The Effects of Live Attenuated Influenza Vaccine on Nasopharyngeal Bacteria in Healthy 2 to 4 Year Olds

A Randomized Controlled Trial

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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

(Received in original form October 14, 2015; accepted in final form January 6, 2016)

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Supported by the European Society for Pediatric Infectious Diseases and AstraZeneca. The funding bodies had no role in the study design, data collection, data analysis, data interpretation, or writing or approval of the manuscript. A.F. and H.C. were supported by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Evaluation of Interventions at University of Bristol. This work was produced by the authors under the terms of the research training fellowships issued by the NIHR.

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.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 193, Iss 12, pp 1401–1409, Jun 15, 2016

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Originally Published in Press as 10.1164/rccm.201510-2000OC on January 7, 2016

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Epidemiological data have suggested a link between respiratory viral and invasive and severe respiratory bacterial infections, whereas animal studies using wild-type and attenuated influenza strains have shown increases in the density of *Streptococcus pneumoniae* and *Staphylococcus aureus* in the nasopharyngeal mucosa. There is no experimental evidence on this subject in humans.

What This Study Adds to the

Field: This randomized trial provides evidence that administration of live attenuated influenza vaccine to healthy children, after a short delay, increases the colonization density of *S. pneumoniae* in individuals carrying this bacterial species at the time of vaccination. It may also transiently increase rates of nasal colonization with *Haemophilus influenzae*. Comparison of pre- and postvaccine samples by multivariate analysis confirms these findings and suggests that vaccination may also transiently increase the density of *Moraxella catarrhalis* among carriers.

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 Belshe 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

Figure 1. Consolidated Standards of Reporting Trials flow diagram of the study. LAIV = live attenuated influenza vaccine.

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

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 χ^2 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

	<i>Streptococcus pneumoniae</i>		<i>Moraxella catarrhalis</i>		<i>Haemophilus influenzae</i>		<i>Staphylococcus aureus</i>	
	OR* (95% CI)	P Value	OR* (95% CI)	P Value	OR* (95% CI)	P Value	OR* (95% CI)	P Value
Unvaccinated	1.00 (Reference)	0.777	1.00 (Reference)	0.091	1.00 (Reference)	0.015	1.00 (Reference)	0.868
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)	
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.

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

	<i>Streptococcus pneumoniae</i>		<i>Moraxella catarrhalis</i>		<i>Haemophilus influenzae</i>	
	Arithmetic Mean Ratio* (95% CI)	P Value	Arithmetic Mean Ratio* (95% CI)	P Value	Arithmetic Mean Ratio* (95% CI)	P Value
Unvaccinated	1.00 (Reference)	0.064	1.00 (Reference)	0.029	1.00 (Reference)	0.161
One LAIV dose	1.24 (0.69–2.23)		2.21 (1.22–4.00)		1.89 (0.98–3.65)	
Two LAIV doses	2.68 (1.11–6.47)		1.99 (0.84–4.73)		1.69 (0.69–4.17)	

Definition of abbreviations: CI = confidence interval; LAIV = live attenuated influenza vaccine.

*Arithmetic mean ratio (95% CI) of carriage density adjusted by months of age, sex, 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). Too few children carried *Staphylococcus aureus* for statistical analysis. Data for the second LAIV dose relate only to the group that received vaccine at visits 1 and 3.

(3.1%), and respiratory syncytial virus (2.8%). Adenovirus, human metapneumovirus, and parechovirus were found in less than 2.5% of samples. Fifteen children (2.3%) were found to have influenza virus, 5 cases of which were influenza A and 10 of which were detected 7 days after vaccination and so likely represented vaccine virus. (None of these children reported influenza-like illness.) We found no evidence for a difference in noninfluenza viral detection rates between groups at baseline (Table 3) or at V2 and V3 (22 of 66 or 24 of 64, respectively; $P = 0.859$; 21 of 65 or 15 of 72, respectively; $P = 0.130$). Rates of detection of rhinovirus, the most frequently detected virus, were also similar in both groups.

Bacterial Prevalence at V1–3

At baseline, carriage prevalence was high for *S. pneumoniae*, *M. catarrhalis*, 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).

Bacterial Density at V1–3

Changes in bacterial density were investigated in those children who were positive for carriage at baseline (V1). Among these children, there was no evidence to suggest a difference in mean *S. pneumoniae* density (GC/ml) between the study groups at baseline (V1) or 7 days (V2) after the intervention. However, at V3 (28 d after vaccination), a sixfold higher mean bacterial density was found in children positive for carriage in the vaccinated group, whose values had risen, compared with controls, whose values appeared to have fallen slightly over this period (Table 5). There was no evidence of a difference at either V2 or V3 (7 or 28 d after vaccination) for *M. catarrhalis* or

H. influenzae. There were too few children with positive results for *S. aureus* at baseline to permit statistical analysis ($n = 15$), although there was a trend toward higher density carriage in vaccinees (Figure E1).

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. catarrhalis*, 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 3. Clinical Characteristics of the Two Study Groups at Randomization

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

*Values are mean (SD) or number/total (percent).

Table 4. Carriage Prevalence of Bacterial Species in Early Vaccine and Control Groups by Visit

	Streptococcus pneumoniae				Moraxella catarrhalis				Haemophilus influenzae				Staphylococcus aureus			
	Children with Carriage (n/N [%])		P Value	OR* (95% CI)	Children with Carriage (n/N [%])		P Value	OR* (95% CI)	Children with Carriage (n/N [%])		P Value	OR* (95% CI)	Children with Carriage (n/N [%])		P Value	OR* (95% CI)
	Controls	Vaccine			Controls	Vaccine			Controls	Vaccine			Controls	Vaccine		
Visit 1 (baseline)	51/72 (70.8)	46/70 (65.7)	0.512	0.79 (0.39–1.60)	52/66 (78.8)	52/68 (76.5)	0.810	0.90 (0.39–2.08)	39/72 (54.2)	43/70 (61.4)	0.376	0.89 (0.30–2.63)	8/72 (11.1)	7/70 (10.0)	0.836	
Visit 2 (~7 d)	48/69 (69.6)	45/66 (68.2)	0.862	0.94 (0.45–1.94)	56/66 (84.9)	54/66 (81.8)	0.641	0.80 (0.32–2.01)	36/69 (52.2)	46/66 (69.7)	0.042	0.83 (0.21–3.30)	5/69 (7.3)	4/66 (6.1)	0.792	
Visit 3 (~28 d)	52/72 (72.2)	41/65 (63.1)	0.255	0.66 (0.32–1.36)	52/68 (76.5)	50/65 (76.9)	0.983	1.01 (0.45–2.28)	47/72 (65.3)	44/64 (68.8)	0.639	1.62 (0.49–5.37)	5/72 (6.9)	7/65 (10.8)	0.433	

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

	Streptococcus pneumoniae				Moraxella catarrhalis				Haemophilus influenzae			
	Mean Carriage Density* (GC/ml [SD])		P Value	Mean Ratio† (95% CI)	Mean Carriage Density* (GC/ml [SD])		P Value	Mean Ratio† (95% CI)	Mean Carriage Density* (GC/ml [SD])		P Value	Mean Ratio† (95% CI)
	Controls	Vaccine			Controls	Vaccine			Controls	Vaccine		
Visit 1 (baseline)	3297 (6394)	2687 (4635)	0.501	0.78 (0.38–1.61)	15,399 (58,725)	13,673 (40,268)	0.830	0.91 (0.38–2.19)	18,379 (41,310)	18,379 (41,310)	0.846	1.11 (0.39–3.20)
Visit 2 (~7 d)	3484 (9640)	4202 (8481)	0.146	1.81 (0.81–4.04)	19,589 (90,043)	19,871 (42,525)	0.165	1.82 (0.78–4.27)	29,985 (73,937)	29,985 (73,937)	0.145	2.19 (0.76–6.30)
Visit 3 (~28 d)	1935 (5275)	16,687 (56,920)	<0.001	6.35 (2.30–17.61)	25,597 (83,640)	18,640 (42,995)	0.845	0.92 (0.38–2.20)	24,550 (46,652)	24,550 (46,652)	0.167	2.15 (0.73–6.34)

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 reconfirmed 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, nor were any taken more than 28 days after a vaccine dose, the timing of the peak of this effect remains unclear. Because bacteria can divide very rapidly, one can speculate that the time lag that rendered the density rise undetectable at 7 days, by when vaccine viral infection would have been well established, indicates that bacterial proliferation is triggered by the host response rather than by the viruses themselves. It is also possible that the usually asymptomatic infections caused by attenuated cold-adapted vaccine viral strains induce relatively slow, small bacterial responses that were hard to detect at the earlier time point. Future studies should include additional sampling times to clarify the duration of this effect and the timing of its peak.

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 to an extent on the interruption of transmission of infections, so our findings should encourage further human studies of transmission dynamics. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the European Society for Pediatric Infectious Diseases and AstraZeneca for funding support. The authors also thank Darryl Hill, Caroline Trotter (University of Bristol), and Jolanta Bernatoniene (University Hospitals Bristol) for advice on study design. In addition, the authors thank Leon Danon for help with the statistical analysis. The authors thank Professor Asgeir Haraldsson (Children's Hospital, Iceland) for comments on the manuscript. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the NIHR, the Department of Health, or Public Health England.

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