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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.

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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 to cause 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].

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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 outcome 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 of age for determining NP colonization of *Spn*, *Hi* and *Mcat* by standard culture as described previously [12,18], and serum samples determining antibody 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 antibody 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 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 Pro version 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.