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Co-colonization by *Haemophilus influenzae* with *Streptococcus pneumoniae* enhances pneumococcal-specific antibody response in young children

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Abstract

Background—*Streptococcus pneumoniae* (*Spn*), *Haemophilus influenzae* (*Hi*) and *Moraxella catarrhalis (Mcat)* are common bacterial pathogens of respiratory infections and common commensal microbes in the human nasopharynx (NP). The effect of interactions among theses bacteria during co-colonization of the NP on the host immune response has not been evaluated. The objective of this study was to assess the impact of co-colonization by *Hi* or *Mcat* on the systemic antibody response to vaccine protein candidate antigens of *Spn and similarly the impact of co-colonization by Spn and Mcat on antibody responses to Hi vaccine protein candidate antigens*.

Methods—Serum samples were collected from healthy children at 6, 9, 15, 18, and 24 months of age when they were colonized with *Spn, Hi, Mcat* or their combinations. Quantitative ELISA was used to determine serum IgA and IgG against three *Spn* antigens and three *Hi* antigens, and as well as whole cells of non-typeable (NT) *Spn* and *Hi*.

Results—NP colonization by *Spn* increased serum IgA and IgG titers against *Spn* antigens PhtD, PcpA and PlyD and whole cells of *NTSpn*, and co-colonization of *Hi or Mcat* with *Spn* resulted in further increases of serum pneumococcal-specific antibody levels. NP colonization by *Hi* increased serum IgA and IgG titers against *Hi* antigens P6, Protein D and OMP26 and whole cells of *NTHi*, but co-colonization of *Spn* or *Mcat* with *Hi* did not result in further increase of serum *NTHi*-specific antibody levels.

Conclusion—Co-colonization of *Hi* or *Mcat* with *Spn* enhances serum antibody response to *NTSpn* whole cells and *Spn* vaccine candidate antigens PhtD, PcPA and PlyD1. Co-colonization appears to variably modulate pathogen species-specific host adaptive immune response.

Keywords

Streptococcus pneumoniae; *Haemophilus influenzae*; *Moraxella catarrhalis*; Polymicrobial co-colonization; Antibody response

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1. Introduction

Respiratory tract infections are the most common pediatric disease associated with significant morbidity and socioeconomic cost [1-3]. *Streptococcus pneumoniae* (*Spn*), *Haemophilus influenzae* (*Hi)* and *Moraxella catarrhalis (Mcat)* are common bacterial pathogens tocause pneumonia, acute exacerbations of bronchitis, acute sinusitis, and acute otitis media (AOM) [1]. The first step of respiratory bacterial infection is nasopharyngeal (NP) colonization [4,5], and NP colonization must precede upper and lower respiratory infections [3,6]. Bacterial NP colonization is determined by many ecological factors including bacterial–bacterial and bacterial-host immune response interactions [4].

There are numerous commensal microbiota and potential bacterial pathogens in the gastrointestinal tract [7,8], and the role of gastrointestinal commensal microbiota in normal and pathogenic host immune response has been well studied [7-9] However, although a similar situation exists in the NP [3,10], little is known about role of NP microbiota in host immune response. According to a recent metagenomic analysis of NP microbiota, there are approximately one million sequences of microbiome in the human NP representing 13 taxonomic phyla and 250 species-level phyla [2]. *Spn, Hi* and *Mcat* are common among the NP microbiota in healthy children [2,10,11]. More than half children at age 6 to 24 months, at times of good healthy may be colonized with these potential bacterial pathogens [5,11]. Co-colonization occurs in approximately 18% of healthy children and 46% of children with AOM [11]. When co-colonization occurs, *Hi* predominates over *Spn* except serotype 19A strains, and *Spn* predominates over *Mcat* to cause AOM when both are present in the NP prior to AOM [12]. The interaction between *Spn, Hi* and *Mcat* is contradictory and relevant mechanism to explain outcomes of co-colonization remain unclear [3,11,13-16].

Host immune responses may influence interactions among microbes and therefore influence the composition of the colonizing flora and invading bacteria [3]. In a mouse model host innate immune responses has been shown to play an important role in out-come of co-colonization of *Spn* and *Hi* [17]. It is unclear whether host adaptive immune response influences the outcome of colonization as well when polymicrobial co-colonization occurs. No prior work has focused on differences in human antibody responses following *Snp, Hi* and *Mcat* co-colonization. The objective of this study was to assess the impact of NP co-colonization of *Spn* with *Hi* or *Mcat* on the systemic antibody responses of young children to vaccine candidate antigens expressed by the organisms. Serum IgA and IgG against pneumococcal antigens PhtD, PcpA and PlyD1 and whole cells of *Spn*, and against *Hi* surface proteins P6, protein D, OMP26 and whole cells of *Hi* were compared among cohorts of children during *Spn* and *Hi* NP colonization and co-colonization.

2. Materials and methods

2.1. Subjects and study design

This study was part of a 5-year prospective, longitudinal evaluation of human child immunity to *Sp* and *Hi* supported by the National Institute of Deafness and Communication Disorders as described previously [11,12,18-21]. NP, oropharyngeal (OP), hereafter referred to as NP samples, and serum samples were collected from healthy children at 6–24 months

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of age for determining NP colonization of *Spn*, *Hi* and *Mcat* by standard culture as described previously [12,18], and serum samples determining anti-body response by quantitative ELISA. Sole colonization was defined as detection of one potential otopathogen, and co-colonization was defined as detection of greater than one potential otopathogen in the NP at a sampling point. The data here involve children who had not received antibiotics for at least 3 weeks prior to sampling. All of the children received standard vaccinations including PCV7 (Prevnar, Wyeth Pharmaceuticals) as appropriate for age. The study was approved by the Institutional Review Board (IRB) of University of Rochester and Rochester General Hospital.

To investigate the influence of co-colonization on serum anti-body responses, the samples from children were divided into age-matched three groups: (1) non-colonization (culture-negative for *Spn*, *Hi* and *Mcat*), (2) sole colonization (culture-positive for *Spn* or *Hi* or *Mcat*), and (3) co-colonization (culture-positive for both *Spn* and *Hi* or *Mcat*).

2.2. Quantitative ELISA for antigen-specific antibody

Spn antigens histidine triad protein D (PhtD), choline-binding protein A (PcpA) and detoxified pneumolysin D1 (PlyD1) were provided by Sanofi Pasteur (Canada) [22]. The *Hi* antigens Protein D was kindly provided as a gift from GlaxoSmithKline Biologicals (Rixensart, Belgium). P6 and OMP26 were recombinant proteins that were expressed in and purified from *Escherichia coli* using P6 plasmid provided by Dr. Tim Murphy (University of Buffalo, US) and OMP26 plasmid provided by Dr. Jennelle Kyd (University of Canberra, Australia).

An adult serum with high endpoint titer of IgA and IgG against all three *Spn* antigens was used as an in-house reference serum for *Spn* antigen-specific ELISA. A sera pool from three adult donors with high endpoint titers of IgA and IgG against all three *Hi* antigens was used as in-house reference serum for *NTHi* antigen-specific ELISA. Antigen-specific IgA and IgG against each individual antigen in the in-house reference sera were quantified using Human IgA and IgG ELISA Quantitation Sets (Bethyl Laboratories, Inc) according to manufacturer's protocol with some modification [23]. The wells of a 96-well microtiter plate for generating a standard curve were coated with 100 ng/well of affinity purified human IgG or IgA capture antibodies in coating buffer (carbonate-bicarbonate, pH 9.6). The wells for measuring antigen-specific antibodies were coated with 100–500 ng/well of the corresponding individual antigens in 100 μl of coating buffer. Antigen specific antibody was calculated with the standard curve generated with SoftMax Pro version 5.2 (Molecular Devices Corp., Sunnyvale, CA) using commercial reference sera containing known amounts of total IgG and total IgA (Bethyl RS10-101).

Seum antigen specific IgG and IgA were determined as described previously with modification [24]. The IgA were run on 96-well microtiter plates with 100 μl of coating and reaction volume and IgG on 384-well microtiter plates with 20 μl of coating and reaction volume. Antigen coating concentrations were 100 ng/well for *Spn* antigens, and 50 ng/well for *Hi* antigens. The initial dilutions of sera were 1:25 for IgA and 1:200 for IgG. The lower detection limits of the IgA were 1.8 ng/ml for PhtD, 2.2 ng/ml for PcpA, 1.5 ng/ml for PlyD1, 2.7 ng/ml for P6, 8 ng/ml for protein D, and 10.5 ng/ml for OMP26. The lower

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detection limit of the serum IgG was 9.2 ng/ml for PhtD1, 41.1 ng/ml for PcpA, 6.1 ng/ml for PlyD, 1 ng/ml for P6, 3.5 ng/ml for protein D, and 4 ng/ml for OMP26.

2.3. Quantitative whole cell ELISA for pneumococcal, and NTHi-specific antibody

Serum IgA and IgG levels against non-typeable *Spn* (*NTSpn) and NTHi* whole cells were determined by quantitative ELISA as described previously with mini modification [24-28]. The *NTSpn* strain RX01, and *NTHi* strain 86-028NP were grown at 37°C to mid-log phase in Todd Hewitt media with 0.5% yeast extract [29], and brain heart infusion with hemin and NAD (each at 4 μg/ml) [28], respectively. The cells were harvested by centrifuge, washed twice with PBS, and then re-suspended in PBS to an OD_{600} (for *NTSpn*) or OD_{490} (for *NTHi*) of 1.0. The cells was diluted 1:10 (for *NTSpn*) or 1:50 (for *NTHi*) with coating buffer and 100 μl per well of diluted cells were fixed in 96-well microterplates at 4°C overnight. The antibodies were quantified using a four-parameter logistic-log standard curve generated with SoftMax Proversion 5.2 (Molecular Devices Corp., Sunnyvale, CA) by using affinity purified human IgG or IgA capture antibodies and a reference serum containing a known amount of total IgG and IgA (Bethyl). The lower detection limits were 18.6 ng/ml for *NTSpn* IgA, 41.8 ng/ml for *NTSpn* IgG, 76.0 ng/ml for *NTHi* IgA, 11.5 ng/ml for *NTHi* IgG.

2.4. Statistical analysis

Geometric means (GM) of antibody concentrations with 95% confidence intervals (CI) were calculated for each group. For the purpose of statistical analysis, the antibody titers below the lower limit of detection were arbitrarily assigned to values equivalent to half the lower detection limit. Comparisons of antibody titers between groups were done with GraphPad Prism 5 software using the Mann–Whitney test after base 10 logarithmic transformations [30,31]. $P < 0.05$ was considered to indicate statistical significance[30,31].

3. Results

This analysis involved 455 serum samples from 494 visits of 213 children between ages of 6 and 24 months. The characteristics of the children are shown in Table 1.

3.1. Influence of NP colonization by Spn on serum antigen specific IgA and IgG antibody responses to Spn

When *Sp* colonized the NP, serum specific IgA and IgG antibody were significantly higher than when Sp NP colonization was not detected (Table 2). There was a 6.8-fold increase in GM serum anti-PhtD IgA ($p < 0.0001$), a 2.2-fold increase in anti-PhtD IgG ($p = 0.0008$), a 11.5-fold increase in anti-PcpA IgA ($P < 0.0001$), and a 3.6-fold increase in anti-PcpA IgG ($p < 0.0001$), a 3.0-fold increase in anti-PlyD1 IgA ($p = 0.008$), and a 2.9-fold increase in anti-PlyD1 IgG ($p = 0.003$). The serum antibody titers to whole cells of *Spn* strain RX01 increased by 2.0-fold in IgA ($p = 0.0002$) and 1.8-fold in IgG ($P = 0.0003$).

3.2. Influence of co-colonization of Hi or Mcat with Spn on serum pneumococcal protein specific IgA and IgG antibody responses to Spn

When co-colonization by *Spn* and *Hi* occurred, compared with sole colonization by *Spn*, Serum IgA further increased by 2.9-fold inanti-PhtD ($p = 0.041$), 1.6-fold in anti-PcpA ($p =$

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0.32) and 2.0-fold in anti-PlyD1 ($p = 0.039$). The serum IgA to *NTSpn* RX01 whole cells further increased by 1.6-fold ($p = 0.035$). There was no significant difference in serum IgG against PhtD, PcpA or PlyD1 antigens, or whole *Spn* cells between sole *Spn* colonization and co-colonization by *Spn* and *Hi* ($p > 0.05$ for all, Table 2).

When co-colonization by *Spn* and *Mc* occurred, compared with sole colonization by *Spn*, serum pneumococcal-specific IgA and IgG responses were significantly increased further excepting anti-whole cell IgG (Table 2). The serum antibodies further increased by 3.0-fold in anti-PhtD IgA ($p = 0.0022$), 1.8-fold in anti-PhtD IgG ($p = 0.013$), 2.0-fold in anti-PcpA IgA ($p = 0.021$), 1.8-fold in anti-PcpA IgG ($p = 0.037$), 2.1-fold in anti-PlyD1 IgA ($p = 0.01$), and 2.7-fold in anti-PlyD1 IgG ($p = 0.0004$). The serum antibody to whole cells of *NTSpn* strain RX01 significantly increased further by 1.5-fold in IgA ($p = 0.002$), but not in IgG ($p = 0.54$). The results suggested that co-colonization of *Hi* or *Mcat* with *Spn* enhanced host pneumococcal protein-specific antibody response.

Co-colonization by *Hi* with *Spn* or co-colonization of *Mcat* with *Spn* were not significantly different in their impact on serum pneumococcal protein-specific IgA or IgG responses to *Spn* antigens or whole *NTSpn* cells (all $p > 0.05$, Table 2).

3.3. Influence of NP colonization by Hi on serum IgA and IgG responses to Hi proteins

When *Hi* colonized the NP, compared with no colonization, serum specific IgA and IgG titers were significant higher excepting anti-OMP26 antibodies (Table 3). There was a 2.5-fold increase in serum anti-P6 IgA ($p = 0.0007$), a 2.4-fold increase in anti-P6 IgG ($p = 0.0001$), a 1.5-fold in increase in anti-protein D IgA ($P = 0.036$), a 1.6-fold increase in anti-protein D IgG ($p = 0.0021$), but no significant difference in anti-OMP26 IgA ($p = 0.50$) or IgG ($p = 0.52$). The serum antibody titers to whole cells of *Hi* increased by 3.7-fold in IgA ($p < 0.0001$) and 2.1-fold in IgG ($p < 0.0001$). Colonization by *Hi* significantly elicited serum IgA and IgG responses against *H*i protein antigens and whole cells as well.

3.4. Influence of co-colonization of Spn or Mcat with Hi with on serum protein specific IgA and IgG responses to Hi

Compared with sole colonization by *Hi*, co-colonization by *Hi* and *Spn* did not influence the serum levels of IgG or IgA against *Hi* surface proteins, or *Hi* whole cells ($p > 0.05$ for all, Table 3). There was no difference in either serum IgA or IgG responses to *Hi* antigens studied, or whole *Hi* cells between co-colonization by *Hi* with *Spn* and co-colonization by *Hi* with *Mcat* (all $p > 0.05$, Table 3).

4. Discussion

In this study, we investigated influence of NP co-colonization by *Spn* and *Hi* or *Mcat* on host adaptive serum immune responses in young children. We found that NP colonization with either *Spn* or *Hi* elicited serum protein-specific IgA and IgG responses to the homologous bacterial species; co-colonization by *Spn* and *Hi* further increased serum antibody responses to *Spn*, but not to *Hi*, compared with sole colonization with *Spn* or *Hi*. The results suggest that interaction of bacterial respiratory pathogens modulates pathogen-specific host adaptive serum immune responses.

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Elicitation of antibody response by bacterial colonization has been documented in previous studies by our group [19,20] and others [32-34], but the knowledge is limited regarding impact of co-colonization on host immune responses to pathogens. Lysenko et al. [17] has studied role of co-colonization by *Spn* and *Hi* in host innate immune response in mouse model. They found that the bacterial density of *Spn* but not *Hi* was lower when *Spn* and *Hi* colonized than when *Spn* or *Hi* colonized alone. They also showed that *Hi* activated the host innate immune response involving complement and neutrophils to kill *Spn*, resulting in rapid clearance of *Spn* from the NP when *Spn* and *Hi* co-colonized the mouse NP [4]. They also found co-colonization with *Spn* and *Hi* resulted in synergetic proinflammatory response [35]. Margolis E et al. have found in infant rat that *Hi* strain-specifically promoted an immune response which limited the invasion of *Spn*. Pro-colonization of *Spn* increased the intensity of *Hi* [4]. Bacterial interactions may influence the antibody response as well. Hollsing AE found that in cystic fibrosis patients, dual colonization with *Pseudomonas aeruginosa* and *Staphylococcus aureus* inhibits serum antibody to *P. aeruginosa* exoproteins, alkaline protease, exotoxin A, and elastase, but enhances serum antibodies to phospholipase C, compared to sole *P. aeruginosa* colonization. Dual colonization also enhances serum antibody titers to *S. aureus* teichoic acid, compare to sole *S. aureus* colonization [36]. Both *Spn* and *Hi* express phosphorycholine, but phosphorycholine is necessary for survival of *Spn*, not *Hi*. Since pre-exposure to one of the two species can induce antibodies against phosphorycholine, thereby co-colonization can promote clearance of *Spn*, not *Hi* [37]. We have been studying three *Spn* vaccine candidate antigens PhtD, PcpA and PlyD1 because they are moving forward at this time in clinical trials as a tri-component vaccine (clinical trial # NCT01764126). The three *NTHi* vaccine candidate antigens P6, Protein D and OMP26 have been the targets of our research for the past 8 years for possible inclusion in a tri-component *NTHi* vaccine. In present report, we found that co-colonization of *Spn* and *Hi* enhanced serum anti-body responses to the three *Spn* vaccine candidate antigens, but *Spn* co-colonization did no enhance serum antibody responses to *Hi* antigens. We intend to further study this unexpected finding.

The mechanism to account for co-colonization of bacterial pathogens influencing the host adaptive immune responses may involve activation and crosstalk of host multiple pattern-recognition receptors (PRRs) in innate immune cells, and components bridging innate and adaptive immunity. The host innate immune system detects microorganisms and responds to their stimuli mainly through Toll-like receptors (TLRs) [38]. *Spn, Hi* and *Mcat* can all be recognized by TLR2, 4 and 9 [39-41]. Clement CG et al. have found that innate immunity induced by *NTHi* can provide protection against *Spn* infection in mouse lung, probably through multiple innate immune mechanisms including formyl peptide receptors, complement receptors, and TLRs [42]. Lim JH et al. have reported that *Spn* synergizes with *NTHi* to induce inflammation via up-regulating TLR2 [40]. *Spn* and *Hi* synergistically activate NF-κB as well as the subsequent inflammatory TNFα, IL-1β, and IL-8 responses via IKKβ-IκBα and p38 MAPK pathway [43]. Such effects of co-colonization by *Spn* with *Hi* on innate immune immunity may lead to influence adaptive immunity. Dendritic cells (DC) play important roles in bridging innate and adaptive immunity [44]. TLRs and cross talk between TLRs, and complement receptors in antigen present cells (APC) control adaptive immune responses [45-47]. TLR signaling not only helps to initiate adaptive

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immune response, but also is required for it [45,48]. Activation of TLR in DCs results in elevated levels of pro-inflammatory cytokines or chemokines leading to the induction of the adaptive immune response, where both T and B-lymphocytes play a crucial role [49]. Further study of the mechanism responsible for increased adaptive immune responses associated with respiratory bacterial co-colonization is warranted and underway.

We also found that co-colonization of *Mcat* with *Spn* increased pneumococcal protein specific antibody responses. Effect of co-colonization of *Mcat* with *Spn* reached statistical significances for both IgA and IgG responses to *Spn*, whereas co-colonization of *Hi* with *Spn* reached statistical significances only in IgA responses to *Spn*, not in IgG probably due to sample size effect. A previous study in the same population by our group showed that the frequency of co-colonization of *Mcat* with *Spn* is much higher than that of *Hi* with *Spn*, and when co-colonization by *Spn* and *Mcat* occurs, *Spn* predominates over *Mcat* to cause AOM [12]. Since we did not have reagents or resources to study the effect of co-colonization by *Spn* and *Mcat* on antibody responses to *Mcat*, we could not correlate antibody responses and outcomes of AOM when *Spn* and *Mcat* co-colonized.

This study has limitations. Sole colonization and co-colonization were defined at a moment in time when samples were collected. We cannot exclude the possibility that children had been colonized with one or co-colonized with two or more potential otopathogens just before the samples were collected. Missed colonization or co-colonization may also have occurred if the density of a second organism was very low and not detected in culture. If the frequency of missed colonization or colonization events were high, the possibility that antibody differences at a moment in time of sampling might be stochastic and cannot be excluded. The NP is the ecological niche for a variety of commensal microbiota and potential respiratory bacteria as well viruses. There are complicated interactions among potential respiratory pathogens. Investigation of *Spn, Hi a*nd *Mcat* co-colonization interactions with the commensal microbiota or other potential bacterial or viral pathogens is of interest to our group [50] but was not studied here. We did not quantify bacterial density and that parameter may be a critical variable influencing immune responses. We excluded children with recent antibiotic exposure so we did not assess the impact of antibiotic treatment on NP colonization outcomes and antibody responses.

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Table 1

Characteristics of children (*n* = 213).

	Number (%)
Female	107 (50.2)
Male	106 (49.8)
Caucasian	149 (70)
Non-Caucasian	74 (30)
Breast feeding	128 (60)
Formula	83 (40)
Smoking exposure	29 (14)
No	184 (87)
Daycare	72 (33.8)
No	141 (66.2)
PCV-7 up-to-date	211 (99)
No	2 (1)

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Table 2

Impact of co-colonization of *Hi* or *Mcat* with *Spn* on serum antibody responses to *Spn*.

	Culture negativeGM (95% CI, *n*)	***Spn*GM (95% CI, *n*)**	***Spn* + *Hi*GM (95% CI, *n*)**	***Spn* + *Mcat*GM (95% CI, *n*)**
IgA (μg/ml)				
Anti-PhtD	0.10**** (0.058–0.178, 118)	0.68 (0.400–1.158, 80)	1.97* (0.897–4.396, 29)	2.07** (1.155–3.712, 65)
Anti-PcpA	0.098**** (0.057–0.170, 118)	1.13 (0.716–1.771, 81)	1.765 (0.894–3.486, 29)	2.232* (1.299–3.835,65)
Anti-PlyD1	0.02** (0.015–0.040, 118)	0.07 (0.043–0.117, 81)	0.143* (0.066–0.308, 29)	0.15* (0.091–0.247, 64)
Anti-whole cell	0.07*** (0.055–0.092, 125)	0.14 (0.113–0.178, 100)	0.22* (0.144–0.328, 45)	0.22** (0.171–0.282, 115)
IgG (μg/ml)				
Anti-PhtD	4.86*** (3.55–6.66, 117)	10.64 (7.726–14.673, 81)	15.42 (8.931–26.616, 29)	18.66* (12.597–27.644, 65)
Anti-PcpA	3.83**** (2.524.5–5.819, 102)	13.80 (10.040–18.971, 78)	17.48 (10.332–29.563, 27)	24.73* (17.115–35.331, 55)
Anti-PlyD1	0.38** (0.242–0.597, 103)	1.10 (0.753–1.604, 76)	2.03 (1.050–3.907, 27)	2.96*** (1.969–4.455, 55)
Anti-whole cell	3.51*** (2.840–4.331, 116)	6.17 (5.105–7.449, 95)	6.90 (5.096–9.336, 42)	6.16 (5.029–7.546, 107)

Spn, S. pneumoniea; *Hi, H. influenzae*; *Mcat, M. catarrhalsi*. GM, geometric mean; CI, confidence interval.

* $P < 0.05$ compared with sole *Spn*.

** $P < 0.01$ compared with sole *Spn*.

*** $P < 0.001$ compared with sole *Spn*.

**** $P < 0.0001$ compared with sole *Spn*.

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Table 3

Impact of co-colonization of *Spn* or *Mcat* with *Hi* on serum antibody responses to *Hi*.

	Culture NegativeGM (95% CI, *n*)	***Hi* GM (95% CI, *n*)**	***Hi* + *Spn*GM (95% CI, *n*)**	***Hi* + *Mcat*GM (95% CI, *n*)**
IgG (ng/ml)				
Anti-P6	42.4*** (34.1–52.6, 124)	106.1 (65.9–170.8, 44)	140.3 (82.8–238.0, 47)	140.6 (85.9–230.2, 42)
Anti-D	82.2* (66.4–101.7, 125)	121.0 (92.8–157.9, 44)	122.5 (89.4–167.9, 47)	129.7 (92.8–181.2, 42)
Anti-OMP26	71.7 (56.6–87.7, 121)	79.3 (56.6–111.3, 42)	104.5 (77.0–141.8, 46)	116.4 (82.8–163.7,41)
Anti-whole cell	22.5**** (17.7–8.5, 122)	83.2 (51.1–135.6, 43)	89.4 (49.3–162.2, 46)	121.6 (59.2–249.7, 42)
IgG (ng/ml)				
Anti-P6	684.9*** (554.0–846.7, 127)	1630.0 (1115.2–2382.3, 44)	1265.7 (879.4–1821.6, 48)	1337.0 (907.8–1969.1, 43)
Anti-D	715.2** (610.0–838.6, 119)	1151.6 (910.8–1456.2, 44)	945.1 (715.8–1247.8, 43)	1007.8 (734.3–1383.2, 36)
Anti-OMP26	873.5 (749.9–1017.5, 125)	1003.6 (798.5–1261.5, 44)	973.7 (777.1–1220.0, 48)	1043.3 (831.9–1308.5, 43)
Anti-whole cell	1517.6**** (1251.4–1840.3, 126)	3165.1 (2413.6–4150.7, 43)	2531.5 (1936.8–3309.0, 47)	2644.5 (1737.2–4025.5, 43)

Spn, S. pneumoniea; *Hi, H. influenzae*; *Mcat, M. catarrhalis*. GM, geometric mean; CI, confidence interval.

* $P < 0.05$ compared with sole *Hi*.

** $P < 0.01$ compared with sole *Hi*.

*** $P < 0.001$ compared with sole *Hi*.

**** $P < 0.0001$ compared with sole *Hi*.