of 69, respectively), or V3 (3 of 65 and 3 of 72, respectively).

**Virology Results for the Whole Study Period**
Respiratory viral nucleic acid was detected (Ct <35) in 216 of 670 samples (from 144 children), among which 37 samples had more than one virus. Rhinovirus was the most frequently detected (16.8%), followed by coronavirus (4.4%), parainfluenza virus (3.3%), bocavirus (3.1%), enterovirus

| Table 1. Odds Ratios (Generalized Linear Mixed Models) for Carriage Prevalence of Bacterial Species after Vaccination, Including All Visits in a Modified Intention-to-Treat Population |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | *S. pneumoniae*             | *M. catarrhalis*            | *H. influenzae*             | *S. aureus*                 |
|                             | OR* (95% CI)                | OR* (95% CI)                | OR* (95% CI)                | OR* (95% CI)                | OR* (95% CI)                | OR* (95% CI)                |
| Unvaccinated                | 1.00 (Reference)            | 1.00 (Reference)            | 1.00 (Reference)            | 1.00 (Reference)            | 1.00 (Reference)            | 1.00 (Reference)            |
| One LAIV dose               | 0.84 (0.52–1.36)            | 1.15 (0.62–2.15)            | 2.23 (1.29–4.21)            | 0.81 (0.34–1.90)            | 0.868                       |
| Two LAIV doses              | 0.90 (0.44–1.81)            | 2.59 (0.94–7.12)            | 3.46 (1.33–9.02)            | 0.73 (0.20–2.65)            |

**Definition of abbreviations:** CI = confidence interval; LAIV = live attenuated influenza vaccine; OR = odds ratio.

*OR of carriage adjusted by months of age, sex, presence of smoker in the home, number of siblings, presence of viral nucleic acid, and time (week in the study), with nursery and subject ID as random effects (intercept). Data for the second LAIV dose relate only to the group that received vaccine at visits 1 and 3.
respectively) and lower for vaccination, respectively), there was no difference between the groups (Table 4). There was no evidence to suggest a difference between the two study groups in the detection of these bacterial species, apart from an apparent modest and transient increase in *H. influenzae* prevalence seen at V2. Forty-six (69.7%) of 66 were positive among the early vaccinees, and 36 (52.2%) of 69 among the controls were positive (P = 0.042, adjusted for clustering by nursery).

### Bacterial Prevalence at V1–3

At baseline, carriage prevalence was high for *S. pneumoniae*, *M. catarrhals*, and *H. influenzae* (68.3%, 77.6%, and 57.8%, respectively) and lower for *S. aureus* (10.6%). There was no evidence to suggest a difference between the groups (Table 4). At V2 and V3 (7 and 28 d after early vaccination, respectively), there was no evidence to suggest a difference between the two study groups in the detection of these bacterial species, apart from an apparent modest and transient increase in *H. influenzae* prevalence seen at V2. Forty-six (69.7%) of 66 were positive among the early vaccinees, and 36 (52.2%) of 69 among the controls were positive (P = 0.042, adjusted for clustering by nursery).

### Carriage over the Whole Study Period

At the first visit, 45 children tested negative for carriage of *S. pneumoniae* (samples at V1 were unavailable for 9 children). Over the course of the study, 42 of these children subsequently tested positive, including 9 children before receiving a vaccine, 20 after receiving one dose, and 10 after receiving two doses; the 3 remaining children left the study. Similarly to the findings of the between-groups analysis, in the before versus after vaccine analysis across all visits, there was little to suggest any difference in carriage prevalence after vaccination (one or two doses of LAIV) for *S. pneumoniae*, *M. catarrhals*, or *S. aureus* (Table 1). However, once again, there was evidence for an effect of vaccine to increase *H. influenzae* prevalence, with higher odds of carriage after both one and two doses of LAIV, after controlling for confounding.

### Table 2. Arithmetic Mean Ratio (Generalized Linear Mixed Models) for Carriage Density of Bacterial Species after Vaccination, Including All Visits in Modified Intention-to-Treat Population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Vaccine (95% CI)</th>
<th>P Value</th>
<th>Controls (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year sex</td>
<td>38.3 (7.8)</td>
<td>0.11</td>
<td>40.3 (6.7)</td>
<td>0.03</td>
</tr>
<tr>
<td>Male sex</td>
<td>35/74 (47.3)</td>
<td>0.93</td>
<td>37/77 (48.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean number of siblings</td>
<td>0.69 (0.77)</td>
<td>0.02</td>
<td>1.04 (1.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>Underlying medical condition</td>
<td>11/74 (14.9)</td>
<td>0.25</td>
<td>17/77 (22.1)</td>
<td>0.25</td>
</tr>
<tr>
<td>Parental smoking</td>
<td>6/73 (8.2)</td>
<td>0.74</td>
<td>5/74 (6.8)</td>
<td>0.74</td>
</tr>
<tr>
<td>National vaccination schedule not complete</td>
<td>2/73 (2.7)</td>
<td>0.53</td>
<td>1/77 (1.3)</td>
<td>0.53</td>
</tr>
<tr>
<td>Given influenza vaccine in previous two seasons</td>
<td>4/73 (5.5)</td>
<td>0.67</td>
<td>3/75 (4.0)</td>
<td>0.67</td>
</tr>
<tr>
<td>Noninfluenza viral nucleic acid detected</td>
<td>24/69 (34.8)</td>
<td>0.51</td>
<td>21/71 (29.6)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*Values are mean (SD) or number/total (percent).
### Table 4. Carriage Prevalence of Bacterial Species in Early Vaccine and Control Groups by Visit

<table>
<thead>
<tr>
<th>Visit</th>
<th>Children with Carriage (n/N [%])</th>
<th>Controls</th>
<th>Vaccine</th>
<th>OR* (95% CI)</th>
<th>P Value</th>
<th>OR* (95% CI)</th>
<th>P Value</th>
<th>OR* (95% CI)</th>
<th>P Value</th>
<th>OR* (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visit 1 (baseline)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>51/72 (70.8)</td>
<td>46/70 (65.7)</td>
<td>0.79 (0.39–1.60)</td>
<td>0.512</td>
<td>52/66 (78.8)</td>
<td>52/68 (76.5)</td>
<td>0.90 (0.39–2.08)</td>
<td>0.810</td>
<td>39/72 (54.2)</td>
<td>43/70 (61.4)</td>
<td>1.36 (0.69–2.66)</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>46/70 (65.7)</td>
<td>45/66 (68.2)</td>
<td>0.94 (0.45–1.94)</td>
<td>0.862</td>
<td>56/66 (84.9)</td>
<td>54/66 (81.8)</td>
<td>0.80 (0.32–2.01)</td>
<td>0.641</td>
<td>36/69 (52.2)</td>
<td>46/66 (69.7)</td>
<td>2.13 (1.03–4.44)</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>39/72 (54.2)</td>
<td>43/70 (61.4)</td>
<td>1.36 (0.69–2.66)</td>
<td>0.376</td>
<td>8/72 (11.1)</td>
<td>7/70 (10.0)</td>
<td>0.89 (0.30–2.63)</td>
<td>0.836</td>
<td>5/69 (7.3)</td>
<td>4/66 (6.1)</td>
<td>0.83 (0.21–3.30)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>52/66 (78.8)</td>
<td>52/68 (76.5)</td>
<td>0.90 (0.39–2.08)</td>
<td>0.810</td>
<td>52/68 (76.5)</td>
<td>50/65 (76.9)</td>
<td>1.01 (0.45–2.28)</td>
<td>0.983</td>
<td>47/72 (65.3)</td>
<td>44/66 (68.8)</td>
<td>1.19 (0.57–2.48)</td>
</tr>
</tbody>
</table>

Definition of abbreviations: CI = confidence interval; OR = odds ratio.
*OR of carriage at each visit in early vaccine group compared with control group with nursery (random effect, intercept) in the model.

### Table 5. Carriage Density of Bacterial Species in Early Vaccine and Control Groups, in Individuals Who Were Carriage Positive at Baseline

<table>
<thead>
<tr>
<th>Visit</th>
<th>Mean Carriage Density* (GC/ml [SD])</th>
<th>Controls</th>
<th>Vaccine</th>
<th>Mean Ratio† (95% CI)</th>
<th>P Value</th>
<th>Controls</th>
<th>Vaccine</th>
<th>Mean Ratio† (95% CI)</th>
<th>P Value</th>
<th>Controls</th>
<th>Vaccine</th>
<th>Mean Ratio† (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visit 1 (baseline)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>3297 (6394)</td>
<td>2687 (4636)</td>
<td>0.78 (0.38–1.61)</td>
<td>0.501</td>
<td>15,399 (58,725)</td>
<td>13,673 (40,268)</td>
<td>0.91 (0.38–2.19)</td>
<td>0.830</td>
<td>17,286 (38,941)</td>
<td>18,379 (41,310)</td>
<td>1.11 (0.39–3.20)</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>4869 (99.6)</td>
<td>45/66 (68.2)</td>
<td>0.94 (0.45–1.94)</td>
<td>0.862</td>
<td>56/66 (84.9)</td>
<td>54/66 (81.8)</td>
<td>0.80 (0.32–2.01)</td>
<td>0.641</td>
<td>36/69 (52.2)</td>
<td>46/66 (68.7)</td>
<td>2.13 (1.03–4.44)</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>39/72 (54.2)</td>
<td>43/70 (61.4)</td>
<td>1.36 (0.69–2.66)</td>
<td>0.376</td>
<td>8/72 (11.1)</td>
<td>7/70 (10.0)</td>
<td>0.89 (0.30–2.63)</td>
<td>0.836</td>
<td>5/69 (7.3)</td>
<td>4/66 (6.1)</td>
<td>0.83 (0.21–3.30)</td>
<td>0.792</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>52/66 (78.8)</td>
<td>52/68 (76.5)</td>
<td>0.90 (0.39–2.08)</td>
<td>0.810</td>
<td>52/68 (76.5)</td>
<td>50/65 (76.9)</td>
<td>1.01 (0.45–2.28)</td>
<td>0.983</td>
<td>47/72 (65.3)</td>
<td>44/66 (68.8)</td>
<td>1.19 (0.57–2.48)</td>
<td>0.639</td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: CI = confidence interval; GC = gene copies.
*Mean carriage density in those who were positive.
†Arithmetic mean ratio. Estimates of arithmetic mean ratio allow for clustering by nursery (random effect, intercept) and so is not the simple quotient of the respective means. Too few children carried Staphylococcus aureus for statistical analysis.
and allowing for clustering by nursery and repeated measures in children over time. Analyses of bacterial density were again restricted to those children with detectable carriage at baseline (to assess changes in density as opposed to acquisition). Again, there was evidence of an increase in *S. pneumoniae* density with increasing doses of vaccine. In particular, compared with prevaccine levels, a 2.5-fold increase in density was observed after two vaccine doses when samples were considered over all time points in the study (Table 2). There was also evidence of vaccine-associated increases in *M. catarrhalis* density in carriers, with a more than doubling observed after the first vaccine dose. Although point estimates postvaccine were also raised relative to prevaccine values for *H. influenzae*, the confidence intervals were wide. Again, too few children were carriers of *S. aureus* to permit analysis of density using this approach. The relationship between nasal discharge score (Symptoms of Nasal Outflow Tally score) and bacterial density was investigated by including nasal discharge scores in the *S. pneumoniae* model, and evidence was found for an independent positive association with pneumococcal density (Table E3).

**Discussion**

To our knowledge, this study represents the first exploration of the effects of experimental respiratory viral infection on bacterial upper respiratory colonization in humans. We show that infections with attenuated viruses can result in increases in carriage density of common bacteria or increases in acquisition, raising the possibility that WT influenza and other viruses may have more marked such effects. Children carrying more bacteria are probably more infectious than those with fewer, and it makes biological sense that such bacteria should evolve to sense viral rhinitis or the host response to it and exploit it as a transmission opportunity. In addition to proliferating, bacteria might change phenotype to one better suited to successful transit. Our findings also suggest that LAIV could be used as an experimental tool with which to elucidate relationships between nasal bacterial abundance and transmission rates between children. Because the effectiveness of pneumococcal conjugate vaccines depends on their impact on bacterial transmission both within and beyond the pediatric population, there are good practical reasons why the biology of this process should be elucidated in detail.

Does this study raise safety concerns about LAIV? We did not detect any safety signals associated with increased bacterial colonization density, but the study was not powered to do so. Upper respiratory carriage of bacteria is not an illness, and many authors assume it to be asymptomatic (22, 23). We previously reported an association between carriage of *H. influenzae* and rhinitis in healthy children (4), and in the present study we demonstrate an association for *S. pneumoniae* density independent of detectable viral nucleic acid and age. However, these symptoms are mild, and the vaccine has been studied in clinical trials (10–13). In addition, it has been widely used in children aged 2 years and older without any safety signals that one might attribute to higher bacterial density such as otitis media, pneumonia, or bacteremia, and overall the vaccine protects against the former (10). One might expect evolutionary pressures to select for bacteria that exploit the ubiquitous respiratory viral infections of early childhood to promote colonization and transmission while minimizing serious pathology in their hosts.

An apparent short-term effect of LAIV on carriage prevalence of *H. influenzae* in the randomized comparison was confirmed in multivariable analysis, where it was also observed for the second vaccine dose. However, measurement of colonization rates with this and other bacterial species was not the primary study aim and absolute numbers of carriers constituting the difference were small, so it is possible that this finding may have occurred by chance. If it is a genuine increase in bacterial acquisition induced by the vaccine, it has echoes of the observation of pneumococcal acquisition in ferrets experimentally infected with influenza (2). In a recent adult pneumococcal challenge study, colonization was more likely in the presence of a respiratory virus (24), although this was not associated with higher carriage density, possibly reflecting differences between adults and children. Confirmatory studies of acquisition of *H. influenzae* and other bacterial species are needed. A twofold rise in *M. catarrhalis* density was seen in the comparison of pre- and postvaccine doses but not in the randomized comparison, most likely due to a population average effect (specifically, the big impact of those with very high colonization densities). We also found that colonization at the time of vaccination was associated with greater overall rises in density, suggesting that increases in bacterial load were due not to acquisition of new strains but to proliferation of those already present (data not shown). This could be confirmed in future studies. We also found no effects of number of siblings (Table 3) on the observed differences between groups (data not shown). There were too few *S. aureus* carriers to analyze the effects of the vaccine on density, although we saw rises in density at V3 (28 d) apparently similar to those seen for *S. pneumoniae* (Figure E1), observations that are concordant with those made in animal models (15).

More generally, the use of randomization and the blinding of the laboratory analysis make it unlikely that our results were subject to bias or confounding, and the size of the effects seen was sufficiently large to allow for possible biological effects on transmission rates. Although several bacterial species were studied, increasing the possibility of apparent differences arising by chance, individual upward trends were consistently seen, while secondary, larger, nonrandomized but conservative multivariable analysis confirmed the positive findings of the randomized comparisons and extended them to other species when potential confounding factors, in particular age and clustering by nurseries, were taken into account.

The stepped wedge design that we used reduced the period over which randomized comparisons could be made but allowed more children and samples to be included in a confirmatory before versus after vaccine multivariate analysis and had the ethical and practical advantage of offering all participants protection against influenza. The higher dropout rate in the early vaccine group was evident mostly after the third visit (Figure 1) and thus did not influence the findings of the between-groups analysis (V1–V3). That no placebo was used is a limitation, but an administration device identical to the vaccine could not be obtained. Less than 30% of the available parents consented to participate, which is high for a pediatric vaccine study but raises the possibility of selection bias. We could not collect information on parents who
refused or on their children and so cannot exclude this possibility, but we have no reason to believe that our subjects were unrepresentative of the population.

Our estimate of the effect of LAIV on bacterial carriage may be conservative. Available resources permitted us to conduct an individually randomized study. Through exposure to vaccinated children with elevated rates or density of colonization, control subjects may also have had their values elevated, diluting the observed effect of the intervention. Future confirmatory studies should preferably have a cluster randomized design.

We detected and quantified species-specific bacterial DNA using PCR rather than viable bacteria by conventional culture techniques and thus, in part, detected DNA from nonviable organisms. Had cultures been done, doubtless some PCR-positive, culture-negative individuals would have been found. However, we have recently shown that individuals with discordant PCR and culture results tend to have low density colonization (16). Although DNA may be present in the absence of living organisms, it nevertheless represents a footprint of recent bacterial presence, which is what this study was designed to measure. Nevertheless, it will be important to perform follow-up confirmatory studies and to elucidate how the bacterial density measurements we report here relate to efficiency of transmission between colonized children.

On the basis of murine studies (15), our expectation was that changes in nasal bacterial colonization density would be evident around 7 days after immunization, and our observations of increases at 28 days were unexpected, raising questions about the time course of this effect in children. As no intermediate samples between 7 and 28 days were taken, we were unable to tell whether the observed effect at 28 days was due to the vaccine. Nevertheless, it is possible that the viral presence caused bacterial colonization to increase rapidly.

Although we present results on several bacterial species that commonly colonize children, we offer no information about serotypes of the studied species or the many others that are commonly present (25). We are now undertaking further analysis of these samples using microbiomic techniques to further explore viral-bacterial interactions.

In conclusion, we show that viral infections may induce species-specific changes in both bacterial density and acquisition and that it is important to study bacterial colonization as a continuous variable. Although not associated with disease, these effects may be important at the population level, especially in the context of more virulent WT respiratory virus infections, which may have potent effects on bacterial transmission between individuals. Nearly all childhood vaccination programs rely on an extent of the interruption of transmission of infections, so our findings should encourage further human studies of transmission dynamics.

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References


